Self-Double-Emulsifying Drug Delivery Systems: A novel approach to topical fixed-dose anti-tubercular drug delivery

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Thesis submitted in fulfilment of the requirements for the degree Doctor of Philosophy in Pharmaceutics at the North-West University

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DECLARATION BY RESEARCHER

I, **Daniélle van Staden**, hereby declare that this **thesis**, titled "**Self-Double-Emulsifying Drug Delivery Systems: a novel approach to topical fixed-dose anti-tubercular drug delivery"**, submitted in fulfilment of the requirements for the degree **Doctor of Philosophy in Pharmaceutics at the Potchefstroom Campus of the North-West University**, is my own original work and has not been previously submitted for examination to any other institution of higher education. I also declare that the resources used in this dissertation have been referenced and acknowledged accordingly.

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PREFACE

Outline of the thesis

This thesis is submitted in article format according to North-West University guidelines. Included articles conform to the requirements preferred by the appropriate journals. The thesis is written according to UK English spelling, with the article chapters written according to the Author Guidelines provided by the applicable journals.

After the introductory **Chapter 1**, **Chapter 2** is a **review article** titled: **'*Adapting Clofazimine for Treatment of Cutaneous Tuberculosis by Using Self-Double-Emulsifying Drug Delivery Systems*'** and was published in '*Antibiotics*' (DOI: 10.3390/antibiotics11060806). This review publication was written to provide background to examiners on the relevancy of this study by discussing clofazimine as an adjuvant drug to tuberculosis regimens, comparing other lipid-based drug delivery systems to spontaneous emulsions and establishing the global challenge created by antimicrobial drug resistance in parallel to the ongoing tuberculosis pandemic. In order to fulfil the requirement of the North-West University a complete literature overview should be included. Hence, this review article is presented as the first literature overview of this study and no separate literature overview was thus included in this thesis as this review was seen as fulfilment of the above requirement.

Chapter 3 is presented as the second **review article** titled: '***The Science of Selecting Excipients for Dermal Self-Emulsifying Drug Delivery Systems***' published in '*Pharmaceutics*' (DOI: 10.3390/pharmaceutics15041293). This review publication was written as a separate literature overview which describes the factors one needs to consider when selecting excipients for formulation of dermal spontaneous emulsions. A second literature overview was included since the research conducted to select excipients prior to commencement of laboratory experiments was based on an intensive literature study of topical/transdermal formulations and self-double-emulsifying drug delivery systems (SDEDDSs). Therefore, a formulation recommendation system is presented in the discussion section of this article as a guide to other researchers considering the inclusion of more than one drug in spontaneous emulsions based on the Biopharmaceutical Classification of the considered drug(s).

Chapter 4 is a **research article,** titled: '***The Development of Dermal Self-Double-Emulsifying Drug Delivery Systems: Preformulation Studies as the Keys to Success***', published in '*Pharmaceuticals*' (DOI: 10.3390/ph16101348). Here the methods and results of preformulation studies are discussed. Firstly, preformulation studies were conducted to generate and select excipients to produce potential primary emulsions. Primary emulsions were then considered for

the purpose of establishing suitability for inclusion into SDEDDSs. In conclusion, the selected primary emulsion and SDEDDS were subjected to droplet size and polydispersity index measurement as well as examination via microscopy and transmission electron microscopy.

Chapter 5, titled: '***Development of a HPLC method for analysis of a combination of clofazimine, isoniazid, pyrazinamide, and rifampicin incorporated into a dermal self-double-emulsifying drug delivery system***' is included as a **technical note** submitted for publication in '*Methods and Protocols*' and is under revision (minor changes) at the time when this thesis was submitted. Chapter 5 describes analytical method development conducted to successfully analyse the four selected anti-tubercular drugs simultaneously with an accurate, reliable and repeatable method.

Chapter 6 concludes the findings of the thesis and recommends future studies to further develop and define this work. All author guidelines as well as different data sets can be found in the appendices at the end of the thesis.

Outsourcing and consultants

Dr HJR Lemmer provided a report explaining the outcomes of **isothermal micro calorimetry compatibility experiments** at the Centre of Excellence for Pharmaceutical Sciences (Pharmacen™) of the North-West University, Potchefstroom Campus, South Africa.

Prof Frank van der Kooy assisted with development of the **HPLC** method at the Centre of Excellence for Pharmaceutical Sciences (Pharmacen™) of the North-West University, Potchefstroom Campus, South Africa.

Microscopic observations were performed by **Dr Anine Jordaan** at the Laboratory for Electron Microscopy, North-West University, Potchefstroom, South Africa.

Ethics

An ethics application for a minimum risk level study was submitted to the ethics committee of the North-West University, which was approved (approval certificate nr: **NWU-00111-17-A1-15**). The ethics approval letter of this study is included as **Annexure N**.

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ABSTRACT

Mycobacteria cause numerous skin infections that are difficult to treat due to intrinsic difficulties of drug permeation into mycobacteria, and their ability to develop resistance to antibiotics. Therefore, the incidence of nontuberculosis mycobacteria (NTM) related dermal infections and cutaneous tuberculosis (CTB) are increasing drastically as a consequence of antimicrobial resistance. This can also be attributed to the inability to carry out vaccinations, proper diagnosis, and treatment of TB during the Corona Virus Disease of 2019 (COVID-19) pandemic. Furthermore, currently no topical dosage form is marketed to locally treat mycobacterial skin infections. Therefore, this work introduces the possibility of exploring self-double emulsifying drug delivery systems (SDEDDSs) as dermal drug delivery vehicles through incorporation of four anti-tubercular agents, namely clofazimine, isoniazid, pyrazinamide, and rifampicin. Clofazimine is a highly lipophilic drug (log P of 7.66), followed by rifampicin with a log P value of 3.80. Isoniazid and pyrazinamide, on the other hand, are polar compounds with log P values of −0.64 and −1.88, respectively. The differences in lipophilicity of the four drugs present a unique challenge to choose excipients capable of solubilising all of the selected drugs while also exhibiting excipient-excipient compatibility, drug-excipient compatibility, and dermal drug delivery properties such as skin penetration enhancement characteristics and skin tolerability of excipients. Hence, during this study, a 'Formulation Recommendation System' (FRS) was developed to enable the optimal selection of excipients during the development of dermal SDEDDSs and self-emulsifying drug delivery systems (SEDDSs) based on the biopharmaceutical classification of drugs intended for incorporation into SEDDSs or SDEDDSs. This represents a novel contribution to the development of dermal spontaneous emulsions. The usefulness of considering auxiliary excipients such as emollients, humectants, preservatives, antioxidants, and cosolvents is discussed. A high-performance liquid chromatographic (HPLC) method was developed to enable the reliable, repeatable, and accurate simultaneous quantification of the selected anti-tubercular drugs. As the highlight of this study, intensive preformulation studies enabled the development of a dermal SDEDDS with the smallest droplet size yet reported in the literature. The droplet size was an average of 92.46 ± 5.52 nm, and the polydispersity index (PDI) value was 0.391 ± 0.05. Furthermore, the transmission electron microscopy (TEM) images revealed that the small droplets contained within the larger droplets of the SDEDDS were smaller than 92.46 nm since droplet size measurement only allowed detection of the larger droplets. Last, the mechanism of self-emulsification was captured at a nanoscale via TEM observation, which confirmed the generation of the first dermal nano-SDEDDS described in the literature. Therefore, use of SDEDDSs represents a novel approach for topical delivery of fixed-dose anti-tubercular drugs.

Keywords: *COVID-19; Cutaneous tuberculosis; Fixed dose combinations; Non-tuberculosis mycobacteria; SDEDDS; SEDDS; Skin; Topical*

UITTREKSEL

Mykobakterieë veroorsaak verskeie velinfeksies wat moeilik behandel word as gevolg van hul intrinsieke vermoë om geneesmiddel (GM) diffusie tot in die bakterieë self te belemmer. Boonop kan dié bakterieë ook weerstandigheidsmeganismes teen toegediende antibiotika(s) ontwikkel. Daarom is daar weens antimikrobiese weerstandigheid ‘n duidelik waarneembare toename van nie-tuberkulose mykobakterieë (NTM) verwante velinfeksies sowel as kutaneuse tuberkulose (KTB). Die duidelike toename kan ook aan die Corona Virus Siekte van 2019 (COVIS-19) pandemie toegeskryf word. Die ontwrigting wat deur die pandemie veroorsaak is, het gelei tot minder getroue inenting teen tuberkulose (TB) asook tot swak of geen diagnosering van TB gevalle. Verder is behandeling van TB-verwante velinfeksies beperk tot sistemiese behandeling aangesien geen topikale doseervorm tans op die mark beskikbaar is om mykobakteriële velinfeksies lokaal te behandel nie. Dus stel die werkstuk die moontlikheid voor om vier TB bestrydende GMs, naamlik: klofasimien, isoniasied, pirasienamied en rifampisien deur middel van self-dubbel-emulsifiserende GM afleweringsisteme (SDEGMASe) topikaal af te lewer. Klofasimien is ‘n hoogs lipofiele GM (log P waarde van 7.66) en kort op sy hakke volg die lipofiele rifampisien met ‘n log P waarde van 3.80. Teenstrydig word isoniasied en pirasienamied, na aanleiding van hul afsonderlike log P waardes van −0,64 en −1,88, as polêre GMs beskou. Die formuleerder word uitgedaag deur hierdie merkbare lipofilisiteitsverskille van die vier GMs aangesien oplosbaareid tesame met vereenigbaarheid tussen GMs en hulpstowwe, GM-GM interaksies en ‘n keuse van hulpstowwe wat gunstige en veilige dermale aflewering van GMs sal bevorder, oorweeg moet word. Daarom is daar tydens die studie ‘n ‘Formulering Aanbevelingsisteem’ (FAS) ontwikkel om formuleerders toe te rus om optimale hulpstof keuses uit te oefen volgens die biofarmaseutiese klassifikasie van verlangde GM(s) om in SDEGMASe of self-emulsifiserende GM afleweringsisteme (SEGMASe) ingesluit te kan word. Die werkstuk lewer ‘n nuwe bydra tot die ontwikkeling van spontane emulsies, wat vir vel aanwending bestem is, deur die FAS wat die insluiting van hulpstowwe soos versagmiddels, humektante, preserveermiddels, anti-oksidante en hulpoplosmiddels vir spontane emulsies oorweeg. ’n Hoë werkverrigting vloeistofchromatografie (HWVC) metode is ontwikkel om betroubare, herhaalbare en akkurate gelyktydige kwantifisering van die vier GMs te verseker. As die hoogtepunt van die studie word die resultate van intensiewe preformuleringstudies voorgehou as die sleutel tot sukses om die dermale SDEGMAS met die kleinste druppelgrootte, van 92.46 ± 5.52 nm (polidispersiteitsindeks (PDI) van 0.391 ± 0.05), nog in die literatuur te vervaardig. Verder word die studie afgesluit met transmissie elekromikroskopie (TEM) foto’s wat toon dat groot druppels van die SDEGMAS kleiner druppels versteek wat kleiner as 92.46 nm is aangesien druppelgrootte bepalings oorheersend die groot druppels waargeneem en dus gemeet het. Op

die TEM foto’s word die meganisme van spontane-dubbel-emusifisering vir die eerste keer op nanovlak vasgevang wat dan die vorming van ‘n SDEGMAS bevestig. Dus kan die formulering van SDEGMASe as ’n nuwe benadering ten opsigte van topikale vaste dosis kombinasie GM aflewering van TB bestrydende GMs beskou word.

Sleutelwoorde: *COVID-19; Kutaneuse tuberkulose; Nie-tuberkulose mykobakterieë; Vaste dosis kombinasies; Vel; SDEGMAS; SEGMAS; Topikaal*

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LIST OF ABBREVIATIONS

%RSD	**Percentage relative standard deviation**
AIDS	**Acquired Immunodeficiency Syndrome**
AVO	**Avocado oil**
BCS	**Biopharmaceutical Classification System**
CFZ	**Clofazimine**
cGMP	**Good Manufacturing Practice**
COVID-19	**Corona Virus Disease of 2019**
CTB	**Cutaneous tuberculosis**
DE	**Delaware**
DNA	**Deoxyribonucleic acid**
EC	**European Commission**
EPO	**Evening primrose oil**
EPTB	**Extra-pulmonary tuberculosis**
ESI	**Emulsion stability index**
EU	**European Union**
FDA	**United States Food and Drug Administration**
FDC	**Fixed dose combination**
FRS	**Formulation Recommendation System**
GI	**Gastro-intestinal**

GIT	**Gastrointestinal tract**
HIV	**Human immunodeficiency virus**
HLB	**Hydrophilic–Lipophilic Balance**
HPLC	**High performance liquid chromatography**
ICH	**International Conference on Harmonization**
INH	**Isoniazid**
IVR	***in vitro* release**
LCTs	**Long-chain triglycerides**
LOD	**Limit of detection**
LOQ	**Limit of quantification**
LTBI	**Latent tuberculosis infection**
MCTs	**Medium-chain triglycerides**
MDR-TB	**Multi-drug resistant tuberculosis**
Mtb	***Mycobacterium tuberculosis***
n-SDEDDS	**nano-self-double-emulsifying drug delivery system**
NTM	**Non-tuberculosis mycobacteria**
OLV	**Olive oil**
O/O	**Oil-in-oil**
O/O/W	**Oil-in-oil-in-water**
OR	**Oregon**

o/w	**Oil-in-water**
PALM	**Palm oil**
PDI	**Polydispersity index**
PE	**Primary emulsion**
PEG	**Polyethylene glycol**
PZY	**Pyrazinamide**
Q1	**Qualitatively**
Q2	**Quantitatively**
Q3	**Arrangement of matter and the microstructure of topical formulations**
r^2	**Correlation coefficient**
RH	**Relative humidity**
RIF	**Rifampicin**
RLD	**Reference listed drug**
SAFF	**Safflower oil**
SC	**Stratum corneum**
SD	**Standard deviation**
SDEDDS	**Self-double-emulsifying drug delivery system**
SEDDS	**Self-emulsifying drug delivery system**
SFAs	**Saturated fatty acids**
SNEDDS	**Self-nano-emulsifying drug delivery system**

SPIN	**Solid-state Pharmaceutical Innovation and Nanotechnology**
STREAM trial	**Evaluation of a Standardized Treatment Regimen of Anti-tuberculosis Drugs for Patients with Multidrug-resistant Tuberculosis**
TAM	**Thermal Activity Monitor**
TB	**Tuberculosis**
TCS	**Topical Drug Classification System**
TEM	**Transmission electron microscope**
TEWL	**Trans epidermal water loss**
TPSA	**Topological surface area**
UFAs	**Unsaturated fatty acids**
UK	**United Kingdom**
US	**United States**
USA	**United States of America**
WHO	**World Health Organization**
w/o	**Water-in-oil**
W/O/O	**Water-in-oil-in-oil**
XDR-TB	**Extensively drug resistant TB**

Chapter 1

Introduction, Research Problem, Aims and Objectives

1.1 Introduction

1.1.1 Cutaneous tuberculosis, a cascade of anti-microbial resistance escalated by the COVID-19 pandemic

Tuberculosis (TB) is currently considered a pandemic disease according to the World Health Organisation (WHO) (Ockenga *et al*., 2023; Seki *et al*., 2021). This highly infectious, airborne, droplet-spread disease is caused by *Mycobacterium tuberculosis* (Mtb) (Brito *et al*., 2022; Seki *et al*., 2021). Epidemiological studies conducted in South Africa indicated that TB was the fourth most likely single cause of premature deaths in the North West Province and the sixth most probable cause in Mpumalanga, regardless of the gender of the patient (Semenya & Maroyi, 2019). Moreover, data collected from the Limpopo Province indicated that TB was the fifth highest cause of death among all races (Semenya & Maroyi, 2019). South Africa is also considered one of the countries with the highest incidence of multidrug-resistant TB (MDR-TB) cases (Bhembe & Green, 2020). MDR-TB refers to resistance against at least two first-line therapy drugs, isoniazid, and rifampicin. This resistance renders the first-line treatment regimen ineffective in patients with MDR-TB (Ockenga *et al*., 2023; Garcia *et al*., 2021; van Zyl *et al*., 2015). Therefore, second-line drugs are receiving increasing attention from the anti-tubercular drug research community despite their conspicuous adverse-effect profiles (Lange *et al*., 2019).

In addition to the increased incidence of MDR-TB, co-infection in immunosuppressed patients, such as those who are infected with the human immunodeficiency virus (HIV) or have diabetes mellitus, contributes to the increased TB burden in South Africa (Berkowitz *et al*., 2018). However, as demonstrated during the Corona Virus Disease of 2019 (COVID-19) pandemic, the world is transformed into a global village where migrants, visitors, and refugees from TB-endemic countries present an enhanced opportunity to spread TB to non-endemic countries (Kabir *et al*., 2021). Conversely, during the strict lockdown responses in an effort to limit COVID-19 transmission, an upsurge in TB infections is now predicted. This may also have been partially attributed to a greater focus on improving critical care facilities during the battle against COVID-19 instead of maintaining general services, including TB-vaccination programmes and TB-

screening (Jain *et al*., 2020). This prediction was confirmed with a decrease of up to 47% in reported TB-case notifications during 2020 at the peak of the COVID-19 pandemic (Williams *et al*., 2023; Hogan *et al*., 2020). Furthermore, an impact modelling study indicated that a potential increase of up to 20% in TB related deaths due to delayed diagnosis, can be expected over the next five years (Williams *et al*., 2023). Furthermore, studies have linked latent tuberculosis activation with coinfection with COVID-19, which will therefore enhance the vulnerability of TB-endemic countries such as South Africa (Gerstein *et al*., 2021; Khayat *et al*., 2021; Takahashi *et al*., 2020).

It is well-known that TB is not limited only to pulmonary infection (Costa & Veasey, 2021). Thus, increased presentation of extrapulmonary TB (EPTB) manifestations is expected to accompany antimicrobial drug resistance (Kebede *et al*., 2021). Cutaneous tuberculosis (CTB), a relatively rare form of EPTB, is becoming more common due to anti-tubercular drug resistance (Bhandari *et al*., 2022; van Staden *et al*., 2020a). Furthermore, CTB is frequently misdiagnosed due to its various clinical forms and similarity to other dermal conditions (Soto-Ferbes *et al*., 2020). This infection is two to three times more common in women compared to men (van Zyl *et al*., 2015). It can target any part of the skin; however, the face is the most common area where CTB occurs (van Zyl *et al*., 2015), complicating the emotional well-being of patients. At this time, CTB is treated with general pulmonary TB infection regimes because currently there is still no commercial topical treatment available to treat CTB (Brito *et al*., 2022; Garcia *et al*., 2021; van Staden *et a*l., 2020a; van Zyl *et al*., 2015). Moreover, TB treatment involves protracted drug treatment regimens, and it is costly. Such treatment regimens are based on drugs with acceptable and serious adverse event profiles (Churchyard, 2019). The first-line treatment regimen consists of an intensive phase (isoniazid, rifampicin, pyrazinamide, and ethambutol) for a period of 8 weeks, followed by a maintenance phase (isoniazid and rifampicin) for 16 to 28 weeks, depending on the HIV status of a patient (Brito *et al*., 2022; Garcia *et al*., 2021; van Zyl *et al*., 2015). Thus, CTB treatment is a long and challenging regimen to complete, with a high incidence of patient nonadherence. However, the development of topically applied dosage forms for the assisted treatment of CTB can eradicate gastro-intestinal (GI) side effects by evading liver metabolism (Ramadon *et al*., 2022; Bellefroid *et al*., 2019). In the same way, topical drug delivery will allow treatment at the affected area without interfering with the oral treatment of TB, as most CTB patients also suffer from pulmonary TB (van Staden *et al*., 2020a; Chen *et al*., 2019; van Zyl *et al*., 2015).

1.1.2. Reasoning anti-tubercular, fixed-dose drug combinations

The focus on anti-tubercular drugs has moved towards repurposing known drug entities, with relatively safe side effect profiles to form part of new regimens that can resolve prolonged treatment times and improve the efficacy against TB (Rao *et al.*, 2018). Together with this shift in

treatment regimens, the concept of fixed-dose combination (FDC) therapy, which is based on the synergistic or additive potential of two or more drugs within a single dosage form to improve therapeutic efficacy and also to delay the development of resistance to the individual components of the combination (Zhao *et al*., 2023; Godman *et al.,* 2020; Lange *et al.*, 2019; Lima *et al*., 2017), is considered essential. Patient adherence is imperative to achieve effective anti-tubercular drug concentrations for a sufficient exposure time in all target populations, as well as to overcome drug resistance in order to achieve enhanced cure rates. Many groups of patients, especially younger children, have been found to not comply with the required individual dosage regimens; this compromises efficacy and increases resistance. FDC therapy offers an alternative means to overcome recrudescent infections in non-compliant patients by shortening the dosing regimen and most often decreasing the doses of the individual drugs due to the synergistic potential offered (Zhao *et al*., 2023; Lange *et al.*, 2019; John *et al.*, 2015). Various studies have indicated that with communicable diseases such as TB, adherence to dosing regimens markedly influences the achievement of positive health outcomes (Hutchins *et al.*, 2011). For example, a meta-analysis of treatment outcomes in patients treated with antiretroviral, TB, hypertension, and diabetes drugs indicated that FDC therapy resulted in a 26% reduction in patient noncompliance compared to the cases in which free-drug component treatment regimens were used (Zhao *et al*., 2023; John *et al.*, 2015). However, it is necessary to understand the relationship between drug dose, pharmacokinetics, as well as consequential pharmacodynamics (therapeutic efficacy) and drug safety (Godman *et al.,* 2020; Salahudeen & Nishtala, 2017). Furthermore, cost and access remain formidable complications regarding FDC treatments. Even so, despite these limitations, there is a reasonable probability of future improved availability and use of FDC therapies in lower and middle- income countries as more evidence is acquired (Godman *et al.,* 2020; Wu *et al.*, 2019).

This study utilises FDC therapy together with repurposed components that can provide a commercially viable topical drug delivery system capable of treating CTB. First, clofazimine, which is an orphan drug originally developed as an anti-tubercular agent but was later used in the triple regimen treatment of multibacillary leprosy (van Staden *et al*., 2022; Sousa *et al*., 2020; van Staden *et al*., 2020a), is considered as an adjuvant agent to first-line anti-tubercular drugs. Interest in this repurposed riminophenazine antibiotic agent was revived after it was established through a clinical trial in Bangladesh and in the more recent STREAM trial to shorten the TB treatment regimen (Tiberi *et al*., 2018). Clofazimine is listed by the WHO as a group 5 drug that alleviates MDR-TB (Li *et al.*, 2019). It is a complementary candidate displaying *in vivo* and *in vitro* efficacy against MDR-TB strains with limited toxicity (Dey *et al*., 2013; Jagannath *et al.*, 1995). Its notably high lipophilicity and its redox potential engender antimicrobial effectiveness through oxidation of reduced clofazimine followed by the formation of reactive oxygen species

(van Zyl *et al.*, 2019). Moreover, recently, clofazimine has been considered as a potential agent to aid in the treatment of non-tuberculosis mycobacteria (NTM) related skin infections that are equally and significantly impacted by the global increase of antimicrobial drug resistance (Wang *et al*., 2023). However, the physicochemical properties of clofazimine are not suitable for dermal drug delivery. For example, it is just sparingly soluble in water and has a log P value of 7.66 (van Staden *et al*., 2022; de Castro *et al*., 2021; van Staden *et al.*, 2020a; van Zyl *et al*., 2019). Nonetheless, possible synergism between clofazimine and anti-tubercular drugs has been reported (Li *et al.*, 2019; Mirnejad *et al.*, 2018). In this project, we consider the possibility of synergism between clofazimine and pyrazinamide for dermal drug delivery. This premise is based on the finding that the dose of clofazimine administered orally can be substantially reduced from 25 mg/kg to 6.25 mg/kg when used in combination with pyrazinamide (Ammerman *et al*., 2018). This synergism will be of advantage for dermal application. Clofazimine induces dose-related red discolouration of the skin and therefore reducing the amount of clofazimine will result in reduced discolouration (Duan *et al*., 2019; Dheda *et al*., 2017). This will also be of benefit due to the limited aqueous solubility of this highly lipophilic compound (da Castro *et al*., 2021). For these reasons, clofazimine and pyrazinamide are to be used as an FDC paired with two other anti-tubercular drugs to generate a FDC treatment.

For this study, an FDC comprising clofazimine, isoniazid, pyrazinamide, and rifampicin is investigated. This follows from the successful treatment of multibacillary leprosy with the triple regimen comprising clofazimine, dapsone, and rifampicin (Andrade *et al*., 2021). Furthermore, there is ample evidence that rifampicin is capable of enhancing the efficacy of clofazimine when used in combination against Mtb *in vitro* (Mukonzo *et al*., 2019; Zhang *et al*., 2017). Recent studies revealed an increase in efficacy of traditional first-line regimens when adding clofazimine (Mashele *et al*., 2022). Hence, the development of a SDEDDS comprising clofazimine, pyrazinamide, isoniazid, and rifampicin can potentially improve the therapeutic efficacy of first-line anti-tuberculosis drugs. Moreover, the generation of SDEDDSs capable of incorporating different FDCs represents a novel approach to individualised therapy due to the drug resistant nature of mycobacterial skin infections (Wang et al., 2023; Zheng & Lupoli, 2023; van Staden *et al*., 2022). The physicochemical properties of the anti-tubercular drugs considered for this study are displayed in **Table 1**.

Table 1: *Physicochemical properties of anti-tubercular drugs considered for this study* (van Staden *et al*., 2022; Husseain *et al*., 2021; Sutradhar & Zaman, 2021; Trousil *et al*., 2020; van Staden *et al*., 2020a; Mehta & Jindal, 2013; Brennan & Young, 2008; Becker *et al*., 2006)

Drug	Log P	Molecular weight	Aqueous solubility	Elimination half-life	Metabolism (elimination)
Clofazimine	7.66	473,40 Da	Sparingly soluble	70 days	Hepatic
Isoniazid	-0.64	137,14 Da	125 mg/mL	45–110 min (rapid metabolisers) 2–4.5 h (slow metabolisers)	Hepatic
Pyrazinamide	-1.88	123,11 Da	15 mg/mL	3-5 h	Hepatic
Rifampicin	3.80	822,90 Da	Sparingly soluble	3-4 h	Hepatic

From **Table 1**, the selected drugs can be ranked according to their lipophilicity when reflecting on their unique drug properties as:

Clofazimine >> Rifampicin >> Isoniazid > Pyrazinamide

Thus, well-known anti-tubercular drugs have been considered for this study. However, clofazimine is known for its severe adverse reaction events that range from substantial discolouration to gastric accumulation. For this reason, the dermal route of administration should contribute to improved patient compliance, since gastric and hepatic metabolism, as well as painful administration pathways, are bypassed (Bellefroid *et al*., 2019). Additionally, attenuation of other side effects and improved bioavailability of drugs can be achieved through targeted drug delivery via the topical route of administration (Bellefroid *et al*., 2019; Machado *et al*., 2018).

1.1.3. Overcoming challenges experienced during dermal drug delivery

Normally, use of dermal drug delivery bestows improved patient compliance due to the non-invasive nature of this particular route of administration (Zakaria *et al*., 2023; Bellefroid *et al*., 2019). However, the skin must not be underestimated due to the formidable barrier properties provided by the outermost layer of the skin, the stratum corneum (SC) (Sparr *et al*., 2023; Zakaria *et al*., 2023; Bellefroid *et al*., 2019). Although the strict lipid

arrangement of the SC can be disrupted by employing skin penetration enhancers in formulations (Sparr *et al*., 2023; Zakaria *et al*., 2023; Bhat *et al*., 2021), care should be taken when choosing these compounds as they are often needed in high concentrations and thus may furthermore either permanently affect the SC, or be notably toxic (Lane, 2013). Consequently, for dermal drug delivery, natural oils are preferred over synthetic skin penetration enhancers due to their increased compatibility with the skin and decreased tendency to cause skin irritation (van Staden *et al*., 2020a; van Staden *et al*., 2020b). Furthermore, numerous natural oils can be considered as potential skin penetration enhancers depending on their fatty acid composition and combining them with compatible, yet effective system stabilising surface-active agents (van Staden *et al*., 2022; Bhat *et al*., 2021, van Staden *et al*., 2020a; van Staden *et al*., 2020b). Natural oils are used here as solubilising agents due to the lipophilic nature of the selected anti-tubercular drugs, clofazimine and rifampicin. Although pyrazinamide and isoniazid are less lipophilic **(Table 1)**, it is anticipated that these drugs would be compatible with one or more of the oils chosen. The increased solubility of these polar compounds should be achieved by their inclusion in an internal oil phase capable of solubilising these drugs.

In relation to drug delivery systems that effectively penetrate the SC, much research has been conducted on the drug properties that lead to enhanced dermal drug delivery. For example, it was concluded that drugs deemed most suitable for dermal drug delivery should have a low molecular weight (≤ 500 D_a), a log P value ranging between 1 and 3, and at least moderate aqueous solubility (≥ 1 g/mL) (Naik *et al.*, 2000). According to the drug properties indicated in **Table 1**, it is clear that the selected drugs do not comply with these guidelines. However, anti-tubercular drugs tend to violate most general medicinal chemistry rules used in the rational design of drugs (Machado *et al*., 2018). Fortunately, it appears that lipophilicity, normally considered a drawback, is a favourable property for anti-tubercular drugs in view of the protective barrier provided by the lipophilic cell wall of mycobacterium (Machado *et al*., 2018). Hence, lipophilic drugs can cross the barrier presented by the lipophilic cell wall more easily compared to hydrophilic anti-tubercular drugs (Machado *et al*., 2018). Consequently, judicious selection of excipients can crucially enhance the efficiency of topical anti-tubercular drug delivery. For example, the inclusion of skin penetration enhancers with potent lipid fluidisation properties can allow these lipophilic drugs to successfully diffuse across the SC (Sparr *et al*., 2023; van Staden *et al*., 2020b; Lane, 2013). In addition, surface-active agents can assist in enhancing skin penetration through lipid disruption (Sakdiset *et al*., 2023; van Staden *et al*., 2020b; Danby *et al*., 2018). However, as noted above, these excipients should not be included in large amounts, since they retain a potent skin irritation potential due to concentration-dependent lipid disruption (Danby *et al*., 2018). In this study, the vehicle chosen to deliver anti-tubercular drug combinations via the dermal route of administration represents the novel aspect of this work, as it enables the evaluation of the potential of traditional

oral self-double-emulsifying drug delivery systems (SDEDDSs) to be used as an FDC dermal drug delivery system.

1.1.4. Self-double-emulsifying drug delivery systems

SDEDDS formulations surpass general self-emulsifying drug delivery systems (SEDDSs) since they are able to encapsulate lipophilic and hydrophilic drugs in increased quantities compared to traditional SEDDSs (van Staden *et al*., 2023a; van Staden *et al*., 2022; Hu *et al*., 2016). However, the selection of excipients is considered more complex as an internal and external oil phase must be selected on the basis of immiscibility so as to achieve spontaneous emulsification of a multiphase delivery system (van Staden *et al*., 2023a; Wang *et al*., 2020; Hu *et al*., 2016). This multiphase drug delivery system consisted of a primary water-in-oil emulsion into which both hydrophilic and lipophilic anti-tubercular drugs are incorporated. An external oil phase is subsequently added in order to achieve increased solubilisation and enhanced skin penetration, followed by the disruption of the lipophilic mycobacterial cell wall. This can be considered challenging, as generally, SDEDDSs should provide increased stability in terms of oral drug delivery because primary emulsions (known for increased stability compared to double emulsions) can be administered to patients and a multiple emulsion will be generated by exposure to GI-fluids (Bhattacharjee *et al*., 2017). However, in terms of dermal drug delivery, excipients must be carefully selected, as multiple emulsions cannot be generated after administration and SDEDDSs must remain stable until dermal application (van Staden *et al*., 2023a; van Staden *et al*., 2023b). Interestingly, SDEDDSs have been reported to show remarkable stability due to their ability to withstand phenomena such as phase separation, flocculation, and Ostwald ripening for periods longer than 6 months without the presence of any preservatives (Bajracharya *et al*., 2019). Therefore, this current work should enable generation of guidelines for future studies aiming to select excipients for dermal SDEDDSs.

1.2. Research problem

CTB is a relatively rare manifestation of EPTB (Costa & Veasey, 2021; Soto-Febres *et al*., 2020). However, increased incidence of CTB and NTM related skin infections is expected due to a global increase in antimicrobial drug resistance and patient noncompliance. The latter is attributed to the protracted treatment regimens and adverse event profiles of anti-tubercular drugs. The lockdown response to the COVID-19 pandemic that has focused on improving critical care facilities instead of maintaining general health care services such as TB vaccination and screening has markedly exacerbated the problem (Williams *et al*., 2023; Deng *et al.*, 2021). Currently, CTB treatment relies on oral or intravenous treatment regimens similar to first-line TB treatment guidelines comprising a minimum treatment duration of six months and may include potential skin graft procedures (Brito *et al*., 2022; Garcia *et al*., 2021; van Zyl *et al*., 2015). As a result of the

complicated and lengthy treatment regimen, there are significant problems due to a lack of patient compliance. As only limited last-line anti-tubercular agents are available to treat MDR-TB and extensively drug resistant TB (XDR-TB) (Tiberi *et al*., 2018), it is imperative to ensure patient compliance and avoid the development of resistance to all first-line anti-tubercular agents. To alleviate both the antimicrobial and TB burden of South Africa, this study presents a potential solution through the development of a novel FDC SDEDDS comprising four anti-tubercular drugs destined for dermal application and topical drug delivery (supplementary route) to specifically target CTB and NTM skin infections.

1.3. Aims and objectives

1.3.1. Research aim

The aim of this research is to develop a topical SDEDDS to deliver the highly lipophilic clofazimine combined with the markedly less lipophilic pyrazinamide together with a third and fourth compound. First, a primary emulsion is generated by considering different internal oil phases or mixtures of internal oil phases for the purpose of selecting an internal oil phase that is immiscible with water and potential external oil phases. This immiscibility between phases favours formation of SDEDDSs. Hereafter, selected natural oils are considered as critical solubilisation components of the four drugs in order to decide which natural oil should be included as the external oil phase in the optimised SDEDDS formulation while providing attractive dermatological benefits (Lin *et al*., 2018). In terms of surface-active agents, Span®83, and Tween®60 are incorporated into the FDC SDEDDSs as the surfactant phase to decrease tension at the interface between the oil and water components to minimise the input energy required for self-emulsification, as well as to improve the thermodynamic stability of the optimised formulation.

1.3.2. Objectives

For the reasons mentioned above, the activities and objectives for this study include the:

- Conducting of compatibility studies of excipient combinations by means of isothermal microcalorimetry;
- Selection of lipophilic components to represent the internal and external oil phase, respectively, and of surface-active agents that provide the required hydrophilic-lipophilic balance (HLB) value of primary emulsions;
- Development of analytical method(s) (i.e., high performance liquid chromatographic method) capable of simultaneously determining the concentrations of the FDC, namely clofazimine, isoniazid, pyrazinamide, and rifampicin;
- Determination of the solubility of the different drug combinations in the selected oils;

- Formulation of primary emulsions that are subjected to emulsion stability index (ESI) studies and self-emulsification performance experiments;
- Establishment of the optimum region of self-double-emulsification for different excipient combinations by employing pseudo-ternary phase diagrams;
- Selection of a checkpoint formulation in a self-double emulsifying region suitable for dermal drug delivery through reflecting the influence of increased oil, water, and surfactant phase concentrations;
- Subjection of both the selected primary emulsion and optimised SDEDDS to droplet size and polydispersity index analyses;
- Examination via microscopic and transmission electron microscopic (TEM) observation of primary emulsions and SDEDDSs to confirm droplet formation and/or establishment of double spontaneous emulsification.

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2 Chapter 2

Review Article published in '*Antibiotics*'

Chapter 2 is a **review article** titled: **'*Adapting Clofazimine for Treatment of Cutaneous Tuberculosis by Using Self-Double-Emulsifying Drug Delivery Systems*'** and was published in '*Antibiotics*' (DOI: 10.3390/antibiotics11060806). This review publication was written to provide background to examiners on the relevancy of this study by discussing clofazimine as an adjuvant drug to tuberculosis regimens, comparing other lipid-based drug delivery systems to spontaneous emulsions and establishing the global challenge created by antimicrobial drug resistance in parallel to the ongoing tuberculosis pandemic. In order to fulfil the requirement of the North-West University a complete literature overview should be included. Hence, this review article is presented as the first literature overview of this study and no separate literature overview was thus included in this thesis as this review was seen as fulfilment of the above requirement.

antibiotics

MDPI

Review

Adapting Clofazimine for Treatment of Cutaneous Tuberculosis by Using Self-Double-Emulsifying Drug Delivery Systems

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Abstract: Although chemotherapeutic treatment regimens are currently available, and considerable effort has been lavished on the development of new drugs for the treatment of tuberculosis (TB), the disease remains deeply intractable and widespread. This is due not only to the nature of the life cycle and extraordinarily disseminated habitat of the causative pathogen, principally *Mycobacterium tuberculosis* (*Mtb*), in humans and the multi-drug resistance of *Mtb* to current drugs, but especially also to the difficulty of enabling universal treatment of individuals, immunocompromised or otherwise, in widely differing socio-economic environments. For the purpose of globally eliminating TB by 2035, the World Health Organization (WHO) introduced the "End-TB" initiative by employing interventions focusing on high impact, integrated and patient-centered approaches, such as individualized therapy. However, the extraordinary shortfall in stipulated aims, for example in actual treatment and in TB preventative treatments during the period 2018–2022, latterly and greatly exacerbated by the COVID-19 pandemic, means that even greater pressure is now placed on enhancing our scientific understanding of the disease, repurposing or repositioning old drugs and developing new drugs as well as evolving innovative treatment methods. In the specific context of multidrug resistant *Mtb*, it is furthermore noted that the incidence of extra-pulmonary TB (EPTB) has significantly increased. This review focusses on the potential of utilizing self-double-emulsifying drug delivery systems (SDEDDSs) as topical drug delivery systems for the dermal route of administration to aid in treatment of cutaneous TB (CTB) and other mycobacterial infections as a prelude to evaluating related systems for more effective treatment of CTB and other mycobacterial infections at large. As a starting point, we consider here the possibility of adapting the highly lipophilic riminophenazine clofazimine, with its potential for treatment of multi-drug resistant TB, for this purpose. Additionally, recently reported synergism achieved by adding clofazimine to first-line TB regimens signifies the need to consider clofazimine. Thus, the biological effects and pharmacology of clofazimine are reviewed. The potential of plant-based oils acting as emulsifiers, skin penetration enhancers as well as these materials behaving as anti-microbial components for transporting the incorporated drug are also discussed.

Keywords: tuberculosis; multi-drug resistant TB; clofazimine; cutaneous tuberculosis; drug delivery; permeation enhancers; self-double-emulsifying drug delivery system; topical administration

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1. Introduction

Control of the global tuberculosis (TB) burden has become a considerable concern in both developing as well as developed countries [1,2]. The countries with the highest TB incidence are India, Indonesia, Nigeria, the Philippines, Pakistan and South Africa [3]. It has been recently noted that there has been an increase in TB cases in Germany and Switzerland [4–6], a phenomenon that may be linked to the flow of African refugees to Europe [4,6–8]. In general, however, there has been an overall decrease of approximately 10% per annum in TB incidence in Europe since 2010. Nonetheless, it does appear that as a

proportion of all individuals who have TB, Europe has the highest percentage of infections due to drug-resistant TB [9].

However, the drive to eliminate TB is not only limited to the aforementioned countries [3]. A recent publication describes the daunting challenge of maintaining control of TB in Australia [10]. Statistics indicate that 90% of TB cases reported in Australia during 2013 were of infected individuals that originated from other countries [11]. This occurrence is linked to the high mobility of modern populations, in this case, disease carriers coming in from neighboring countries such as Papua New Guinea [12]. On the other hand, the United States (US) experienced 22 years of declining TB incidence until 2015 when the number of TB cases increased for the first time since 1983. The incidence of multi-drug resistant TB (MDR-TB) in the US is described as stable, but special attention has to be paid to the vulnerability of infected children as MDR-TB affects both adults and minors alike [13].

Largely through difficulties in diagnoses and a generally active physical demeanor, TB in children is frequently misdiagnosed or overlooked. There is nonetheless a vast number of children infected with TB and MDR-TB, and more concerning is the fact that this number is increasing [14]. In TB endemic areas, it furthermore appears to be one of the ten main causes of death in children younger than 5 years of age [15]. Additionally, TB deaths in children are often misdiagnosed as being due to meningitis, pneumonia, malnutrition and/or HIV/Acquired Immunodeficiency Syndrome (AIDS). If these aspects are taken into consideration, deaths due to TB in children are considerably higher than as based on the initial correct diagnoses [14,16]. In addition, accurate estimates of the true MDR-TB burden among children are unavailable as TB displays a paucibacillary nature in children, which complicates confirmation of the disease [13]. Therefore, this disease is now receiving much closer attention in both developing and developed countries; this is mandatory for acquiring control over, and eventual elimination of TB [17,18].

For these reasons, it is recommended that the focus of TB treatment must shift to the global antimicrobial resistance crisis as less than 50% of patients treated for drug-resistant TB infections are efficiently cured. It is furthermore advocated that individualized therapy should be implemented [9,19]. Currently, poor adherence to anti-tubercular treatment regimens is attributed to prolonged treatment schedules and adverse reactions accompanied by extended administration [3]. In turn, poor adherence to first-line anti-tubercular regimens leads to the development of resistant TB isolates [3]. Therefore, the potential of including existing anti-tubercular agents, such as clofazimine, to first-line regimens might shorten treatment time and improve patient adherence [3].

The World Health Organization (WHO) implemented the "End-TB" campaign in September 2018 that had as its central aim the elimination of TB by the year 2035. To achieve this objective, three pillars of the WHO End TB Strategy were developed, where "intensified research and innovation" was included as one of the pillars. The two key components of this pillar are "discovery, development and rapid uptake of new tools, interventions and strategies" and "research to optimize implementation and impact, and promote innovations". Moreover, at the first ever United Nations high level meeting on TB held in 2018, the commitment was made to increase research and innovation, with the development of new tools for TB treatment [20–25]. Therein, emphasis has now been placed on finding unique drug delivery systems, while utilizing "familiar drugs", in addition to using more traditional approaches of discovering new and active compounds.

However, a drastic shortfall in aims of the End-TB Strategy has lately and starkly become evident, as is described in the WHO 2021 TB report [26]. The report clearly states that the global progress of the End-TB Strategy has been reversed to a 18% decline in newly diagnosed TB cases [26], thus representing a stark regression back to the situation as was noted in 2012 [26]. This drastic decline is attributed to the COVID-19 pandemic as the lock-down response greatly revoked the provision of essential TB services [26]. Therefore, it is clear that research into TB as outlined in the 2018 United Nations report must now be greatly intensified in order to now fulfil the aims of the WHO End-TB strategy. Therefore, this review aims to discuss the potential of adapting current anti-tubercular drug delivery

by presenting clofazimine in Self-Double-Emulsifying drug delivery systems (SDEDDSs). Dermal drug delivery is suggested for the purpose of limiting adverse reactions associated with clofazimine while improving patient compliance as an additional therapy to provide topical treatment options for cutaneous TB (CTB). The possibility of plant-based oils acting as emulsifiers, skin penetration enhancers as well as these materials behaving as antimicrobial components for transporting the included drug(s) are also considered.

2. Anti-Tubercular Drug Resistance

TB is one of the principal epidemics that cause global morbidity and mortality. It is a fatal airborne infection, which is generated by *Mycobacterium tuberculosis (Mtb)* [26]. *Mtb* is categorized as an acid-fast bacillus with a slow growing rate [1]. Furthermore, this organism is able to survive in a quiescent state whilst within the human body without eliciting any TB symptoms [27]. This inactive TB state is referred to as latent TB infection (LTBI) [14,27]. Current research indicates that at least one in four people worldwide have latent TB. In almost 10% of the population infected with latent TB, this will develop into active TB. However, numerous individuals, including children, who may be living with TB patients in poor, overcrowded conditions, as well as immune-compromised patients, are especially vulnerable to LTBI. Subsequently, upon development of an immunocompromised situation such as through infection with HIV, development of diabetes mellitus, through an organ transplant, by becoming pregnant, or living in overcrowded and unhygienic environments coupled with poor nutritional intake, the inactive TB state may transform into an active TB infection. Persons, for example, infected with HIV, are 20 to 30 times more prone to contract active TB from LTBI [14,28–31].

In short, active TB is currently systemically treated and consists of two phases. The first being termed the intensive phase, which is about 8 weeks long. During this stage, first-line anti-tubercular agents namely, isoniazid, rifampicin, ethambutol and pyrazinamide, are concurrently administered. Following, once patients are no longer deemed infectious, they still require more treatment to successfully eliminate the infection, therefore, a maintenance phase is employed that entails concurrent administration of isoniazid and rifampicin. This stage is continued for at least 16–28 weeks, depending on the HIV status of a patient. It can even last up to 12 months. Importantly, patients need to adhere strictly to this treatment regimen to avoid drug resistance [32–34].

What is more concerning however is that the WHO indicated that 458,000 of the 10 million active TB cases reported in 2017 were triggered by *Mtb* with specific resistance towards rifampicin and isoniazid, which are two of the four first-line drugs normally used against active TB [25,26,35]. MDR-TB is defined as resistance to at least two of the front line anti-tubercular agents and is more prevalent in patients not adhering to anti-TB therapy. Multi-drug resistance is furthermore predictive of a poor outcome since these drugs are the key constituents of anti-TB treatment [25,35–40]. Estimates from the WHO 2021 TB report indicated a decline in the number or patients that were enrolled for treatment of MDR-TB from 2018 to 2020. However, this is by no means a good report as it only indicated that fewer patients registered for MDR-TB treatment and adhered to the treatment guidelines. This was probably due to the global COVID-19 pandemic lock-down responses, which in turn, significantly prevented essential TB services from being performed [24]. Thus, this extremely infectious disease is rendered even more complex due to the increasing occurrence of antimicrobial drug resistance.

Nevertheless, how does drug resistance occur given that TB is both preventable and treatable? As stated, TB drug resistance principally means that *Mtb* is resistant to first line anti-tubercular agents including isoniazid, rifampicin, pyrazinamide, streptomycin and ethambutol [36,41]. In order for a TB regimen to be effective, the simultaneous administration of various bactericidal and sterilizing anti-tubercular agents must be maintained for a sufficient period of time [1,42] in order to guarantee anti-microbial effectiveness together with the prevention of *Mtb* mutations that might lead to drug resistance [40]. Resistance

generally emerges because of inadequate TB management when anti-tubercular drugs are misused or mismanaged. Examples include:

- patient noncompliance (failure to complete the correct full course of treatment),
- reinfection with *Mtb* post treatment,
- the inappropriate utilization of medicines such as when health-care providers prescribe the incorrect treatment regime (incorrect employment of antibiotics),
- prescribing an incorrect dosage, or length of time for treatment (incorrect treatment regime),
- an inconsistent treatment (drug availability),
- restricted access to treatment, or
- when the requisite drugs to be used are of poor quality [31,43,44].

It has even been found that clinical and genetic risk factors may also encourage the development of MDR-TB in non-HIV infected patients [45].

Even so, it has been established that the risk of development of drug-resistant TB is higher among HIV-infected individuals compared to HIV-seronegative patients. A study conducted in 1993–1994 in the USA found that HIV-infected patients with TB who had not previously been treated for TB were already infected by bacterial isolates with an 11.3% incidence of isoniazid resistance and an 8.9% incidence frequency of rifampicin resistance. These numbers were almost double those recorded from an HIV-negative population [46]. Although the mechanisms involved in the development of rifampicin resistance are not yet clearly understood, the poor absorption of rifampicin and ethambutol in HIV-infected patients may be associated with acquired drug resistance leading to treatment failure [45]. As portrayed in Figure 1, *Mtb* is combated with multiple anti-tubercular agents attacking this deadly infection with different mechanisms of action.

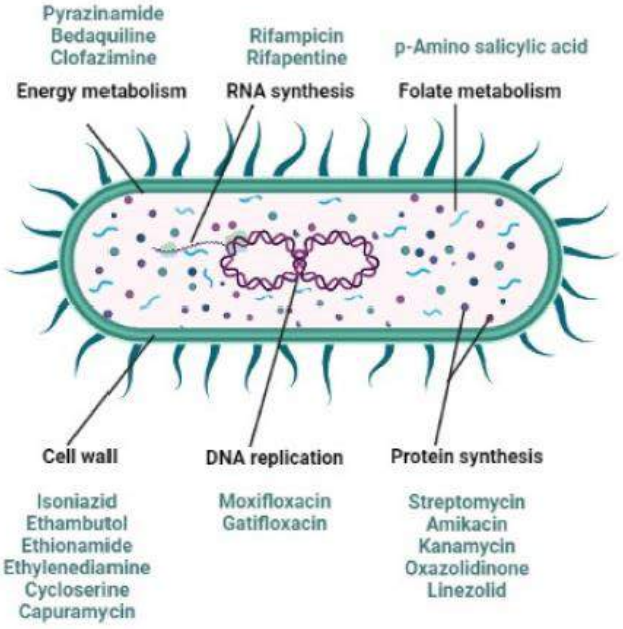


Figure 1. Mechanism of action of numerous anti-tubercular drugs (adapted from [47]).

MDR-TB is generally treated with fluoroquinolones (moxifloxacin and ofloxacin) and injectable agents (amikacin, capreomycin, kanamycin or streptomycin), normally employed as last line anti-tubercular agents, in combination with p-aminosalicylic acid, cycloserine or ethionamide for at least 18 months [37,44]. Table 1 indicates the latest classification system provided by the WHO to initiate a prolonged, but effective MDR-TB treatment

regimen. Clearly, this represents a complex treatment regimen that is costly and significantly extended, which furthermore tends to enhance lack of compliance. Moreover, in 8.5% of all patients that contracted MDR-TB, *Mtb* is also found to be resistant to at least one fluoroquinolone and/or one of the second-line injectable aminoglycosides. Resistance to both fluoroquinolones and aminoglycosides is described as extensively drug resistant TB (XDR-TB), whereas pre-XDR refers to resistance either to fluoroquinolones or aminoglycosides, respectively [25,35,40,48–50].

Table 1. Recent MDR-TB drug classification system for promoting an extensive MDR-TB treatment regimen as established by the WHO, adapted from [51].

Priority Drug Groups	TB Drug
Group A: Incorporate all three drugs as part of regimen	Levofloxacin OR Moxifloxacin Bedaquiline Linezolid
Group B: Add one or both drugs to regimen	Clofazimine Cycloserine OR Terizidone
Group C: Add to complete regimen and include when drugs from Group A and B cannot be included as influenced by resistance, toxicity, or tolerability	Ethambutol Delamanid Pyrazinamide Imipenem–cilastatin OR Meropenem AND Amoxicillin/Clavulanate Amikacin OR Streptomycin Ethionamide OR Prothionamide Aminosalicylic acid

The emergence and transmission of these types of TB are again partly driven by the association between TB and HIV (TB/HIV coinfection). Evidence shows that populations infected with HIV are more prone to contract XDR-TB, which is fundamentally untreatable with normal TB treatment regimens. XDR-TB is a critical health hazard, predominantly in communities with a high incidence of HIV [44]. According to the 2018 WHO global TB statement, treatment is only effective in 55% of individuals that contracted MDR-TB, and is only effective in 34% of XDR-TB patients [25,35]. The presence of MDR-TB and XDR-TB furthermore enhance the occurrence of extra-pulmonary TB infections [52]. Therefore, treatment is even more complicated as 5% of all new TB cases are considered multi-drug resistant, with the global estimate being that 3.3% of first time TB cases and 20.5% of previously treated TB patients display MDR-TB [32,38].

3. Cutaneous Tuberculosis, an Extra-Pulmonary Infection

TB presents normally as a pulmonary infection. However, approximately 20% of TB cases are extrapulmonary (EPTB) and this number is rising; consequently, it cannot be ignored any longer. EPTB includes ocular TB, hepatobiliary and splenic TB, TB spondylodiscitis, pleural TB, TB encephalitis, breast TB, TB pericarditis, TB lymphadenitis, gastrointestinal and peritoneal TB, genitourinary TB, osteoarticular TB, TB meningitis and cutaneous TB (CTB).

The incidence of EPTB increases with associated HIV infection, an increase in MDR-TB and immunosuppressive treatment, specifically utilizing inhibitors of tumor necrosis factor-alpha TNFα [1,32,33,53–62].

CTB is a relatively uncommon disease where merely 1–2% of all EPTB infections involve cutaneous association and of these cases only about 0.1–1% of all cutaneous disorders can be related to CTB [32,54,59,63]. CTB was first described in 1826 by Théophile Laennec, inventor of the stethoscope, who reported a lesion on his hand that was evidently triggered through mycobacterial penetration through his skin. The causative organism was however only identified in 1882 when Robert Koch first identified the bacterium *Mtb* as the TB pathogen [32,33,54,55,59,63–65].

Skin manifestations due to EPTB are normally caused by *Mtb* and are known as true CTB. However, other atypical *Mycobacterium* species may also account for cutaneous manifestations. These Mycobacteria can be divided into two sub-categories, namely: slow growers (*M. haemophilum*, *M. leprae*, *M. marinum*, *M. ulcerans*, and less regularly the *M. avium* complex, *M. kansasii*, *M. malmoense*, *M. scrofulaceum*) and rapid/fast growers (*M. chelonae*, *M. fortuitum*, and infrequently *M. abscessus*). Rapidly growing organisms have a 7 day or less incubation period, whereas slow growers have a longer incubation time [32,66–71]. *M. bovis* and the Bacille Calmette-Guérin vaccine, an attenuated strain of *M. bovis*, have moreover been reported to cause CTB [70]. Infections by means of atypical mycobacteria appear primarily in immunocompromised populations and there is a tendency for dissemination, whereas CTB infection in immunocompetent hosts follows skin penetration. This type of CTB is normally described as localized [55].

True CTB is more prevalent among women and young adults. The most frequently affected area of the skin is the face, although it commonly appears on the neck and torso as well [71]. Factors contributing to the diverse manifestations of the different CTB sub-categories include age and sex. For example, scrofuloderma is more common amongst pediatric patients, whereas erythema induratum of Bazin presents more frequently in females, and TB verrucosa cutis is predominantly observed in males [72–74]. The incidence of divergent CTB manifestation is displayed in Figure 2.

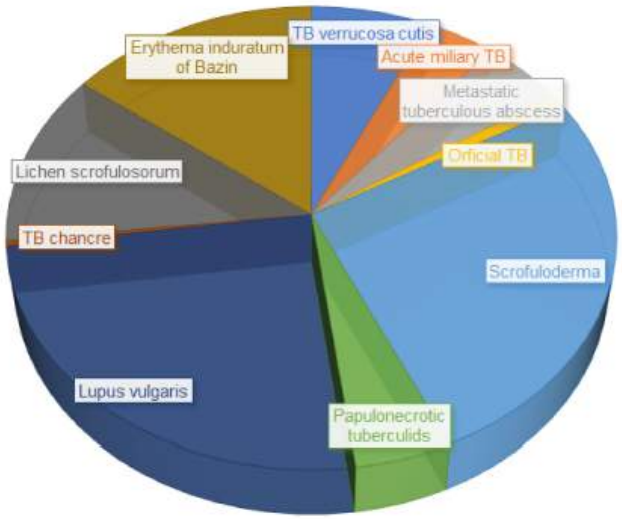


Figure 2. Incidence of different CTB manifestations (adapted from [75]).

Individuals exposed to malnutrition or unhygienic conditions, or suffering from diabetes mellitus, end-stage renal disease, malignancies, HIV co-infection, or through submission to immunosuppressive therapy, and infants and pregnant women, amongst others, are at risk of contracting active CTB [26,76,77]. Overall, a diverse collection of individuals is vulnerable, especially due to the global increase in MDR-TB and XDR-TB [78].

Currently three criteria are used to classify CTB, namely, pathogenesis of the causative organism, clinical presentation, and histology. CTB is therefore generally categorized as either an endogenous or exogenous propagation, considering the bacterial basis and the infection means, as well as a multibacillary or paucibacillary configuration, depending on the bacterial load in the lesion. The exogenous mechanism refers to direct inoculation into the skin, whereas the endogenous mechanism is secondary to a previous infection where transmission arises by means of contiguous, lymphatic or hematogenous dissemination [79–83]. This variety of clinical CTB manifestations severely complicates diagnoses. Moreover, CTB is an elusive dermal condition as it resembles a number of visually similar dermal disorders [32,63,76,79]. In addition, the microbial basis of CTB infection (Figure 3) may be

difficult to define, even though improved detection techniques including culture smears, polymerase chain reaction examinations and enzyme-linked-immunosorbent serologic assays are available [33,34,63,79,83,84].

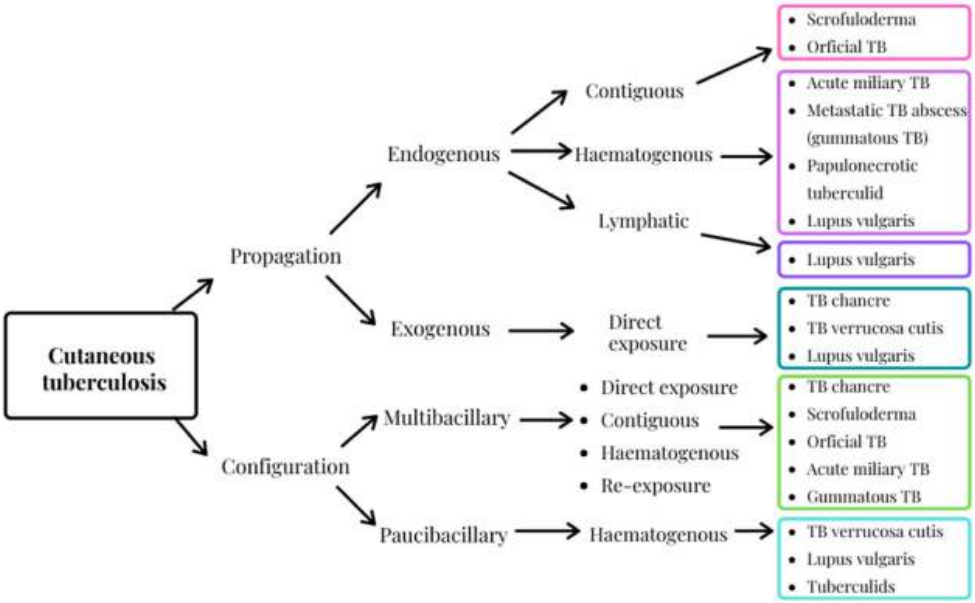


Figure 3. Classification system of CTB (adapted from [71,83]).

Currently, treatment options for CTB remain the same as for general systemic TB due to the involvement of systemic infections in most cases. Furthermore, surgical interventions involving excision of lesions and correction of deformities in certain indications are sometimes necessary [32,55,85]. CTB treatment also comprises two phases. The first phase is the intensive phase and lasts approximately 8 weeks. This phase consists of simultaneous administration of first line anti-tubercular agents. Following this regimen, patients are no longer considered infectious, but do require more treatment in order to eradicate the infection. Subsequently, a maintenance phase is implemented consisting of simultaneous administration of isoniazid and rifampicin. This phase is sustained for a period of at least 16 weeks for HIV negative patients and 28 weeks for HIV positive patients, but nonetheless may even last for 9–12 months. For the treatment to be successful, patients need to adhere stringently to this treatment regimen [32–34]. However, due to these long-term treatment phases, patient compliance may be problematic, and in order not only to improve patient compliance, but also enhance positive treatment outcomes, alternative solutions are required. Firstly, anti-microbial agents other than conventional TB drugs must be explored in order to shorten the treatments required to cure MDR-TB and XDR-TB, and to minimize the adverse effects that are normally associated with first-line anti-tubercular compounds. Likewise, novel therapeutic combinations of existing antibiotics and first line TB agents may be more beneficial as recommended by the WHO and United Nations [2,19–23].

Recently, interest in so-called "orphan drugs", i.e., drugs intended for rare diseases that for example affect less than 200,000 people per annum in the USA, has increased despite the fact that these drugs are considered orphans both therapeutically and commercially due to the minor patient population in need of these compounds [80]. Interestingly, orphan drugs are attracting attention from researchers aiming to treat dermal conditions since many side effects can be circumvented by utilizing dermal applications [80].

Clofazimine is one such compound that notably displayed a significant efficacy against resistant TB strains in the 'Bangladesh regimen' described below [2,25,81]. Individualized anti-tubercular therapy is furthermore increasingly recommended, as it is seen as the most

effective and safe manner of targeting TB resistance in individual patients without inducing further resistance [82,83]. In order to turn the tide against TB and CTB, reintroduction of agents such as clofazimine should not be disregarded as these can assist in both individualized therapy where drugs are only employed if effective against the infection as indicated by predictive tests, as well as contributing to shortening of the treatment phases [2,48,84].

4. Clofazimine, an Orphan Drug

Clofazimine, initially known as "B663", was originally synthesized in 1954 by Vincent Barry and co-workers to treat TB [85]. It is a highly lipophilic riminophenazine dye, which is significantly active against *Mtb*. However, despite high bactericidal and sterilizing activity in vitro as well as in a mouse model of MDR-TB, and its good oral absorption in micronized form along with its deposition and persistence in adipose tissue and in cells of the reticuloendothelial system, this compound did not initially progress as an anti-tubercular agent as it was found to be ineffective against TB in humans [14,86–91]. Nonetheless, it was concluded that due to the accumulation of clofazimine inside macrophages, it should be effective against intracellular diseases [92]. Efficacy against leprosy was noted at the end of 1959, and after clinical trials during the 1960s in Nigeria and elsewhere, the Swiss pharmaceutical company Novartis marketed clofazimine in 1969 under the brand name Lamprene® as an anti-leprosy agent [25,93,94].

In 1982, a WHO Study Group recommended utilizing clofazimine as a constituent of a triple drug combination with dapsone and rifampicin for treatment of multibacillary leprosy [88,94]. However, Novartis was only granted FDA approval of clofazimine at the end of 1986 and its use as anti-tubercular agent lapsed for several decades [93,95,96]. Moreover, clofazimine was classified as a Group 5 drug by the WHO, indicating that it is a drug with unclear efficacy that is not recommended for routine use [97].

Later, interest in clofazimine for the treatment of TB was awakened following its inclusion in the so-called "Bangladesh regimen" where it was established that approximately 90% of patients that contracted MDR-TB were healed within a 9–11 month period [2,81,98]. This was a substantial improvement over the standard WHO-proposed 24 months regimen that cured less than 50% of MDR-TB patients. Results from a larger clinical trial study in Bangladesh and from other clinical trials in Cameroon and Niger confirmed these conclusions [89,99–101]. The Bangladesh regimen according to the WHO 2016 guidelines comprises an initial treatment phase consisting of 4–6 months' treatment with kanamycin, moxifloxacin, prothionamide, clofazimine, pyrazinamide, isoniazid (in high dosages) and ethambutol. This initial phase is followed by a 5 months treatment period with moxifloxacin, clofazimine, pyrazinamide and ethambutol [2,100]. The specific bactericidal and treatment-shortening action of clofazimine was furthermore noted in MDR-TB mouse models, and it was likewise apparent in a controlled clinical trial performed in China [102–104].

In contrast, the randomized controlled STREAM trial indicated non-inferiority of the short-course-containing regimen, but not superiority [105]. For these reasons, the full potential of clofazimine as an anti-tubercular agent has not yet been established. Nonetheless, clofazimine is listed in the WHO Model List of Essential Medicines as a leprostatic-, anti-tubercular- and anti-inflammatory agent [106]. It is also categorized by the WHO as a Group C agent, that is, other core second line anti-tubercular agents [48]. Therefore, clofazimine may have the capacity to significantly advance the treatment of MDR-TB and CTB.

Oral absorption of clofazimine is variable, fluctuating from 45–62% when taken on an empty stomach; however, absorption is increased when ingested with food. This drug is metabolized by glucuronidation in the liver, incompletely expelled by the biliary route, and only negligibly excreted in urine, sputum, sebum, and sweat. Clofazimine has been reported to cross the placenta and is excreted in breast milk [90,95,107,108]. Second line anti-tubercular drugs are known to be more toxic [109] and clofazimine is no exception as the highly lipophilic nature of this drug leads to accumulation in macrophages [92]. However, it must be noted that human macrophages are the primary host cells for *Mtb*, and

thus, such a property will be of decisive clinical advantage when using clofazimine [92]. Moreover, accumulation of clofazimine in adipose cells such as in the heart, liver, breasts, adrenal glands, pancreas, spleen, bone marrow and lamina propria of the jejunum, extends the half-life to 65–70 days [88–90,95,107,108,110–114]. However, such accumulation leads to adverse reactions including reversible skin, hair and corneal dyschromia to brownish-black, dark red, or even to orange pink. Approximately 46% of patients receiving clofazimine treatment display conjunctival deposition, whereas nearly 53% experience crystal deposition in the cornea and 32% of patients present with clofazimine crystals in their tears [114,115]. Deposition of a dark-brown pigmentation is furthermore sometimes visible on the fingernails. Discoloration is more evident in fair-skinned individuals exposed to sunlight and even though it is reversible, it may take years to be completely resolved. Breast milk, sweat, urine, and feces may also discolor. In addition, dry skin and ichthyosis, defined as an increase in skin thickness due to lack of shedding of dead skin cells, often transpires. Severe gastrointestinal reactions (nausea, vomiting, abdominal pain, diarrhea) are rather common (reported in >10% of patients) and deemed most concerning, as accumulation, as well as precipitation of clofazimine in the wall of the small intestine, ensue after sustained administration [88,95,102,107,108,110,111,116,117]. In 1–10% of patients, weight loss, pruritus, diminished visual perception, and/or eye irritation/dryness occur. Less commonly, more severe adverse effects may be observed, including enteropathy possibly complicated by intestinal obstruction, gastrointestinal bleeding, and splenic infarction [107,108,118,119].

Normally, side effects accompanied by clofazimine treatment are dependent on the dose administered and the duration of administration [87]. Clofazimine up to 300 mg daily is often well tolerated; however, 100 mg per day is normally recommended due to gastrointestinal intolerance. Higher dosage regimens given over longer periods, i.e., beyond 4 months, may lead to crystal enteropathy and malabsorption, which is accompanied by effusive vague and/or severe abdominal distress. Coincidentally, discontinuation of treatment normally leads to a reasonably quick clearing of symptoms. Granting the fact that gastrointestinal side effects are not infrequent and have been observed in roughly one third of patients treated with clofazimine, the severity is usually not substantial enough to evoke termination of the treatment [14,95,102,107,108,113,118–120].

Fortunately, clofazimine is predominantly well-tolerated in children as observed from pediatric leprosy treatment [14]. It is classified as Pregnancy Category C, though little research has been conducted regarding its use to treat MDR-TB during pregnancy and limited data exist for later life teratogenicity. From data obtained from case reports, it was found that only 5 MDR-TB pregnant patients received clofazimine as part of the treatment regimen. All infants born were considered normal. Moreover, no significant adverse reactions due to clofazimine as part of the leprosy treatment regimen during pregnancy have been reported [88,107,108,121,122]. Clofazimine is furthermore safe to co-administer with antiretroviral therapy [123] and no known significant drug-drug interactions have been reported [88,121]. Thus, overall, insight into the physicochemical characteristics of clofazimine enables the design of novel drug delivery systems, and it should be possible to circumvent the most serious adverse effects of severe gastrointestinal reactions and accumulation of clofazimine in the gastro-intestinal tract. Clearly, the topical administration route will be advantageous as gastrointestinal accumulation can be bypassed and patient compliance not compromised [124]. Thus, an alternative route of administration together with a unique clofazimine dosage form will fortify this orphan drug with a more user-friendly profile.

5. An Alternative Strategy to Deliver Clofazimine More Effectively

5.1. Contemplating Topical Delivery

As stated, oral delivery of clofazimine is not only erratic, but also evokes numerous systemic adverse effects. On the other hand, topical drug delivery represents "the application of a drug onto the surface of the body to a localized area, such as the nose, eyes,

rectum, vagina or skin, in order to establish relief of a condition restricted at the area of application, without creating any systemic effect" [125,126].

Over the past three decades, the trend to deliver drugs via the skin has been markedly increasing. It is becoming a considerably more popular alternative administration route, especially when aiming to avoid complications that accompany oral drug delivery [124]. Through implementation of this route to treat various topical ailments, patient compliance is increased, the risk of drug and food interactions is decreased, and hepatic metabolism is circumvented amongst others [127,128], thus rendering the dermal application route conspicuously beneficial [129]. However, the skin is designed to fulfil a barrier function, and subsequently permeation of various drugs, in concentrations that are of clinical relevance, is limited [130,131]. The barrier function of the skin is linked to the lipophilic nature of the outermost layer of the skin, namely the stratum corneum (SC) [131]. Therefore, the main challenge when formulating a topical drug delivery system is to attain an optimum concentration of the encapsulated drug at the site of action for a sufficient period of time [125,132].

The skin can be described as a multi-layered organ [133]. Each layer fulfils its own role in protecting the body, maintaining hydration and allowing sufficient blood perfusion to the skin [134–136]. The skin consists of three layers, namely the epidermis, dermis and hypodermis [137]. Epidermis thickness varies according to the cell layer thickness at the different skin regions of the body [135]. This layer is divided into two distinct fragments, the SC and viable epidermis [134]. The SC forms the outermost layer of the epidermis and is the lipophilic, protective layer of the skin that complicates drug delivery of a vast range of substances [136]. This protecting function of the SC is enforced by its lipophilic nature [134]. The SC consists of lifeless, anucleated cells comprising corneocytes linked by corneodesmosomes surrounded by a lipid matrix containing a mixture of cholesterol, triglycerides, free fatty acids and ceramides [134]. The second skin layer, the dermis is the most biochemically active layer of the skin [134]. It is responsible for body temperature regulation, cutaneous sensation, skin elasticity, blood circulation in addition to skin nutrition, and the removal of toxic substances [134,135]. Below the dermis is the third skin layer known as the hypodermis [137]. Subcutaneous fat is encased within the hypodermis [135,138]. Thereby, it acts as a supportive membrane for both the dermis and epidermis [135,139]. In order for a drug to be delivered through the skin and into the systemic circulation, it must be able to move through the lipophilic SC and the underlying hydrophilic viable epidermis and dermis, respectively. Consequently, a drug must have both hydrophilic and lipophilic properties in order to facilitate successful transdermal drug delivery [134,136].

There are three pathways that enable drug diffusion through the skin to occur [138]. First, a drug enters the skin through the intracellular or intercellular pathway [137]. Highly hydrated keratins are part of the corneocytes that are the main building blocks of the epidermis, where some components are able to traverse these cells. Moreover, hydrophilic drugs predominantly pass through the skin via the intracellular pathway [134]. Secondly, intercellular permeation may occur via diffusion of a compound through the lipid matrix of the skin as mostly observed for lipophilic drugs [134,137]. The third route, the transappendageal pathway, is restricted as a mere 0.1% of the skin surface is occupied by hair follicles and sweat glands that present an opportunity for modest transappendageal permeation of drugs [136,137]. Here, an additional limiting step for permeation of hydrophilic composites is the lipid-rich nature of the sebum that fills the sebaceous glands [140]. On the other hand, however, delivery of lipophilic drugs may be enhanced through this route [134]. Sweat, on the other hand, provides a pathway for the delivery of hydrophilic drugs via the transappendageal pathway [137], although drug delivery in the presence of sweat is limited as diffusion must occur against the diffusion gradient [140]. Hence, the physicochemical properties of a drug are highly important for the development of a drug delivery system that may enable topical and/or transdermal drug delivery [141]. The skin anatomy and dermal drug penetration pathways are displayed in Figure 4.

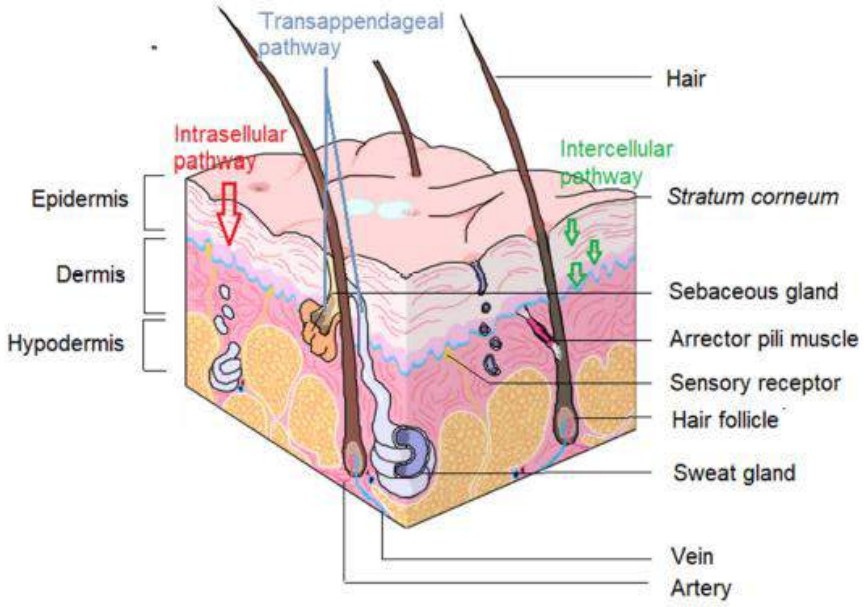
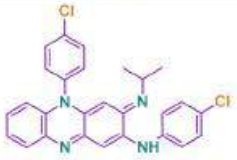


Figure 4. Dermal drug penetration pathways (adapted from [134]).

5.2. *Clofazimine Characteristics Challenging the Topical Route*

The physicochemical characteristics of clofazimine are presented in Table 2. Molecules with molecular weights less than 500 Da, such as clofazimine, with a molecular weight of 473.40 Da [142], are capable of relatively facile diffusion through the skin [141,143]. As skin is a non-homogenous medium, smaller particles will display increased skin permeation [144]. Clofazimine furthermore exhibits an exceptionally long half-life, as noted [94], which is beneficial since an extended half-life may require less frequent dosing. In turn, this characteristic might even reduce the adverse skin discoloration normally associated with the oral absorption of clofazimine [145].

Table 2. Physicochemical Characteristics of Clofazimine [a].

Physicochemical Characteristics	
Chemical formula	$C_{27}H_{22}Cl_2N_4$
Molecular weight	473.40 D_a
Chemical structure	
Melting point	210–212 °C
Appearance	Reddish-brown, fine powder
Log P(Octanol-water partition coefficient)	7.66
UV Detection wavelength	254 nm
Elimination half life	70 days
pK_a value	8.51
Solubility	Soluble in methylene chloride, very slightly soluble in ethanol, aqueous solubility < 0.001 mg/L

[a] from [92,142,146].

Generally, the rate of diffusion through the skin will only occur at a rapid transfer rate if the concentration of clofazimine present on the skin surface is saturated [145]. It has been established that supersaturation on the skin can increase skin penetration and permeation of lipophilic drugs [147]. The diffusion and partition parameters assumed for lipophilic compounds indicate that supersaturation relates to increased thermodynamic activity without apparent effect on the barrier function of the skin. Hence, the clofazimine concentration incorporated into formulations, as well as its solubility in these formulations will significantly contribute toward improving the transfer rate through the skin [146]. Elsewhere, it has been established that the melting point of a compound may be used for evaluation of compound purity, as well as being used as a predictor of compound solubility [148]. In general, a melting point of less than 200 °C is recommended to ensure adequate solubility. Clofazimine has a slightly higher melting point (210–212 °C) than recommended, and although this difference is only marginal, drug solubility might possibly present some challenges [94,141]. The solubility parameter of a drug destined for dermal delivery should be similar to that of the skin (approximately 10 cal/cm^3) [149]. An aqueous solubility of more than 1 mg/mL is deemed most favorable for a drug that needs to diffuse through the skin [94].

Clofazimine possesses a Log P value of 7.66, reflecting its highly lipophilic nature [150,151]. The Log P value is the n-octanol-water partition coefficient, which will reflect the partitioning of a drug between the lipophilic SC and the hydrophilic viable epidermis and dermis [152]. Thus, the high Log P value of clofazimine negatively affects skin permeation as the ideal Log P range, identified for topically delivered drugs, is approximately between 1 and 3 [142]. Overall, the high lipophilicity and very low aqueous solubility add to the challenges of formulating clofazimine into a topical/transdermal drug delivery system [95].

Another property to consider is the pH of the skin where the SC is able to tolerate a pH range from 5–9 [141]. For this reason, the pH of a dermal formulation is an important factor that must be considered in order to avoid skin irritation [153]. Moreover, the pH of a formulation will determine the fraction of unionized molecules available at the skin surface [154]. Drug molecules presented at the skin surface in an ionic form will cross the SC via the transappendageal route [155]. Conversely, unionized drug molecules will enter the skin via the intercellular (lipid) route [156]. The acidic or basic properties of a drug can determine which physicochemical properties are more important during topical or transdermal drug delivery [157]. For weakly acidic drugs, the order of importance is molecular weight > Log P > solubility; whereas the order of importance for weakly basic drugs, such as clofazimine is molecular weight > solubility > Log P.

Overall, although the effective topical delivery of highly hydrophobic compounds for example clofazimine faces various challenges, these may be overcome by utilizing excipients as well as unconventional drug delivery systems. For example, natural oils initiate reversible fluidization and rearrangement of the lipid matrix within the SC that facilitates penetration enhancement of drugs. Thus, the inclusion of natural oils may assist topical drug delivery [158]. In addition, natural oils are frequently employed during the formulation of dermal drug delivery systems as stabilizers, solubility enhancers and emulsifiers. Furthermore, the application of natural oils onto the skin is considered safe and generally acceptable to the public [159,160].

5.3. Auxiliary Natural Excipients to Enhance Topical Clofazimine Delivery

As stated by the WHO [160], approximately 80% of communities located in developing countries deploy ethno therapy, including topical application of natural oils as a more affordable health care and cosmetic alternative where skin pathologies are problematic. Insights provided by current research indicate that the topical application of natural oils may be accompanied by mutually beneficial properties influenced by the composition of the individual natural oil together with its method of extraction from usually a plant, or less commonly, an animal source. These properties may include enhanced skin barrier

homeostasis, anti-oxidative activity, anti-inflammatory action, anti-microbial properties, promotion of wound healing, and possible anti-carcinogenic characteristics [161–166].

Unrefined plant-based oils, cold-pressed or cold extracted, are not exposed to heat or chemical processes during manufacturing, and thus the components remain unaltered [166–170]. Naturally occurring sediments, such as wax, in unrefined plant-based oils may improve dermal occlusive effects that inhibit skin dehydration whilst providing prolonged contact time with the skin to improve drug delivery. Unrefined natural oils retain the highest nutrient concentration together with unaltered fatty acids, and display a decreased tendency towards skin irritation [163,169,170]. On the other hand, cutaneous lipid disruption induced by fatty acids present within natural oils is required for skin penetration for delivery of the drug [167]. Thus, the extent of skin penetration may vary due to the unique composition of the plant oil selected in a topical formulation [158]. Further, the amount of drug which dissolves will be dependent on the nature of the oil, that is, different concentrations of dissolved drug are obtained for different oils, as has been observed for ibuprofen [171].

Free fatty acids are released when natural oils are metabolized within the skin [158]. They are of considerable importance for dermal drug delivery. The fatty acids present in plant-based oils disrupt the lipid structure of the SC enabling entry of the drug into the skin. The chemical structure, degree of unsaturation of the fatty acid and alkyl chain length influence the extent of skin penetration [172]. Interestingly, it has been found that unsaturated fatty acids have improved cutaneous penetration compared to saturated fatty acids with identical alkyl chain lengths [172]. This is attributed to improved capacity to disrupt the dermal lipid structure due to the more rigid chain structure of unsaturated fatty acids [172]. Not unexpectedly, combining different plant-based oils can provide a synergistic enhancement of dermal penetration [172].

Fatty acids can also contribute towards the barrier repair function of the skin. The best-known fatty acid for effecting skin penetration enhancement by means of disrupting the lipid structure of the SC is the essential mono-unsaturated fatty acid oleic acid. In contrast, the essential di-unsaturated fatty acid linoleic acid repairs the barrier function of the skin as it restores the lamellar phase of the lipid matrix within the SC [158,162,163]. In general, oleic and linoleic acids predominate in individual plant-based oils [163]. However, the optimum ratio of oleic acid to linoleic acid for favoring dermal drug delivery is still unknown [163].

Overall, designing and optimising dermal drug delivery systems for drugs as lipophilic as clofazimine present unique challenges. We next consider the potential of SDEDDSs compared to other lipid-based dermal drug delivery vehicles for topical delivery of clofazimine.

6. The Prospect of Self-Double-Emulsifying Drug Delivery Systems for Enhancing Topical Delivery of Clofazimine

6.1. Lipid-Based Carrier Systems Ideal for Lipophilic Drugs

It is estimated that approximately 30% of current commercial drugs including clofazimine and up to 50% of newly discovered drugs are highly hydrophobic [152,153]. Thus, lipid-based carrier systems are receiving special attention in the pharmaceutical industry in order to improve delivery of these drugs, especially those displaying poor aqueous solubility [173–176]. Encapsulation of the lipophilic drugs into inert lipid vehicles is shown to provide reproducible drug concentration profiles [173,177]. Lipid carrier systems include oils, surfactant dispersions, emulsions, nanoemulsions, liposomes, solid lipid carrier systems, nanostructured lipid carrier systems, self-emulsifying drug delivery systems (SEDDSs) and SDEDDSs [173,177–179]. Overall, lipid carrier systems can facilitate targeted drug delivery, thereby improving therapeutic effects and decreasing side effects, which will be important for anti-tubercular treatment [178].

6.2. Why SDEDDSs Are Considered More Appropriate Than Other Lipid-Based Carrier Systems

Liposomes, emulsions and nanoemulsions are frequently employed as drug delivery systems. These lipid-based formulations are also attracting increasing attention for en-

hancing topical and transdermal drug delivery [178–181]. Liposomes are spherical, bilayer structures of 100–200 nm in diameter. They are generally prepared from phospholipids and are able to encapsulate a wide range of diverse drugs, either in their aqueous central compartment or within the lipid bilayer. Their size, number of bilayers and various other components that may be included bestow their distinct characteristics. For example, inclusion of a surfactant results in the formation of transferosomes that are able to cross the SC. Inclusion of ethanol produces more flexible liposomes termed ethosomes. Liposomes were originally developed for parenteral administration of anticancer and antifungal agents. They are not as widely utilized for topical or transdermal drug delivery and are mainly included in cosmetics and long-acting sunscreen formulations, although their membrane structure is similar to that of natural membranes found in skin. Liposomes are expected to remain at the surface of the skin where they fuse with the skin lipids and slowly release the encapsulated drug [179,182–184].

Conversely, emulsions are heterogeneous, thermolabile biphasic liquids consisting of two or more immiscible fluids, which are made miscible through the addition of surfactants or emulsifying agents. They can be further categorized into micro- and nano-emulsions, as well as SEDDSs, depending on the formulation methods that are used. However, the instability of conventional emulsions causes difficulty in drug delivery [180,185]. Microemulsions technically are not emulsions, but are thermodynamically stable, single-phase systems that form spontaneously once the correct oil to water ratios are achieved. They possess a number of different microstructures depending on the nature and concentrations of the components. Alternatively, nanoemulsions are thermodynamically unstable dispersions that contain individual small droplets with a mean diameter of less than 200 nm. However, these dispersions possess reasonably good kinetic stability, as the droplets do not collide as frequently as in ordinary emulsions. The small droplet size furthermore allows for deep penetration into tissues as well as through fine capillaries. Positively charged (cationic) nanoemulsions increase skin permeation of poorly soluble drugs due to their interaction with the negatively charged skin epithelial cells. The main advantages of nanoemulsions include increased drug loading, enhanced drug solubility and bioavailability, reduced variability of drug uptake between different patients, controlled drug release, and protection from enzymatic degradation [178,180,182].

SEDDSs were originally developed from emulsions in order to generate a more physically stable formulation that is easier to prepare and can be used for oral drug delivery, as exemplified by their development specifically for oral administration of the immunosuppressant cyclosporine [178,185,186]. More recent activities focus on developing SEDDSs for other drug delivery routes of administration such as via the ocular-, vaginal- and sublingual routes [186–190]. Additionally, use of SEDDSs for treatment of dermal pathologies have been described [191,192]. SEDDSs are isotropic and thermodynamically stable emulsions containing an oil, surfactant, co-surfactant and drug(s) that spontaneously transform into oil-in-water emulsions upon the addition of aqueous media under gentle agitation [193]. SEDDSs are best suited for the incorporation of lipophilic drugs [194] as they are solubilized in the oily concentrate and then introduced into the aqueous media with mild agitation [178,185,186,194,195]. Interestingly, it has been demonstrated that SEDDSs encapsulating poorly water-soluble drugs elicited higher oral bioavailability in comparison with SEDDSs incorporating hydrophilic drugs; this clearly indicates the potential of SEDDSs to improve lipophilic drug delivery [196,197]. Nonetheless, for topical delivery of a drug such as clofazimine, the amount of clofazimine incorporated into the SEDDSs should be greater than the saturated concentration established in the oil phase in order to maximize clofazimine flux through the skin, as has also been noted for other poorly aqueous soluble drugs [197].

SEDDSs can be formulated in either nano- or micro droplets [198], where nanodroplet formation may occur in the presence of surface-active agents that cause spontaneous emulsification of finely dispersed droplets [194,199]. Formation of these finely dispersed nanosized droplets follows from the intrinsic physicochemical properties of the constituents.

Stability of these droplets depend upon the ratio of oil to surfactant [200]. However, it has been noted that high surfactant concentrations can cause tissue irritation [201]. Thus, spontaneous emulsification has to occur with decreased surfactant concentrations without reducing the stability of the formulations [202].

In view of the advantages of SEDDSs in terms of ease of formulation and solubilizing capacity for lipophilic drugs, SDEDDSs should provide even more benefits given the inclusion of multiple phases to optimize solubility and drug delivery via the dermal route of administration [203]. Drug delivery can be optimized by selecting immiscible oil phases in order to develop oil-in-oil-in water (O/O/W) or water-in-oil-in-oil (W/O/O) emulsions [203]. The employment of O/O/W systems will be beneficial if immiscible oil phases are used to create oil droplet formation in an inner oil phase within an outer oil phase [203]. This will provide a lipid matrix for enhancing solubility of lipophilic drugs while the water phase provides dermal hydration, which in turn establishes increased dermal drug delivery [204]. Conversely, W/O/O systems can provide a matrix for solubilization of both hydrophilic and lipophilic drugs with the added benefit of increased skin penetration as achieved by the outer oil phase that leads to enhanced dermal drug delivery [203–205].

As TB treatment requires combinations of different drugs administered over long periods of time as discussed above [37], the use of SDEDDSs can potentially provide an attractive dermal drug delivery vehicle by enabling simultaneous inclusion of several drugs within the hydrophilic and lipophilic phases of these multi-emulsions. Thereby, use of SDEDDSs represents a novel dermal drug delivery approach when compared to known dermal drug delivery vehicles. The lipid-based drug delivery systems discussed here are presented in Figure 5.

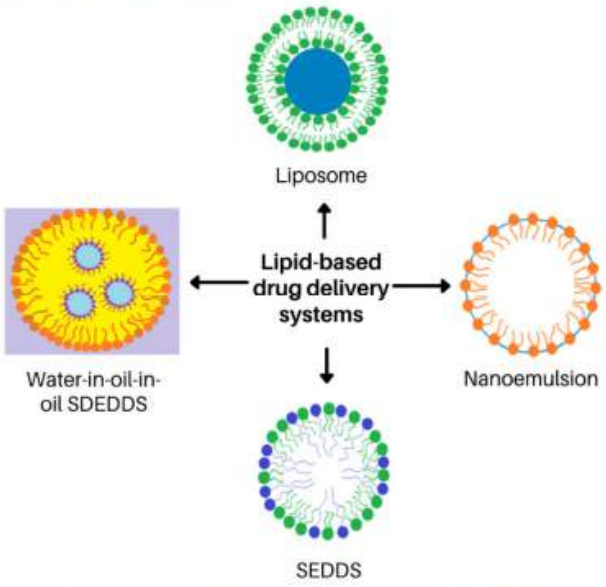


Figure 5. Lipid-based drug delivery systems [204,206].

SDEDDSs are polydispersed systems (Figure 5) [206] that for oral drug delivery have the promise of increasing the oral bioavailability of drugs known to have low water solubility and high permeability, that is of the Biopharmaceutical classification system (BCS) Class II drugs. By transporting drugs dispersed in oil droplets while passing through the gastrointestinal tract (GIT) [206], they enable prolonged drug release within the GIT—the drugs incorporated in the inner-oil phase must partition across multiple phases before reaching the site of action where drug release is desired [206]. For topical application, the formulation in principle does not require prolonged release since the formulation is directly applied to the affected area to establish a localised therapeutic effect [207]. Nevertheless, the capacity for prolonged drug release provided by SDEDDSs can establish

the intended therapeutic effect by controlling drug release as well as enabling the release of multiple drugs [206,207]. Conrolled drug release into the skin will obviate erratic drug release which can result in unwanted systemic uptake of the applied drug [207]. Use of conventional topical formulations that do not have controlled release properties place focus on establishing direct skin penetration of the encapsulated drugs [207]. In general, this is understandable as drugs must cross the protective barrier provided by the skin. This barrier reduces entry of drugs into the deeper skin layers thereby limiting dermal bioavailability which also compromises therapeutic effectiveness [207]. Therefore, optimal selection of excipients accompanied by a refined pre-formulation approach for preparing the SDEDDSs should overcome the challenges posed by drug solubilization and skin lipid disruption, and enable sustained release required to achieve dermal drug delivery [204,205,207].

SEDDSs and SDEDDSs additionally distribute drugs into the lymphatic system [178,180,196,208]. Due to absorption of SEDDSs and SDEDDSs through lymphatic vessels found in the dermis, drug delivery may be further enhanced [209,210]. Lymphatic absorption of clofazimine will provide an advantage as lymphadenitis is the most commonly observed manifestation of EPTB in both adults and children [68]. The lipid oil component is important for effecting lymphatic absorption through the dermis. Note that for oral administration, the oil has to solubilize a minimum of 50 mg/mL drug in order for lymphatic uptake to occur. In this respect, oils containing long chain triglycerides demonstrated increased lymphatic uptake, and the quantity of oil significantly contributes towards successful lymphatic uptake [171,186]. It was established that that a concentration of $\geq$25% oil content provides sufficient lymphatic absorption [131]. Unfortunately, lipids that are highly unsaturated may undergo autoxidation, and thus the addition of anti-oxidants must also be considered for topical drug delivery [208].

Not only is the evolution of SEDDSs moving towards alternative drug administration routes, but these systems are also being modified to advance muco-adhesion in order to extend contact time at the site of application [211–213]. Moreover, SEDDSs are being reformed to possess zeta-potential changing properties to improve drug delivery across mucus membranes [214]. Thus, combining mucolytic enzyme carrier systems with SEDDSs in order to enable local cleavability of mucus membranes resulted in increased drug permeation [215]. Consequently, SEDDSs have the potential to revolutionize drug delivery [187,194].

7. Conclusions

To summarize, the potential advantages of SEDDSs and SDEDDSs are enhanced drug entrapment capacity within physically more stable formulations, especially when compared to liposomes and emulsions. In addition, the formation of submicron droplets leads to an increase in the absorption surface area. SEDDSs elicit increased rates and amounts of drug absorption, thus increasing drug bioavailability. They moreover are able to deliver BCS Class II drugs efficiently. SEDDSs similarly have the potential for effective delivery of BCS class III drugs that display high solubility and decreased permeability properties, BCS class IV drugs that exhibit both low solubility and permeability characteristics, and hydrolytically susceptible drugs. Additionally, they offer consistent progressive diffusion profiles with reduced dosing and dosing frequencies. Moreover, upscaling techniques to prepare SEDDSs and SDEDDSs are simplified and cost effective compared to preparation of other lipid-based drug delivery systems.

Overall, the development of a topical SDEDDSs to aid in CTB treatment is potentially of considerable value as a reduced drug dose will be required to establish bioequivalence with oral drug delivery [201]. Due to the intractable nature of TB, high fixed doses of antitubercular drugs are normally recommended as inter-individual variability can lead to drug concentrations below the effective therapeutic dosage. Consequently, utilizing SDEDDSs for dermal administration of clofazimine will require decreased drug concentrations, will provide consistent drug delivery profiles that will be cytotoxic towards *Mtb* and thereby suppress drug resistance [216]. Importantly, such a route of administration should reduce

the unpleasant discoloration associated with oral administration of clofazimine [87,210]. The improved drug loading capacity of SEDDSs can further support anti-tubercular treatment by either including higher concentrations of clofazimine or incorporating fixed-dose drug combinations with other antitubercular drugs [217,218]. In the latter respect, incorporation of both artemether and lumefantrine in a solid SEDDS double fixed dosage form was used successfully for treatment of malaria [217]. For TB, it should be possible to delay the development of anti-microbial resistance by co-administration of two drugs known to act synergistically [216]. For this reason, clofazimine and pyrazinamide, for example, can be combined into a SDEDDS formulation as synergism between the two drugs has already been established [71,219]. Moreover, recent synergism was reported from evaluating a combination of clofazimine, isoniazid and rifampicin against actively replicating planktonic organisms and slow replicating organisms [220]. Interestingly, first-line antitubercular drugs are not the only drugs that acts synergistically when used in combination with clofazimine. In a model study involving the use of macrophages infected with *Mtb*, synergism was detected between clofazimine and the novel prenylated amino-artemisinin WHN296 [221]. These significant findings support the contention that clofazimine will be a key component to most anti-tubercular drug regimens intended to treat active TB, resistant TB infections, and also CTB.

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Chapter 3

Review Article published in '*Pharmaceutics*'

Chapter 3 is presented as the second **review article** titled: '***The Science of Selecting Excipients for Dermal Self-Emulsifying Drug Delivery Systems***' published in '*Pharmaceutics*' (DOI: 10.3390/pharmaceutics15041293). This review publication was written as a separate literature overview which describes the factors one needs to consider when selecting excipients for formulation of dermal spontaneous emulsions. A second literature overview was included since the research conducted to select excipients prior to commencement of laboratory experiments was based on an intensive literature study of topical/transdermal formulations and self-double-emulsifying drug delivery systems (SDEDDSs). Therefore, a formulation recommendation system is presented in the discussion section of this article as a guide to other researchers considering the inclusion of more than one drug in spontaneous emulsions based on the Biopharmaceutical Classification of the considered drug(s).

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Review

The Science of Selecting Excipients for Dermal Self-Emulsifying Drug Delivery Systems

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Abstract: Self-emulsification is considered a formulation technique that has proven capacity to improve oral drug delivery of poorly soluble drugs by advancing both solubility and bioavailability. The capacity of these formulations to produce emulsions after moderate agitation and dilution by means of water phase addition provides a simplified method to improve delivery of lipophilic drugs, where prolonged drug dissolution in the aqueous environment of the gastro-intestinal (GI) tract is known as the rate-limiting step rendering decreased drug absorption. Additionally, spontaneous emulsification has been reported as an innovative topical drug delivery system that enables successful crossing of mucus membranes as well as skin. The ease of formulation generated by the spontaneous emulsification technique itself is intriguing due to the simplified production procedure and unlimited upscaling possibilities. However, spontaneous emulsification depends solely on selecting excipients that complement each other in order to create a vehicle aimed at optimizing drug delivery. If excipients are not compatible or unable to spontaneously transpire into emulsions once exposed to mild agitation, no self-emulsification will be achieved. Therefore, the generalized view of excipients as inert bystanders facilitating delivery of an active compound cannot be accepted when selecting excipients needed to produce self-emulsifying drug delivery systems (SEDDSs). Hence, this review describes the excipients needed to generate dermal SEDDSs as well as self-double-emulsifying drug delivery systems (SDEDDSs); how to consider combinations that complement the incorporated drug(s); and an overview of using natural excipients as thickening agents and skin penetration enhancers.

Keywords: excipient selection; innovative delivery systems; self-double-emulsifying drug delivery system; self-emulsifying drug delivery system; skin penetration enhancer; surfactant; topical drug delivery systems

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1. Introduction

Self-emulsification is considered a formulation technique that has proven capacity to improve oral drug delivery of poorly water-soluble drugs by advancing both the solubility and bioavailability of these active compounds [1]. The capacity of self-emulsifying drug delivery systems (SEDDSs) to produce emulsions after exposure to moderate agitation and dilution by means of water phase addition provides a simplified method to improve delivery of lipophilic drugs, where prolonged drug dissolution in the aqueous environment of the gastro-intestinal (GI) tract is known as the rate-limiting step, influencing drug absorption [1,2]. Additionally, spontaneous emulsification has furthermore been reported as an innovative topical drug delivery system that enables successful crossing of mucus membranes as well as skin [3–5].

The ease of formulation generated by the spontaneous emulsification technique itself is intriguing due to the simplified production procedure and unlimited upscaling possibilities [1,6]. However, spontaneous emulsification depends solely on selecting excipients that complement each other in order to create a vehicle aimed at optimizing drug

delivery [1,5–7]. If excipients are not compatible or able to trigger the spontaneous emulsification process once exposed to mild agitation, no self-emulsification will be achieved. Therefore, the generalized view of excipients as inert bystanders facilitating delivery of an active compound cannot be accepted when selecting excipients needed to produce self-emulsifying drug delivery systems (SEDDSs) [1,8]. Hence, this review describes the excipients needed to generate more specifically dermal SEDDSs as well as self-double-emulsifying drug delivery systems (SDEDDSs), how to consider combinations that complement the incorporated drug(s), and an overview of using natural excipients as potential preservatives and skin penetration enhancers.

It has been established that self-emulsification depends on the chosen lipid/surfactant combination, surfactant concentration, as well as the ratio of lipid to surfactant [9]. Findings have also confirmed that only extremely precise excipient combinations can lead to the formation of spontaneous emulsions [1,9,10]. Hence, after selecting drug(s) considered for the incorporation into SEDDSs or SDEDDSs, the screening of excipients is significant in terms of compatibility, solubility, and stability [1,9]. However, when systems with multiple excipients are designed, the evaluated excipients should produce overall solubilization instead of only exhibiting sufficient solubility of a single excipient that forms part of the formulation [1]. Therefore, the included surfactant, co-surfactant, and oil phase(s) can be of a synthetic, semi-synthetic, or natural origin as long as toxicity and tissue irritancy are considered during excipient selection [1]. Importantly, excipients are generally selected for oral SEDDSs based on their (1) capacity to attain maximum drug loading; (2) ability to ensure maximum drug absorption as influenced by sufficient duration of self-emulsification, together with minimized droplet size; (3) limiting fluctuation of droplet size influenced by the pH of the aqueous medium as well as electrolyte concentration; and (4) limiting degradation of the administered drugs in the physiological environment [1,2,9]. On the other hand, excipients selected for dermal drug delivery must be considered based on their (1) capacity to attain maximum drug loading, but to a lesser degree when compared to drugs destined for oral absorption since dermal application allows delivery of higher drug concentrations directly to the affected site; (2) ability to ensure maximum dermal drug diffusion by including excipients that facilitate diffusion by limiting trans epidermal water loss (TEWL), through hydration (water), and/or humectant inclusion; (3) limiting droplet size fluctuation and maintain a decreased droplet size to improve dermal drug delivery as achieved by selecting surface active agents capable of providing sufficient stabilization without reaching toxic surfactant concentrations; and (4) including excipients such as skin penetration enhancers in order to achieve dermal lipid disruption required to facilitate drug diffusion into underlying skin layers [5,11–14].

Considering the numerous factors mentioned, when selecting excipients for dermal SEDDSs compared to oral SEDDSs, one can also regard these same factors if aiming to create dermal SDEDDs instead of SEDDSs. Dermal SDEDDSs provide the option of including even more active ingredients in combination as these multiple emulsions can offer more options for solubilization of numerous drugs in a single formulation compared to SEDDSs that are traditionally limited to a water and oil phase supported by the surfactant phase [15]. It has been reported, for example, that incorporating quercetin into an SDEDDS improved skin permeation compared to other dermal drug delivery systems [11]. Moreover, literature concluded that a rutin-loaded non-aqueous SDEDDS exhibited a sustained release profile of up to 12 h [12]. Therefore, studies have clearly indicated the advantages of utilizing SDEDDSs as multiple-emulsion dermal drug delivery systems if approached correctly. However, only a limited literature about dermal SEDDSs and SDEDDSs has already been published, which contributes to the lack of information when aiming to select excipients during the development of these unique systems. Table 1 lists recent publications related to the successful formulation of dermal spontaneous emulsions.

Table 1. Recent developments published on dermal spontaneous emulsions.

Dosage Form	Year of Publication	Drug	BCS [1] Drug Class	Reference
SDEDDS	2015	Nattokinase	Enzyme	[16]
SNEDDS [2]	2018	Curcumin	Class IV	[17]
SEDDS	2020	Clofazimine	Class II	[5]
SEDDS	2020	Rose bengal	Xanthene	[18]
N-SDEDDS [3]	2021	Rutin	Class II	[12]
Nanoemulgel (prepared from SEDDS)	2021	Chrysin	Class II	[19]
SNEDDS [2]	2023	Astaxanthin	Class IV	[20]

[1] Biopharmaceutical classification system; [2] self-nano-emulsifying drug delivery system; [3] non-aqueous self-double-emulsifying drug delivery system.

Considering the summary provided in the table, it can be concluded that spontaneous emulsification is a valuable tool that should be investigated and optimized to the fullest. Therefore, this current work aims to equip the reader with an improved understanding of excipients needed to generate both dermal SEDDSs and SDEDDSs as well as the refinement of these drug delivery systems by discussing the inclusion of excipients such as thickening agents, emollients, humectants, preservatives, antioxidants, and co-solvents [5,12,16–20].

2. Components of Dermal SEDDSs/SDEDDSs

2.1. Drug(s) Inclusion

According to the literature, drugs considered most suitable for oral delivery via SEDDSs should preferably have a log *p* value ≥ 2, include a modest drug dosage to achieve therapeutic efficacy, and not be subjected to substantial first-pass metabolism [1,10]. If inclusion of high drug dosages is considered, remarkable solubility in at least one of the SEDDS phases should be demonstrated [1,21]. Generally, optimizing therapeutic effectiveness of orally administered SEDDSs is achieved by considering the most significant factors such as drug solubility, dissolution rate, and permeability [1]. Dissolution rate is a contributing factor that can essentially alter drug release kinetics as well as drug GI absorption [1]. These factors are best explained by the well-known Biopharmaceutical Classification System (BCS) used to predict drug solubility in the aqueous environment of the GI tract and, on the other hand, the lipophilic features of a drug that allow the crossing of membrane barriers [22]. A summary of the BCS utilized to classify drugs according to their solubility and permeability features is displayed in Figure 1.

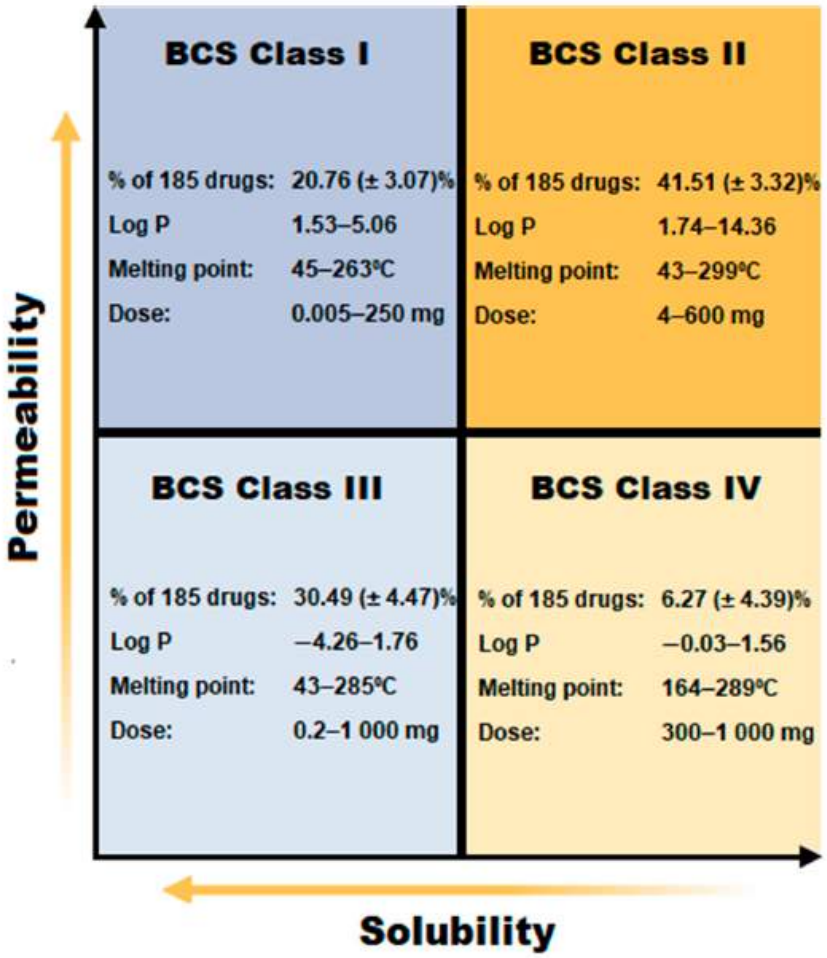

Figure 1. Biopharmaceutical Classification System (BCS) [23].

Similar to the BCS employed during pre-formulation studies of oral dosage form development, the Topical Drug Classification System (TCS) has also been reported in the literature [22,24]. In the United States of America (USA), most generic topical products have qualitatively (Q1) and quantitatively (Q2) similar ingredients compared to the reference listed drug (RLD) [22,24]. The application of in vitro release (IVR) and in vitro characterization is considered for a topical dosage form of differing dosage strengths, such as suspensions, ointments, creams, and gels. Therefore, the TCS is based on the consideration of Q1, Q2, and the arrangement of matter and the microstructure of topical formulations (Q3) [22,24]. In the following, four distinct drug classes are identified by the TCS that provide pre-formulation evidence which indicates if a biowaiver can be granted for generics of topical products based on various potential scenarios that might arise during formulation development [22,24]. This system is depicted in Figure 2.

However, the TCS is limited compared to the BCS when aiming to overview a drug in terms of suitability for topical and transdermal drug delivery. Moreover, according to the authors' knowledge, no clear classification system exists to evaluate drugs when considering drug incorporation into topical SEDDSs or SDEDDSs during the pre-formulation phase. As a lipid-based drug delivery system, SEDDSs are regarded as favorable vehicles for BCS Class II and IV drugs due to the reduced aqueous solubility of these compounds [1]. However, the decreased permeability capacity of BCS Class IV drugs creates an opportunity to put dermal SEDDSs to the test as the drug delivery vehicle must compensate for the poor permeability properties of the BCS Class IV drugs in order to facilitate dermal drug

delivery. Hence, this review will provide a formulation recommendation system (FRS) in the discussion section for drugs considered for spontaneous emulsion incorporation by providing guidelines for creating a SEDDS vehicle that can improve dermal drug delivery of each BCS drug class and provide the option of including more than one drug class into a single SEDDSs or SDEDDSs. In order to present a useful and scientific FRS for SEDDSs and SDEDDSs, general excipients utilized to produce spontaneous emulsions will now be discussed.

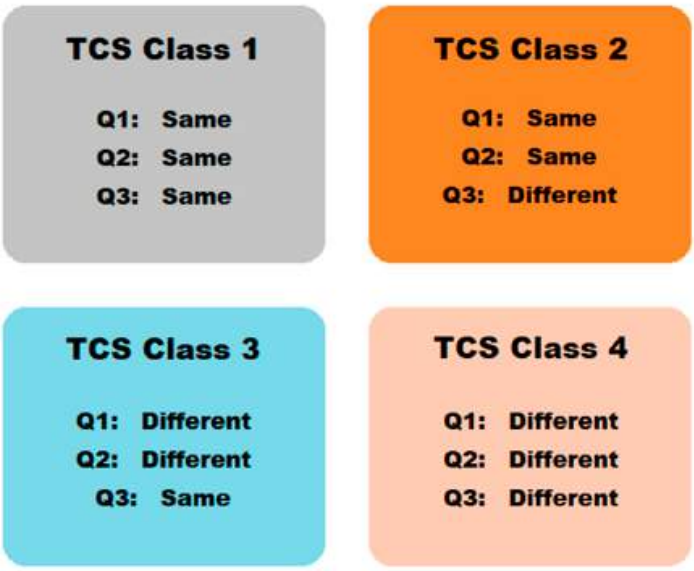

Figure 2. Topical Drug Classification System (TCS) [22].

2.2. Oil Phase(s)

As the lipophilic component of SEDDSs, the oil phase is crucial for solubilizing lipophilic drugs in order to enable drug delivery from either oral or dermal vehicles [14]. Improved drug solubility allows quick availability of a drug(s) for gastric absorption via the lymphatic system [1]. In terms of dermal drug delivery, enhanced solubility of a drug(s) in the oil phase presents an opportunity to introduce supersaturated SEDDSs to the skin to enhance the dermal flux of the active ingredient(s) [5]. However, one must be reminded that the release of a drug from emulsions is regulated by the polarity of the presented lipid matrix [1,13]. The polarity of any surface-active agent or lipid component is influenced by the chain length and the degree of saturation of the fatty acid composition in the lipid constituent, together with the molecular weight that impacts droplet size and size distribution, and therefore, its capacity to inhibit crystallization and sustain a supersaturated state for a longer period of time [1,25]. Moreover, characteristics such as melting point and the hydrophilic–lipophilic balance (HLB) of oils can be linked to the presence of different glycerides [1]. These glyceride features are determined with regard to glycerol content employed to generate mono-, di- or triglycerides dependent on the degree of esterification and the type of fatty acids [1]. Interestingly, medium-chain triglycerides (MCTs), classified as the presence of 6 to 12 carbon chains, render delivery of drugs into the systemic circulation via portal blood during oral administration [1]; whereas, the inclusion of long-chain triglycerides (LCTs), exceeding 12 carbon chains, leads to lymphatic system transport when utilizing oral SEDDSs [1,9]. However, oil phases comprising mainly MCTs such as ethyl oleate and caprylic/capric triglycerides are generally selected above oil phases consisting of LCTs due to improved solubility of the included drug(s), enhanced fluidity, and the improved ability to withstand oxidation reactions [1,9]. Moreover, MCTs generally improve the ease of self-emulsification compared to LCTs as these longer triglyceride chains are of a more hydrophobic nature that impede ease of emulsification [26]. Hence, MCTs

will generally be favored above LCTs during the development of dermal SEDDSs as the ease of spontaneous emulsification is imperative when creating these systems. However, lymphatic drug uptake from dermal SEDDSs cannot be excluded when only employing MCTs as oil components may have access to the lymphatic system after skin application, but this differs from oral administration [5,14].

In the skin, lymphatic vessels configure two networks as part of the one-way lymphatic system [27]. One configuration extends into the dermal papillae at a superficial level known as the superficial lymphatic plexus, whereas the second configuration is found near the subpapillary arterial network, i.e., deep lymphatic plexus [27]. The dermal lymphatic network is initially observed as capillaries of a small, blind-ended nature accompanied by minimal basement membranes that are directly linked to the extracellular matrix [27]. This link is established by anchoring filaments that allow a rapid body response to local changes observed for intestinal fluid pressure due to swelling of the extracellular matrix as brought about by pulling on filaments due to the presence of edemas that loosen the buttonlike inter-endothelial junctions [27]. Next, the described lymphatic capillaries drain into collecting vessels identified by the presence of a continuous basement membrane, coverage of smooth muscle, and a valve system that inhibit retrograde fluid flow [27]. The discussed dermal lymphatic system may be observed in Figure 3.

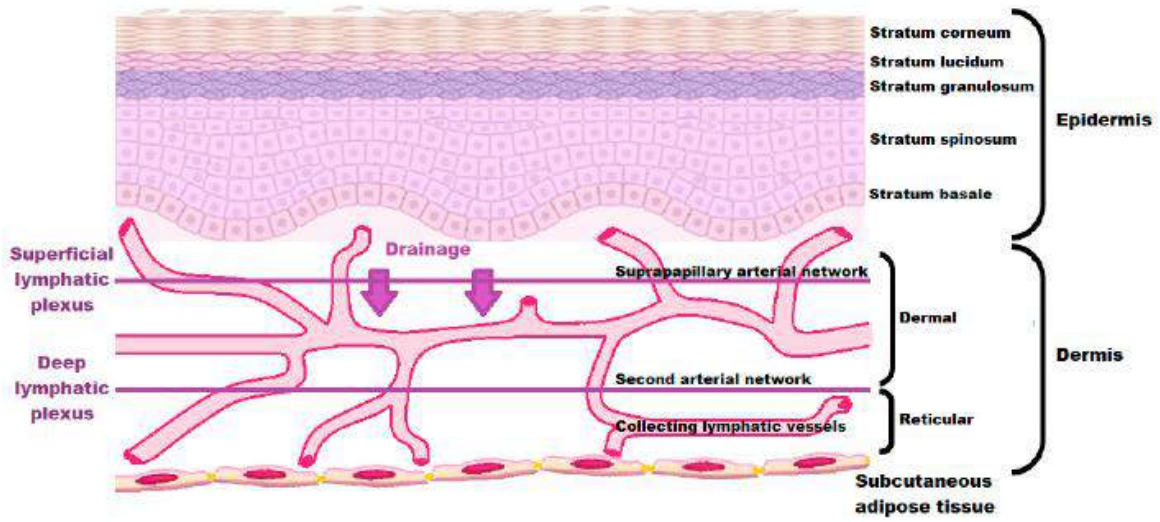

Figure 3. Structure of the lymphatic network of the skin.

As stated, the routes of uptake of MCTs and LCTs from the lymphatic system, accessed by oral administration, differ from dermal applications as these lipid components must travel via the GI tract directly via portal blood to the systemic circulation, as is primarily observed with MCTs. LCTs, on the other hand, are transported via intestinal lymphatics where lipoproteins named chylomicrons, solely produced in the GI tract, transport LCTs to the lymphatic network [9]. Therefore, no clear evidence of the need to use LCTs to gain lymphatic access, in the case of dermal application, can be provided and further investigation is needed. Hence, the priority of generating SEDDSs with intensified ease of self-emulsification is considered more important than dermal lymphatic uptake. However, dermal lymphatic uptake cannot be overruled completely due to the lack of evidence.

When developing SEDDSs or SDEDDSs for dermal drug delivery, one must bear in mind the importance of selecting an oil phase with skin penetration enhancement properties. This is of substantial importance as the outermost skin layer, i.e., the stratum corneum (SC), forms a protective external barrier against toxins, pathogens, and drug molecules [28]. The SC comprises keratin-rich cells inserted in multiple lipid bilayers [28]. Therefore, skin penetration enhancers must exhibit lipid disruption properties to gain drug entry into the

underlying skin layers to achieve therapeutic effects. Lipid disruption can be attained by active or passive means [28,29]. Active lipid disruption refers to the employment of devices, i.e., microneedles, thermal ablation, micro-dermabrasion, electroporation, and cavitational ultrasound, that are used effectively but are restricted to utilization on small skin areas as use on larger skin areas may be difficult [28]. Passive lipid disruption, alternatively, is achieved by chemical permeation enhancers that affect lipid disruption through lipid extraction, fluidization, or introducing other mechanisms of structural reorganization [28]. However, a balance must be maintained between achieving desired lipid-disruptive properties and avoiding dermal irritation due to lipid disruption [28]. Chemical penetration enhancer examples include azone derivates, alcohols, esters, glycols, fatty acids, pyrrolidones, surfactants, terpenes, and sulphoxides [28]. These compounds are deemed small molecules (<500 Da) that can penetrate the skin in increased quantities, which can lead to skin irritation, cytotoxicity, or irreversible skin barrier alteration [28]. Therefore, focus has been shifting towards utilizing skin penetration enhancers obtained from a more natural origin [5]. For instance, olive oil is a rich source of oleic acid and can be included as a natural oily source of this well-known skin penetration enhancing fatty acid that provides dermal lipid disruption without irritation [5,13,30]. Interestingly, the extent of glyceride saturation is considered more important in terms of topical/transdermal drug delivery compared to oral drug delivery, as numerous publications have confirmed that unsaturated fatty acids (UFAs) have superior dermal lipid-disruptive properties compared to saturated fatty acids (SFAs) [14,31,32]. This discovery can be linked to the reduced capacity of SFAs to dissolve within the natural lipids of the SC, which renders diminished lipid disruption accompanied by less effective skin penetration enhancement [14,31,32]. Additionally, during the selection of the oil phase in terms of dermal drug delivery, one must consider the sensation experienced by patients when applying the dosage form onto their skin.

It has been reported that stickiness and greasiness of dosage forms such as ointments decrease patient compliance [33]. Hence, the skin sensation provided by the oil as well as the organoleptic properties of the oil should also be contemplated. For instance, sweet almond oil is referred to in the literature as a lightweight oil accompanied by a mild smell [34]. However, although this oil may possess acceptable organoleptic properties, oils retrieved from nut origin should be used with caution due to the possibility of patients being allergic to nuts [14]. On the other hand, jojoba oil renders an excellent lubricity without greasiness, as is the case with other lipids such as lanolin and petrolatum [35]. Rice bran oil is described as a "natural and value-added healthy ingredient" in terms of enhancing skin moisture and contributing towards improved physical stability of emulsions [36]. Thus, the lipid component of SEDDSs or SDEDDSs can influence patient compliance. Yet, in terms of dermal drug delivery, the challenge remains to select a lipid component with sufficient cutaneous lipid-disruptive properties while also limiting TEWL to assist with dermal drug diffusion by means of occlusivity without leaving behind a sticky and unpleasant skin sensation.

Then again, when considering different oil phases as potential lipid components of SDEDDSs compared to SEDDSs, selection is even further complicated when having to ensure that oils included into SDEDDSs exhibit immiscibility in order to establish an internal and external oil phase when developing water-in-oil-in-oil (w/o/o) emulsions as well as oil-in-oil-in-water (o/o/w) emulsions [37]. Traditional (aqueous) SDEDDSs provide the advantageous capacity to incorporate hydrophilic drugs together with decreased concentrations of hydrophobic drugs as two water phases are present to dissolve water-soluble drugs [37]. However, the development of non-aqueous SDEDDSs, where two oil phases and a single water phase are present, can provide similar advantages compared to traditional SDEDDSs, apart from increasing the concentration of the fat-soluble drug that can be incorporated [37]. Hence, internal oil phases can be described as polar organic solvents combined with non-polar natural oils, utilized as the external oil phase, to maintain distinct phase separation [37]. Moreover, the inclusion of a polar internal oil phase establishes increased drug solubilization achieved by increased drug solubility observed in the internal

oil phase compared to the external oil phase [37]. Additionally, this creates the opportunity to select an internal oil phase that optimizes drug solubility while including an external oil phase with favorable but less desirable solubility properties that optimizes skin penetration enhancement and ensures maximized dermal drug delivery. However, to stabilize these unique multiple emulsions, often large concentrations of surfactants are employed that can disturb physiological skin processes upon chronic application [37].

2.3. *Surfactant(s) and Co-Surfactant(s)*

Surfactants can induce phospholipid emulsification, which leads to cellular damage that results in a cytolytic process accompanied by the release of proteins, lysosomal and cytoplasmic enzymes, as well as inflammatory mediators [38]. Consequently, phospholipid emulsification normally establishes skin irritation, especially if a patient is frequently exposed to increased surfactant concentrations during topical application of topical/transdermal treatment. However, surfactants are essential ingredients included in SEDDSs as well as SDEDDSs for the purpose of stabilizing emulsions. Therefore, a scientific balance must be maintained between including sufficient surfactant concentrations required for stabilization while keeping concentrations low enough to avoid potential skin irritation upon chronic exposure during prolonged treatment periods. A potential solution is to use both a surfactant and a co-surfactant since the literature states that the inclusion of a co-surfactant can simulate similar stabilizing effects compared to increasing the concentration of an individual surfactant [1,39]. This can decrease the amount of individual surfactant needed by approximately 30% [1,39]. Another option is to use non-ionic surfactants during the development of dermal drug delivery vehicles, as these compounds tend to establish moderate, irreversible changes to membranes while being non-toxic [1,14].

The main objective for surfactant inclusion is to decrease interfacial tension by forming an interfacial film that allows dispersion formation [1,14], whereas co-surfactants decrease the transitory negative value of the established interfacial tension even further [1]. This provides flexibility to the interfacial film that enables formation of varied curvatures for creation of different microemulsion concentrations [1]. The contact enlargement results in the scattering of fine droplets [1]. Next, more surfactant or a higher ratio of surfactant to co-surfactant will be absorbed until the film is depleted enough to maintain positive interfacial tension that results in spontaneous emulsification. Co-surfactants generally comprise medium-chain length alcohols (C3–C8) such as ethanol, isopropyl alcohol, 1-butanol, and propylene glycol [1,40].

The most established tool utilized during emulsion formulation is calculating the hydrophilic–lipophilic balance (HLB) values of surfactants and co-surfactants utilized in combination prior to formulation [1]. This tool, though not perfect, is employed to determine the ratio of surfactant to co-surfactant as well as consider different surface-active agent combinations if a fixed ratio is chosen in advance. Fixed ratios of surfactant to co-surfactant can be selected for SEDDSs and SDEDDSs in advance as the literature has confirmed the formation of more stable SEDDSs at a ratio of 1:1 (surfactant:co-surfactant), whereas decreased stability causes precipitation of the incorporated drug despite an enlarged emulsion range [5,41]. Generally, surfactants with an HLB value exceeding 12 are selected to achieve improved emulsification of SEDDSs [1]. The HLB scale together with examples of HLB values of different excipients can be seen in Figure 4.

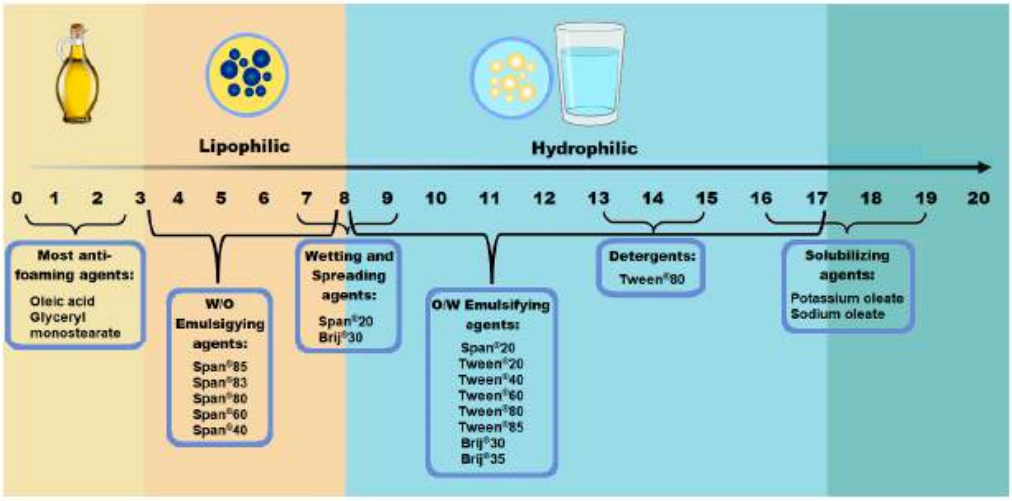

Figure 4. Hydrophilic–Lipophilic Balance (HLB) Scale [42,43].

As illustrated in Figure 4, traditionally the HLB scale ranges from 1 to 20 despite reporting HLB values that exceed 20 [44]. An HLB value ranging from 1 to 10 signifies a more lipophilic compound [44,45], whereas an HLB value between 11 and 20 indicates a more hydrophilic nature [44–46]. This is influenced by the number of hydrophilic groups present in each molecule. Hence, combinations of lipophilic and hydrophilic surface-active agents can generate an optimized HLB value [44–46]. In this case, an optimized HLB value refers to choosing between formulating an oil-in-water (o/w) emulsion or a water-in-oil (w/o) emulsion.

When considering dermal drug delivery, the choice between an o/w or w/o emulsion can be influenced by numerous factors. Firstly, o/w emulsions are more susceptible to oxidation reactions [47]. This increased oxidative susceptibility is linked to a higher extent of interfacial interactions provided by the lipid component and prooxidants such as metal ions in the aqueous phase due to the greater surface area provided by oil droplets [47]. The literature suggests that lipid oxidation observed in w/o emulsions should occur at a rate similar to that of bulk oils since the surface of the oil phase is directly exposed to air [47]. Consequently, the need to discuss and investigate the inclusion of excipients such as antioxidants during formulation of SEDDSs and SDEDDSs will be addressed in another section of this review.

Next, the nature of the incorporated drug(s) can influence the type of emulsion. This statement is based on the principle that a drug should be dissolved in the emulsion to diffuse across the SC into the underlying skin layers [5,14,15]. Therefore, if a lipid-soluble drug is incorporated, it will mainly be dissolved within the lipid phase of an emulsion [48]. The SC is known for its dominant lipid arrangement [49]. Thus, a lipid-soluble drug can benefit from inclusion into an o/w emulsion as the drug would desire escape from the predominantly aqueous environment and diffuse into the lipophilic SC when made possible by the inclusion of skin penetration enhancers and the hydrating effect contributed to water inclusion [5,14,49–51]. However, w/o emulsions are notorious for improved occlusive effects that limit TEWL and prolong contact time between the formulation and the skin that in turn improves dermal drug diffusion [5,14,51]. Therefore, considering these few mentioned factors, a one-size-fits-all approach is insufficient during development of topical/transdermal SEDDSs and SDEDDSs as careful consideration must be given to the nature of the drug(s) as well as the stability of the formulations during selection of surface-active agents. The selection of an intended HLB value prior to formulation is beneficial as it can decrease the number of pre-formulation experiments during development of SEDDSs and SDEDDSs destined for dermal drug delivery.

Finally, the HLB values considered for multiple emulsions such as SDEDDSs will differ as definite inclusion of both a lipophilic and a hydrophilic surface-active agent is needed to establish formulation stability at the different interfaces present within multiple emulsions [52]. For this reason, during development of multiple emulsions, one surface-active agent must provide stabilization of the primary emulsion and a second surface-active agent should provide stabilization of the secondary emulsion formation [52].

2.4. The Water Phase

The final essential component for uncomplicated SEDDSs and SDEDDSs to form spontaneously is the hydrophilic component. Water can be described as the primary skin penetration enhancer as its presence on the skin establishes hydration that leads to loosening of the tightly organized lipid structure of the SC [14,49,53]. Moreover, water is essential in providing an aqueous medium needed to dissolve hydrophilic drugs incorporated into SEDDSs or SDEDDSs. The necessity of including water in spontaneous emulsions destined for dermal application can be considered the most significant difference between oral spontaneous emulsions and dermal spontaneous emulsions [14]. Normally, an oily concentrate together with a surfactant phase is administered orally; and only after administration is it introduced to the aqueous environment of the GI tract, where, once inside the body, spontaneous emulsification can occur [1]. However, contrary to this type of delivery system, spontaneous emulsification of dermal SEDDSs and SDEDDSs must occur prior to application as sweat secretion is erratic and unpredictable. It can therefore not be regarded as the sole water component for spontaneous emulsification upon dermal application due to high inter-patient variability as well as the influence that the dermal condition being treated may have on the delivery system (e.g., secretions) [54,55]. Importantly, different dermal diseases can alter skin structure, either temporarily or permanently, due to factors such as skin hydration, climate effects, and the causative organism [54,55]. Therefore, water inclusion or inclusion of a polar solvent is considered imperative during formulation of dermal spontaneous emulsions.

3. The Addition of Auxiliary Excipients

3.1. Why the Need for Refinement of Topical/Transdermal Spontaneous Emulsions?

After reviewing the main components utilized for the preparation of spontaneous emulsions, it is time to consider the effect of auxiliary additives that can potentially benefit dermal drug delivery as well as emulsion stability. Dermal permeation of drugs can be influenced by the type of emulsion prepared with reference to o/w and w/o emulsions as well as different types of multiple emulsions [56]. Generally, drug(s) dissolved in the external continuous phase of an emulsion should exhibit permeation behavior that differs from drug(s) incorporated into the internal dispersed phase droplets [56]. For instance, droplet size of the internal phase should notably influence dermal permeation [56]. However, the addition of auxiliary excipients during the formulation of both SEDDSs and SDEDDSs can assist in creating stable as well as elegant emulsions while also considering modification of dermal drug delivery [56]. For example, the inclusion of a thickening agent can aid in the formation of a more occlusive, uniform, and stable emulsion [56].

3.2. Some Examples of Auxiliary Excipients That Can Be Included into SEDDSs and SDEDDSs for Topical/Transdermal Drug Delivery

3.2.1. Thickening Agents

The addition of a thickening agent directly impacts the viscosity of a formulation [56–58]. Nevertheless, the literature is still debating whether the thickening of formulations does in fact hinder or rather enhance dermal permeation of drugs [56–58]. It has for example been reported that the inclusion of a thickening agent, i.e., xanthan gum, improved dermal drug permeation due to increased stability and homogeneity of the said emulsion [56]. This can be attributed to a significant reduction in droplet size as drugs dissolved in internal phase droplets were able to diffuse with increased success over the obstacle provided by the SC,

specifically due to the decreased droplet size. The smaller droplet size enlarges the surface area of the oil droplets, which in turn increases the contact area between the oil droplets and the skin, inducing higher skin permeation of the incorporated drugs. Moreover, it was found that the increased viscosity established occlusivity that prolonged the time the skin was exposed to the formulation, which consequently facilitated higher dermal drug permeation [56]. Additionally, this decreased droplet size also exhibited increased emulsion stability [56]. Contrarily, another study established that increased viscosity could restrict diffusion of a drug through the formulation, impeding dermal drug diffusion [58]. Furthermore, another study aimed at delivering berberine from chitosan hydrogel bases concluded that diffusion of berberine through the gel-like base into the aqueous external phase was restricted due to the increased viscosity of the formulation [57]. However, the same study found that increased doses of incorporated berberine rendered enhanced dermal drug delivery despite the use of high-viscosity formulations [57]. Moreover, the inclusion of skin penetration enhancers, in this case the non-ionic surfactant Tween®80, was emphasized to enable drug release from formulations of high viscosity [57]. Lastly, hydrophobic bentonite clay was employed together with non-ionic surfactants to assist with co-stabilization of non-aqueous SDEDDSs [16]. Hence, thickening agents can assist with dermal drug delivery from SEDDSs and SDEDDSs that might be of a low viscosity but easily transpire into emulsions.

3.2.2. Emollients and Humectants

Emollients are added to formulations for the purpose of improving spreadability, improving skin feel, as well as improving the hydration function of the SC [59–61]. The softening of skin brought about by emollients is attributed to the formation of an occlusive film on the skin that renders a reduced TEWL [62]. They are nonetheless mostly considered cosmetic ingredients instead of pharmaceutical ingredients [60]. Consequently, emollients are included in lotions, creams, and ointments to adjust consistency of cosmetic vehicles for the purpose of providing a softer and smoother skin feel [63]. However, the contribution of emollients with regards to dermal drug delivery should not be underestimated. Emollients do not only enhance the character of the applied vehicle but also promote dermal penetration of incorporated drug(s) [63]. Moreover, emollients have demonstrated the capacity to reduce stress experienced by the SC, such as injuries or wounds, by penetrating the SC and replacing the lost water volume [61]. This emphasizes the profound relationship between the transport properties provided by emollients and their beneficial effect on repairing the barrier function of the outermost skin layer [61].

A model was suggested by Berkey et al. [61] to consider both the penetration volume of emollients, i.e., drug transport potential in the SC, as well as the biomechanical stress reduction on the SC upon emollient application. This model can greatly assist during evaluation of emollients for inclusion into spontaneous emulsions. Their study concluded the importance of reviewing viscosity, molecular mass, and diffusivity [61]. Simply stated, small molecules generally demonstrate increased diffusivity, which leads to improved dermal penetration [61]. However, the emollient glycerol is discussed as an example where a decreased molecular mass rendered increased diffusivity as expected but not exceptional SC penetration volume values [61]. This is attributed to the more viscous nature of glycerol as well as its higher water solubility compared to other emollients tested during this study [61]. In addition, topological surface area (TPSA) consideration can also provide valuable information with regards to the determination of penetration volume as it contributes towards understanding emollient bonding interactions with SC components [61]. By taking TPSA into consideration, together with molecule size and polarity, it was concluded that smaller molecules of low polarity tend to demonstrate improved penetration of the SC lipid matrix [61]. Interestingly, this signifies the importance of not exclusively using log p values of emollients as a penetration prediction measurement since log p increases while polarity is reduced and decreases as the molecular weight is reduced [61].

Cosmetic emollients such as dipropyl heptyl carbonate, dibutyl adipate, and propyl heptyl caprylate are often confused with dermatological emollients, which refer to lotions, creams, and ointments utilized to improve skin barrier function during eczema treatment [61]. Similarly, the mechanisms of emollients and humectants are easily confused during formulation of topical preparations. Emollients establish reduced TEWL by generating an occlusive film on the skin that fills cracks and injuries to repair SC barrier function, whereas humectants draw moisture from the environment and lock the attracted moisture in the skin to improve dermal hydration [61,64]. Humectants are useful in countering the drying effect that surfactants tend to have on the skin [65]. Humectants are frequently paired with occlusive agents such as petrolatum to provide protection against drying skin in the winter months when less moisture is available for humectants to lock inside the SC [66]. Examples of humectants include hyaluronic acid, urea, glycerol, and propylene glycol [64,67]. Some excipients, such as glycerol, can be used as either an emollient or humectant based on the concentration of glycerol included in a formulation [68].

3.2.3. Preservatives and Antioxidants

The addition of a hydrophilic component in SEDDSs and SDEDDSs raises the need for preservative inclusion for the purpose of inhibiting microbial growth [69]. Additionally, the presence of a lipophilic phase signifies the importance of including an antioxidant since lipids tend to spontaneously participate in autoxidation with atmospheric oxygen [70]. Moreover, depending on the preservative antioxidant function, photoprotective properties can also be provided [69]. However, the undeniable global concern raised by the toxicity, sensitization, and irritation of preservatives cannot be ignored [69,71]. For example, the preservative triclosan may lead to the disruption of sexual hormones as well as the deregulation of the thyroid function [59,69,72]. Furthermore, the famously phenyl-, benzyl-, pentyl-, isopropyl-, and isobutyl parabens have been banned from cosmetic products by the European Commission (EC)-amended Annexures II and V of the EU Cosmetic Regulation since 2014 due to no submitted safety evaluation data [69,71,73]. Therefore, preservatives mark an ethical area of formulation where safety should be considered above the functionality of ingredients [69]. Fortunately, similar to the employment of natural oils as lipid components, in order to avoid skin irritation and make use of natural skin penetration enhancers, preservatives from a natural origin are widely and successfully researched. An excellent example is with regards to the use of *Nigella sativa* L. seed oil as a natural preservative [74,75], which is already used in the food industry as a natural preservative of Mediterranean cheeses [75]. Another example includes cinnamaldehyde, which is an active monomer isolated from the bark of cinnamon stems and used as a preservative in both the cosmetic and food industries [76]. Likewise, excipients utilized during formulation of mucolytic SEDDSs, such as conventional cationic surfactants, can potentially be used as multi-purpose ingredients if a proper risk-benefit assessment is made [3]. Therefore, inclusion of preservatives into topical/transdermal SEDDSs and SDEDDSs can be beneficial to prolong the shelf-life of these spontaneous emulsions.

3.2.4. Co-Solvents

Co-solvents are frequently included during the formulation of both mucoadhesive and solidified SEDDSs intended for oral drug delivery [77–79]. Therefore, the possibility as well as benefits of utilizing co-solvents during the preparation of dermal SEDDSs and SDEDDSs should be reviewed. This can be of particular importance during the delivery of lipophilic drugs that should be presented in higher concentrations to achieve therapeutic effects. Moreover, inclusion of co-solvents such as ethanol in dermal formulations can lead to the evaporation of the co-solvent during application, which can render an increased flux of drug across the SC due to maintained supersaturated drug concentrations [80]. The choice of solvent can also greatly benefit skin penetration enhancement if it is a solvent known for SC lipid-disruptive properties [80]. Evaporative systems provide non-occlusive, passive delivery of drugs without the disadvantage of causing skin irritation [80]. Moreover, these

are quick drying systems which improve patient compliance [80]. However, supersaturated formulations are known as inherent thermodynamically unstable systems, which in turn may cause drug crystallization [80]. Fortunately, drug crystallization can be prevented by including antinucleating polymers that either inhibit nucleus formation or impede crystallization [80].

Instead of evaluating the addition of a co-solvent, one can rather consider developing SDEDDSs instead of SEDDSs. This statement is made after reflecting on the literature regarding the development of topical/transdermal SDEDDSs by creating non-aqueous multiple emulsions produced by spontaneous emulsification [12,37]. The main aims during formulation of SDEDDSs are to improve the solubility of the incorporated drug(s), use the lowest surfactant concentration possible, and improve skin retention time compared to other dermal vehicles [37]. Hence, the addition of a third emulsion phase can assist in improving solubility while decreasing surfactant concentrations and improving skin retention time. Solubility can be increased by selecting an oil phase with a polar nature such as Transcutol® or glycerol [12,37]. Moreover, surfactant phase concentrations can be limited to a minimum amount if pre-formulation studies are performed by evaluating oil phase immiscibility and determining the self-emulsification capacity of SDEDDSs by constructing pseudo-ternary phase diagrams [5]. Lastly, skin retention time can be improved due to the possibility of utilizing an external oil phase capable of excellent SC lipid disruption to facilitate enhanced dermal drug delivery [15]. In addition, multiple emulsions, such as SDEDDSs, provide the opportunity to achieve sustained release during delivery of both an individual drug as well as more than one drug, since drugs of different solubilities will favor certain phases of the emulsions and remain in these solvents at concentrated quantities compared to other phases [15,81]. For this reason, sustained release can occur according to diffusivity of the inner phases across the outermost phase prior to reaching the SC.

4. Discussion

This paper provides insight into recent innovations in terms of dermal spontaneous emulsions compared to oral spontaneous emulsions. The capacity of these formulations to cross barriers such as mucus membranes and the skin provide evidence of their versatility if designed correctly. However, as seen by the detailed description of general excipients as well as auxiliary compounds, the generalized view of excipients as inert bystanders cannot be accepted when aiming to develop dermal SEDDSs or SDEDDSs. Self-emulsification depends solely on the selected lipid/surfactant combination, surfactant concentration, as well as the ratio of lipid to surfactant. Therefore, extremely precise excipient combinations are essential to achieve formation of spontaneous emulsions. Consequently, after selecting drug(s) that are to be included into spontaneously formed emulsions, excipient screening and selection are important in terms of compatibility, solubility, and stability. Furthermore, excipients selected for SEDDSs and SDEDDSs should (1) have the capacity to attain maximum drug loading, but to a lesser extent compared to oral SEDDSs as dermal application of drugs allow delivery of higher drug concentrations directly to the affected area; (2) optimize dermal diffusion by selecting excipients that facilitate transport due to minimized TEWL by means of hydration and/or humectant inclusion; (3) ensure uniform droplet size distribution and maintain reduced droplet sizes for the purpose of improving diffusivity across the SC; and (4) utilize skin penetration enhancers in order to ensure SC lipid disruption that leads to drug diffusion into underlying skin layers. With the purpose of optimizing the development of dermal SEDDSs and SDEDDSs, this review provides an FRS that offers guidelines in terms of excipient selection for different BCS drug classes as displayed in Figure 5.

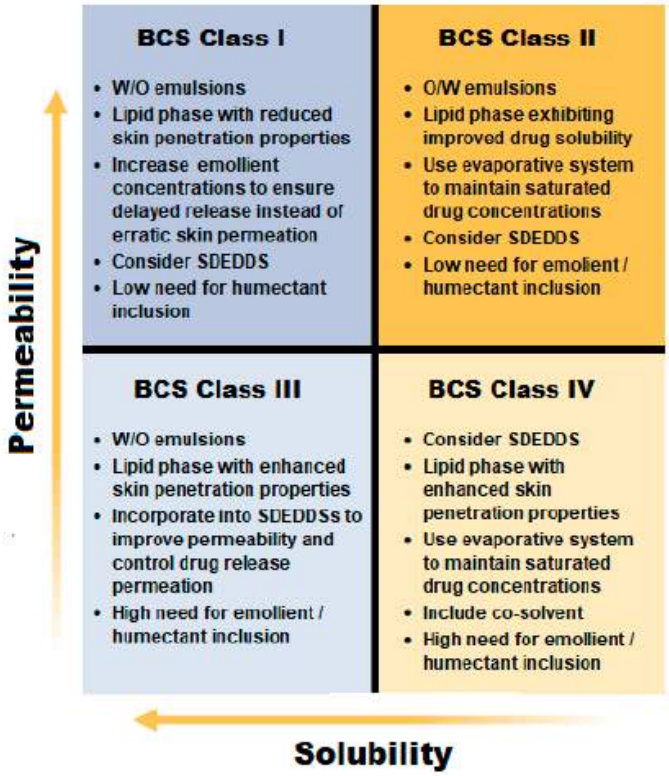

Figure 5. Formulation recommendation system (FRS) when considering drugs belonging to different biopharmaceutical classifications for incorporation into dermal spontaneous emulsions.

As demonstrated in Figure 1, BCS Class I drugs have high permeability and high aqueous solubility [23]. Therefore, this review recommends attempting delivery of these types of drugs from w/o emulsions, as the external lipid phase should provide sufficient skin penetration enhancement properties while the drug is mainly concentrated in the internal water phase. If a BCS Class I drug exhibits excellent permeability across the SC, the external oil phase can be selected to restrict diffusion, which in turn will render controlled drug release during dermal application. This restricted diffusion is also of importance when aiming to deliver a BCS Class I drug into the skin layers (topically) instead of establishing systemic drug delivery (transdermal). Lipids comprising elevated SFA levels can be contemplated as a good choice, as SFAs are known to cause decreased lipid disruption compared to UFAs [14,31,32]. On the other hand, emollient inclusion will influence viscosity and may establish delayed drug release compared to formulations of lower viscosity [57,58]. Moreover, utilizing SDEDDSs instead of SEDDSs can furthermore provide delayed as well as controlled drug release of BCS Class I drugs, especially if therapy with more than one drug is desired. In addition, BCS Class I drug delivery will not necessarily benefit highly from humectant inclusion since these drugs are known to have high permeability properties and humectant inclusion improves dermal drug permeation.

Next, BCS Class II drugs can be recommended for inclusion in either w/o or o/w emulsions. This statement is made bearing in mind the benefits of delivering a lipophilic drug from a w/o system where the external oil phase provides a larger medium to dissolve the drug into compared to o/w emulsions, while also providing skin penetration enhancement due to the presence of skin penetration enhancers in the oil phase. However, it can be reasoned that a lipophilic drug will remain trapped in a mainly lipophilic vehicle instead of crossing the lipophilic SC [82,83], hence risking a reservoir effect in the SC without diffusivity into underlying skin layers. Therefore, despite the advantage of utilizing increased oil concentrations to fully dissolve BCS Class II drugs, an oil phase should be included that renders increased drug solubility in lower concentrations while incorporating BCS

Class II drugs into o/w emulsions. Likewise, co-solvent inclusion as well as generating SDEDDSs instead of SEDDSs can contribute toward increased drug solubility, which leads to enhanced dermal drug delivery [80]. Furthermore, SDEDDSs provide the possibility of optimizing drug solubility while controlling dermal drug permeation, as BCS Class II drugs are limited in terms of aqueous solubility and not drug permeability [84]. Last, evaporative drug delivery systems can similarly be considered in order to maintain saturated drug concentrations at the skin surface to benefit dermal diffusion of a BCS Class II drug [80].

On the other hand, BCS Class III drugs will be more suitable for w/o emulsion inclusion as these drugs have sufficient aqueous solubility while needing assistance with membrane permeation [23]. Therefore, an oil phase with high dermal lipid-disruptive properties should be included [32]. Moreover, a SDEDDS formulation will benefit these drugs as a multiple-emulsion formulation provides the alternative of including powerful skin penetration enhancers in different phases that can establish controlled skin permeation [15]. Emollient or humectant inclusion will greatly benefit BCS Class III drugs since these auxiliary excipients improve dermal drug permeation by limiting TEWL [62,66].

Finally, BCS Class IV drugs are problematic due to both poor permeability and aqueous solubility [23]. However, dermal drug delivery is not considered impossible if the drug delivery vehicle is designed correctly. The inclusion of potent skin penetration enhancers are vital in order to establish SC lipid disruption for the purpose of achieving dermal drug permeation [32]. Alternatively, utilizing an evaporative drug delivery system to improve drug solubility by maintaining saturated drug concentrations at the skin surface can lead to increased dermal flux [80]. Furthermore, co-solvent inclusion and/or SDEDDS development should receive serious consideration [15]. SDEDDSs are of particular advantage during delivery of BCS Class IV drugs, as non-aqueous SDEDDSs can be utilized to optimize drug solubility while achieving effective dermal drug diffusion due to the reversible SC lipid disruption made possible through the inclusion of natural skin penetration enhancers [5,14,32]. In addition, emollient or humectant inclusion should not be forgotten as these excipients, as stated previously, can contribute toward enhanced dermal drug diffusion by limiting TEWL while creating drug delivery channels for drug diffusion between the strict structure of the SC lipids [58]. If BCS Class IV drugs are compared to BCS Class II drugs, it can be reasoned that either w/o or o/w emulsion inclusion will suffice if effective skin penetration enhancers and drug solubilizers are incorporated. However, multiple emulsions such as SDEDDSs seem to be a more promising alternative as both o/w and w/o emulsions are restricted in terms of excipient quantity and compatibility if desiring spontaneous emulsification together with enhanced skin penetration and improved drug solubility. Therefore, SDEDDSs can provide an alternative dosage form to optimize BCS Class IV dermal drug delivery due to their capacity to include more excipients to assist with improved drug delivery.

5. Conclusions

As spontaneous emulsions develop as a dosage form able to cross barriers such as mucus membranes and skin, the importance of developing spontaneous multiple emulsions should not be ignored. Formulation of multiple emulsions is considered complex as o/w and w/o systems must exist in a single system [84]. However, these systems are known to be of a finer texture and smooth touch upon application [84]. Moreover, multiple emulsions can protect drugs from degradation and establish controlled drug release rates [84]. This controlled release can be established by the capacity of multiple emulsions to create internal depots by entrapping drugs from an external diluted continuous phase [84]. Furthermore, double emulsions are also able to improve solubility of drugs [84]. Hence, the above can provide a solution to delivering both drugs of poor permeability and those with increased lipophilicity, which are known to impede successful dermal drug delivery.

This review aimed to provide insight into the main excipients of dermal spontaneous emulsions, such as the lipid, water, and surfactant components, as well as preparation methods of SEDDSs. Additionally, the potential role of auxiliary excipients during formula-

tion of dermal spontaneous emulsions was also discussed. Emphasis must be placed on the importance of spontaneous emulsification and excipient compatibility between the main components of spontaneous emulsions; and that auxiliary excipients are not responsible for establishing spontaneous emulsification but instead are included to provide extended emulsion shelf-life while improving dermal drug delivery. Moreover, future prospects of dermal spontaneous emulsions can include the investigation of different formulation techniques and the effect that these different techniques may have on factors such as droplet size and ease of dermal diffusion. Therefore, to the knowledge of the authors, this is the first review of its kind suggesting refinement of self-emulsified drug delivery systems destined for dermal drug delivery by including auxiliary excipients well known to the pharmaceutical industry.

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Chapter 4

Research Article published in '*Pharmaceuticals*'

Chapter 4 is a **research article,** titled: '***The Development of Dermal Self-Double-Emulsifying Drug Delivery Systems: Preformulation Studies as the Keys to Success***', published in '*Pharmaceuticals*' (DOI: 10.3390/ph16101348). Here the methods and results of preformulation studies are discussed. Firstly, preformulation studies were conducted to generate and select excipients to produce potential primary emulsions. Primary emulsions were then considered for the purpose of establishing suitability for inclusion into SDEDDSs. In conclusion, the selected primary emulsion and SDEDDS were subjected to droplet size and polydispersity index measurement as well as examination via microscopy and transmission electron microscopy.

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Article

The Development of Dermal Self-Double-Emulsifying Drug Delivery Systems: Preformulation Studies as the Keys to Success

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Abstract: Self-emulsifying drug delivery systems (SEDDSs) are lipid-based systems that are superior to other lipid-based oral drug delivery systems in terms of providing drug protection against the gastrointestinal (GI) environment, inhibition of drug efflux as mediated by P-glycoprotein, enhanced lymphatic drug uptake, improved control over plasma concentration profiles of drugs, enhanced stability, and drug loading efficiency. Interest in dermal spontaneous emulsions has increased, given that systems have been reported to deliver drugs across mucus membranes, as well as the outermost layer of the skin into the underlying layers. The background and development of a double spontaneous emulsion incorporating four anti-tubercular drugs, clofazimine (CFZ), isoniazid (INH), pyrazinamide (PZY), and rifampicin (RIF), are described here. Our methods involved examination of oil miscibility, the construction of pseudoternary phase diagrams, the determination of self-emulsification performance and the emulsion stability index of primary emulsions (PEs), solubility, and isothermal micro calorimetry compatibility and examination of emulsions via microscopy. Overall, the potential of self-double-emulsifying drug delivery systems (SDEDDSs) as a dermal drug delivery vehicle is now demonstrated. The key to success here is the conduct of preformulation studies to enable the development of dermal SDEDDSs. To our knowledge, this work represents the first successful example of the production of SDEDDSs capable of incorporating four individual drugs.

Keywords: clofazimine; isoniazid; lipid-based drug delivery; multiple emulsion; pseudoternary phase diagram; pyrazinamide; rifampicin; self-double-emulsifying drug delivery system; self-emulsifying drug delivery system; skin

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1. Introduction

Lipid-based drug delivery systems are under intensive examination for improving the oral bioavailability of lipophilic drugs [1,2]. Self-emulsifying drug delivery systems (SEDDSs) are superior to other lipid-based systems by providing protection against the gastrointestinal (GI) environment, inhibition of drug efflux mediated by P-glycoprotein, increased lymphatic drug uptake, improved control of plasma drug concentration profiles, improved stability, and enhanced drug loading efficiency [3,4]. SEDDS are an isotropic mixture of synthetic and/or natural oils used in combination with a surfactant and co-surfactant that can spontaneously transform into an emulsion when introduced with gentle agitation into aqueous media [1,4]. The original conceptual basis of the manufacture of SEDDSs was via introduction of an isotropic mixture of oil and surface active agents into GI fluids under mild agitation, as achieved by the peristaltic movements of the GI tract, in order to establish self-emulsification [1,4]. SEDDSs have also been shown to penetrate barriers such as the mucus gel layer of the GI tract, and the outermost protective skin barrier [2,5,6].

The mucus gel layer of the GI tract comprises water (90–95%), glycoproteins, and additional minor components such as DNA, lipids, and electrolytes [5]. These components

must be considered when developing formulations that have to cross this barrier [5]. Mucus glycoproteins comprise an entangled visco-elastic network that protects the underlying GI epithelium against xenobiotics and microorganisms [5]. Thus, this barrier will hinder delivery of nanosized drug delivery systems, leading to the reduced oral bioavailability of the incorporated drugs [5]. However, SEDDSs that incorporate a hydrophilic polyethylene glycol (PEG) surface layer (referred to as a PEGylated surface) are able to cross this mucus barrier [5]. Moreover, SEDDSs with a droplet size <50 nm are able to diffuse easily through the mucus network; this layer generally prohibits the entry of substances and organisms of sizes ranging between 100–200 nm [5]. Also, SEDDSs with proteolytic enzymes anchored on the surface can potentially increase mucus permeability [7]. Hence, given the hydrophilic nature of the mucus layer of the GI tract, it is noteworthy that SEDDSs are able to cross this barrier. SEDDSs also have the capacity to deliver drugs through the lipophilic barrier provided by the outermost skin layer, that is, the stratum corneum (SC) [6]. The capacity of a drug delivery vehicle to cross such divergent biological barriers is thus worthy of further investigation.

Compared to conventional drug administration routes, the dermal route is considered safe and non-invasive, and is especially useful for delivering drugs of poor aqueous solubility [8]. Two important questions arise in relation to formulation of SEDDSs: (i) can the mechanism of spontaneous emulsification be defined? (ii) is it possible to modulate the spontaneous emulsification properties of these lipid-based formulations? [9]. Notably, utilization of these emulsions as vehicles for the simultaneous delivery of multiple drugs remains a slowly growing field [10]. The simultaneous delivery of multiple drugs poses several challenges, including drug–drug interactions, and the need to select components of the emulsion that are able to dissolve and incorporate the drugs as well as the excipients [10]. Therefore, although SEDDSs are valuable topical/transdermal drug delivery systems, there do remain certain limitations [4,6]. Nevertheless, the further investigation of self-double-emulsifying drug delivery systems (SDEDDSs) is required in order to develop dermal emulsions as multidrug delivery vehicles [11].

2. Results and Discussion

2.1. Oil Immiscibility Studies

All experiments were carried out in triplicate, and the results are expressed in Figure 1 as mean ± SD (n = 3) values for the different oils tested. No external oil phase mixed with beeswax or linseed oil displayed clear separation. Further, no separation was detected between shea butter and any internal oil phase. Therefore, Transcutol®:PEG (9:1) was selected as the only suitable internal oil phase. Clear separation between Transcutol®:PEG (9:1) and natural oils was detected in the following order: evening primrose oil (EPO) > olive oil (OLV) > avocado oil (AVO) > palm oil (PALM) > safflower oil (SAFF).

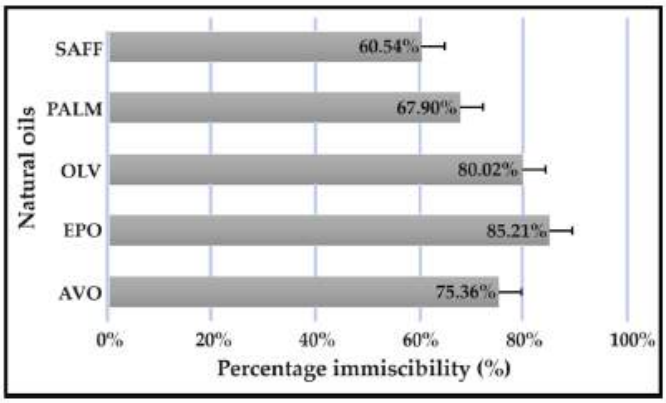

Figure 1. Immiscibility percentage of individual natural oils mixed with a combination of Transcutol®:PEG (9:1).

These results can be explained by the polar natures of Transcutol® and PEG due to their relatively high dielectric constants and hydrogen bonding [2,12]. Transcutol® also known as diethylene glycol monoethyl ether, is a liquid well known to the cosmetic industry due to its favorable solubility properties that improve the solubilization of lipophilic and hydrophilic drugs [13]. Moreover, Transcutol® is frequently included as a co-surfactant and skin penetration enhancer [13]. The addition of Transcutol® to a dermal formulation with an aqueous component will influence the ionization equilibrium of the incorporated drugs, should they be capable of undergoing ionization [12]. This solvent effect occurs when a liquid with a dielectric constant lower than that of water is added to an aqueous solution of a drug. The consequence of solvating drugs with a solvent of a lower dielectric constant suppresses the extent of ionization and enhances ion pair formation at the expense of forming solvent-separated ion pairs [12]. Therefore, the larger amount of neutral, unionized drug and improved formation of ion pairs induced by the addition of Transcutol® will increase the quantity of un-ionized drug able to cross the skin barrier [12].

It is reported that inclusion of both water and Transcutol® in dermal formulations is advantageous, as the absence of water decreases the thermodynamic driving force needed to enable penetration of drug into the skin [12]. The addition of water permits a drug to precipitate from solution, and therefore enhances the driving force required to establish dermal flux [12]. This is especially true for lipophilic drugs, since lipophilic drugs may tend to remain in a lipophilic vehicle or create a reservoir effect where the drug remains in the lipophilic SC instead of entering the underlying hydrophilic layers of the skin [4,6]. Hence, it can be argued that it would be better to include one of the natural oils as the internal oil phase so as to enable Transcutol® to fulfil the role of the external oil phase. Clear phase separation of water-in-oil-in-oil (W/O/O) emulsions will potentially be exhibited due to the hydrophilic nature of Transcutol®–PEG mixtures. However, because addition of water and the formation of binary mixtures of Transcutol® with water favor dermal drug delivery, compared to Transcutol® alone it would be inappropriate to include the Transcutol®–PEG mixture as the external oil phase and separate water and Transcutol® with an internal oil phase. This may prevent the SDEDDSs from releasing the incorporated drugs, despite exhibiting desirable SDEDDSs properties [12]. Moreover, Transcutol® is considered a well-tolerated topical excipient with the ability to solubilize both hydrophilic and lipophilic drugs, which may be highly beneficial when considering the various lipophilicity profiles of model drugs [12].

2.2. Evaluation of the Pseudoternary Phase Diagram to Find Potential Primary Emulsions

A large self-emulsification area was observed, which may signify appropriate excipient combinations. As indicated in Figure 2, four checkpoint formulations were selected as potential primary emulsions (PEs). Checkpoint formulations were selected based on the concept of avoiding regions with poor dermal drug delivery properties. The concentration of the surfactant phase was limited to a ratio of no more than 5 compared to other components of the triplot, because increased surfactant concentrations are known to cause dermal irritation [6]. Moreover, reversed micelle occurrence was avoided by omitting oil ratios > 9 [6]. Lastly, water-rich areas known for micelle formation were avoided by selecting checkpoint formulations of ratios less than 9 in terms of water content. It was consequently decided to select checkpoint formulations with the highest internal oil phase and the highest water content possible due to the capacity of Transcutol® to solubilize both lipophilic and hydrophilic drugs [12]. Furthermore, water is also a crucial component needed to optimize dermal drug delivery facilitated by partnering water and Transcutol®, and the inclusion of hydrophilic drugs such as isoniazid (INH) and pyrazinamide (PZY) can benefit from the presence of a greater aqueous component [12].

As indicated in Figure 2, four checkpoint formulations were selected (PE 1, PE 2, PE 3 and PE 4). Next, the PEs were evaluated by being subjected to self-emulsification performance testing and determination of the emulsion stability index (ESI).

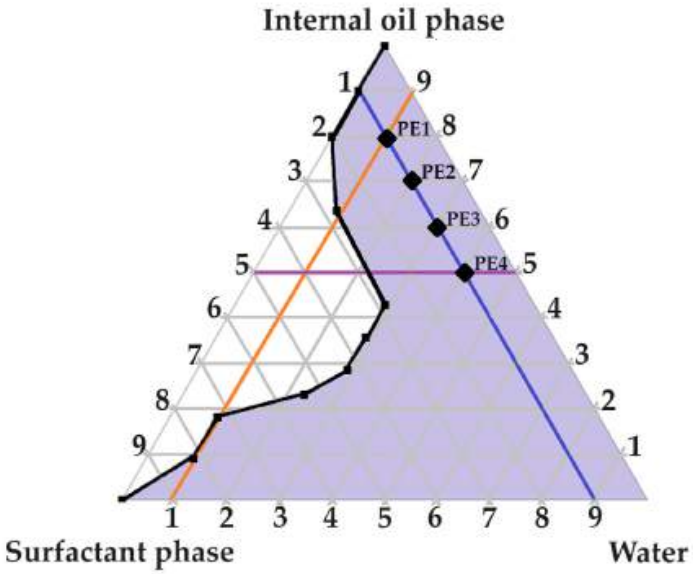

Figure 2. Pseudoternary phase diagram of water, surfactant phase and internal oil phase. The numbering represents the given parts of the different phases included in a ratio at a specific point on the pseudoternary phase diagram.

2.3. Evaluation of Primary Emulsions

2.3.1. Self-Emulsification Performance

Rapid emulsification is highly favorable when developing oral SEDDSs or SDEDDSs, as spontaneous emulsification is a rate-limiting step preceding successful drug absorption [14]. However, drug diffusion through the highly lipophilic SC marks a rate-limiting step during dermal drug delivery [4,6]. Therefore, slower rates of spontaneous emulsification are desired when designing dermal SEDDSs or SDEDDSs, since formulations with elongated self-emulsification rates can predict prolonged contact between the skin and the applied formulations due to the presence of occlusive formulation characteristics [4,6]. Therefore, only formulations that received C or D grading were considered. Fortunately, PE 1, PE 2 and PE 3 received D grading with self-emulsification times of 3 min, 4 min 30 s, and 5 min 10 s, respectively, and were consequently deemed fit for further investigation. However, PE 4 was identified as an E-grade emulsion due to large oil droplet formation, despite a self-emulsification time of 5 min 50 s, only 40 s longer than depicted by PE 3.

2.3.2. Emulsion Stability Index

The PEs were visually inspected after removal from a water bath set at a temperature of 68 °C for a period of 3 h [15]. No phase separation was observed for PE 1, PE 2, or PE 3, which indicated an ESI of 100%. Emulsion instability was found in PE 4 (ESI of 80%), as visible creaming occurred. For this reason, PE 4 was excluded from future characterization experiments.

The poor robustness shown by sudden short exposure to a high temperature may be associated with an increase in the surfactant concentration. Interestingly, an elevation of the surfactant content can reduce the size of the droplets until the surfactant concentration reaches a concentration at which the size of the droplets increases, as established by the aggregates produced from excess surfactant included [16]. The sudden increase in temperature exposure accelerated this instability process, indicating that PE 4 is not suitable for further consideration.

2.4. Construction of Pseudoternary Phase Diagrams for Self-Double-Emulsifying Drug Delivery Systems

Figures 3–7 indicate the self-double-emulsification areas observed for different combinations of the selected excipients. Pseudoternary phase diagram construction is necessary to confirm if spontaneous emulsification is achievable, and especially to establish the ease and degree of self-emulsification made possible by refined excipient selection during preformulation decision making. Excellent self-emulsification was observed during the addition of natural oils to the fixed mixture of water and Transcutol®:PEG together with surfactant. It may seem to be unusual to use the fixed combination of the internal oil phase and the surfactant phase as a single component of the triplot instead of plotting the water and the internal phase together, while plotting the surfactant phase separately. However, the use of decreased surfactant and internal oil phase concentrations are imperative, as the propensity for self-emulsification is intensified with limited surfactant phase content. Decreased surfactant concentrations imply a reduced risk of skin irritation, especially during prolonged use, as well as lowered production costs [4,6]. Moreover, careful attention must be paid to the Transcutol®–PEG content of SDEDDSs, as Transcutol® should be included in moderate amounts compared to water, due to its high affinity for water that limits the dermal diffusion if it is not accompanied by sufficient water concentrations [12].

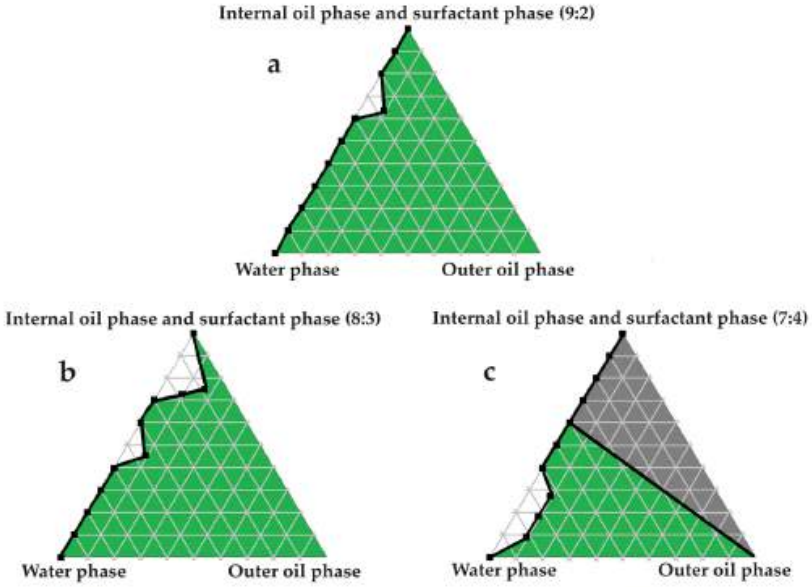

Figure 3. Pseudoternary phase diagrams of water, avocado oil, and fixed proportions of the surfactant and internal oil phases. In (**a**) the internal oil and surfactant phases are combined in a ratio of 9:2; in (**b**) the internal oil and surfactant phases are blended in an 8:3 ratio; and in (**c**) the internal oil and surfactant phases are included as a 7:4 ratio.

Excellent self-emulsification was exhibited during the construction of the pseudoternary phase diagrams of the tested SDEDDSs, as seen in Figures 3–7. Limited variation could be related to the addition of different external oil phases, since the spontaneous emulsification behavior were closely similar However, clear differences can be seen when the region of self-emulsification is compared for different Transcutol®:PEG:surfactant phase ratios. This phenomenon is attributed to the instability of SDEDDSs due to increased surfactant concentrations [17]. It should be noted that Transcutol® can also be employed as a co-surfactant [18]. Therefore, if the ratio of Transcutol®:PEG:surfactant phase is not optimized, Transcutol® can enhance the effect of the surfactant phase and thus further contribute to the emulsion instability. The gray areas indicated in the 7:4 ratio (internal oil phase:surfactant phase) in the pseudoternary phase diagrams signify that self-emulsification was observed, but the consistency of the SDEDDSs changed to high viscosity, eliciting poor formulation

properties such as creaming and excipient precipitation. Again, this is a clear indication that the use of increased concentrations of the surfactant phase are not favorable. Interestingly, this can be related to the reduced difference in the ratio of the internal oil phase compared to the surfactant phase, represented by the 8:3 and 7:4 ratios (internal oil phase to surfactant phase). Therefore, it was decided to maintain an internal oil-phase-to-surfactant-phase ratio of 9:2.

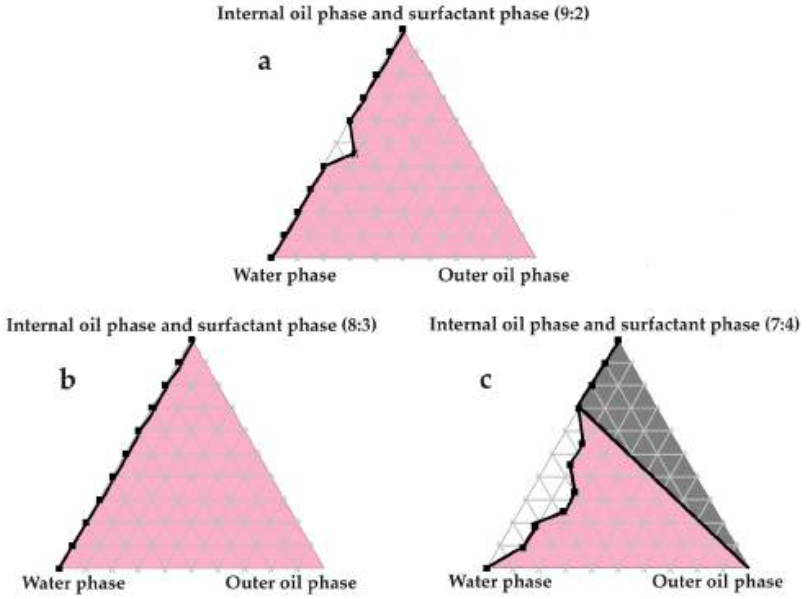

Figure 4. Pseudoternary phase diagrams of water, evening primrose oil, and secure fractions of the surfactant and internal oil phases. In (**a**) the internal oil and surfactant phases consist of a 9:2 ratio; in (**b**) the internal oil and surfactant phases comprise an 8:3 ratio; and in (**c**) the internal oil and surfactant phases are combined in a 7:4 ratio.

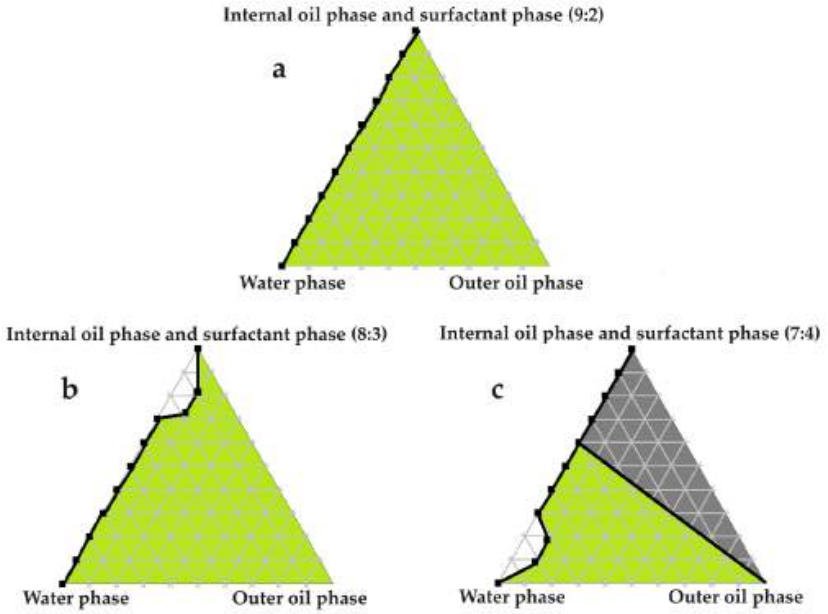

Figure 5. Pseudoternary phase diagrams of water, olive oil, and fixed ratios of the selected surfactant and internal oil phases. In (**a**) the internal oil and surfactant phases comprise a ratio of 9:2; in (**b**) the internal oil and surfactant phases consist of an 8:3 ratio; and in (**c**) the internal oil and surfactant phases are blended in a 7:4 ratio.

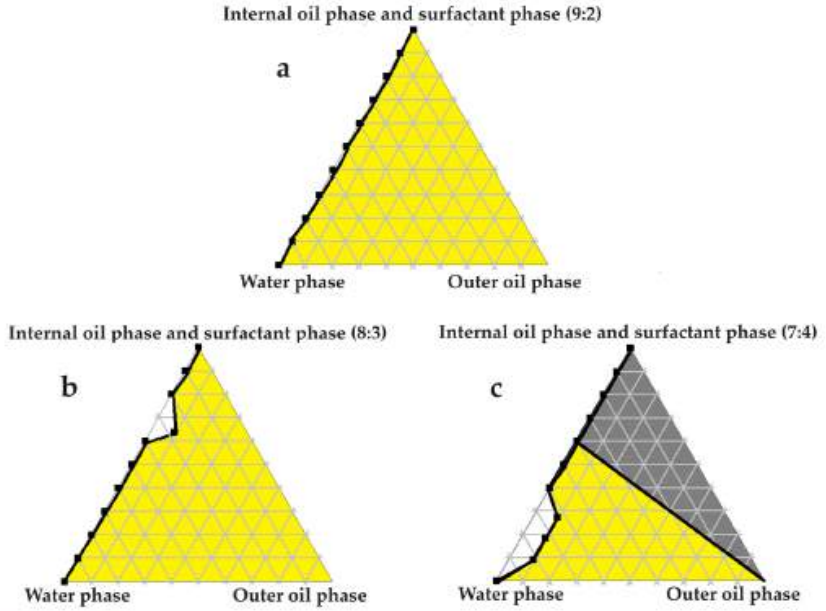

Figure 6. Pseudoternary phase diagrams of palm oil and set ratios of the chosen surfactant and internal oil phases. In (**a**) the internal oil and surfactant phases are combined in a ratio of 9:2; in (**b**) the internal oil and surfactant phases are blended in an 8:3 ratio; and in (**c**) the internal oil and surfactant phases are included as a 7:4 ratio.

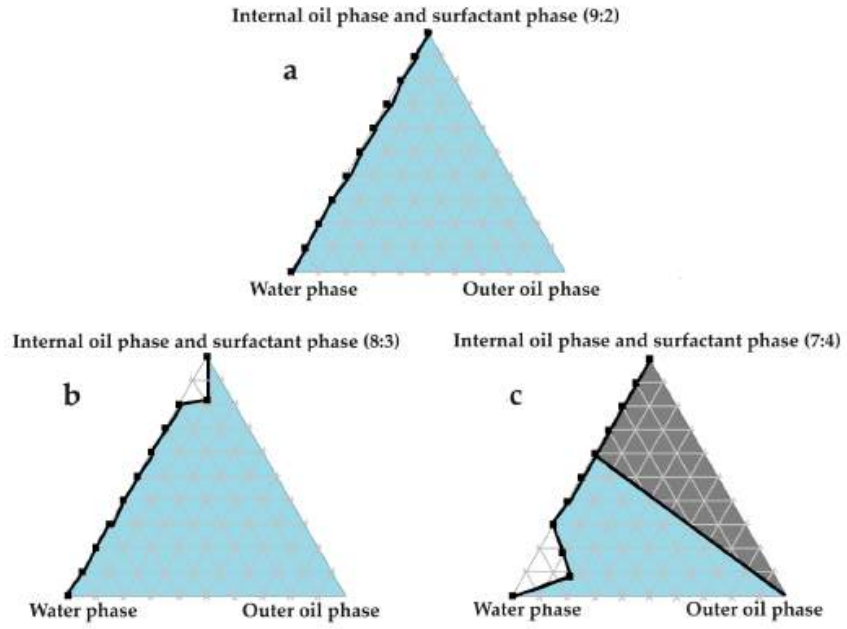

Figure 7. Pseudoternary phase diagrams of water, safflower oil, and set proportions of the surfactant phase as well as the internal oil phase. In (**a**) the internal oil and surfactant phases consist of a 9:2 ratio; in (**b**) the internal oil and surfactant phases comprise an 8:3 ratio; and in (**c**) the internal oil and surfactant phases are combined in a 7:4 ratio.

2.5. Solubility Studies

Solubility studies are crucial for determining the amount of excipients needed to best solubilize different drug combinations, while maintaining concentrations close to saturation to enhance the driving force needed to achieve dermal diffusion [4,6,19]. The solubility

of antitubercular drugs was determined for the following solvents: AVO-, OLV-, EPO-, PALM-, SAFF, and PE, comprising a 9:9:2 ratio (internal oil phase:water phase:surfactant phase), as displayed in Figures 8–11. The results are expressed as mean ± SD (n = 3).

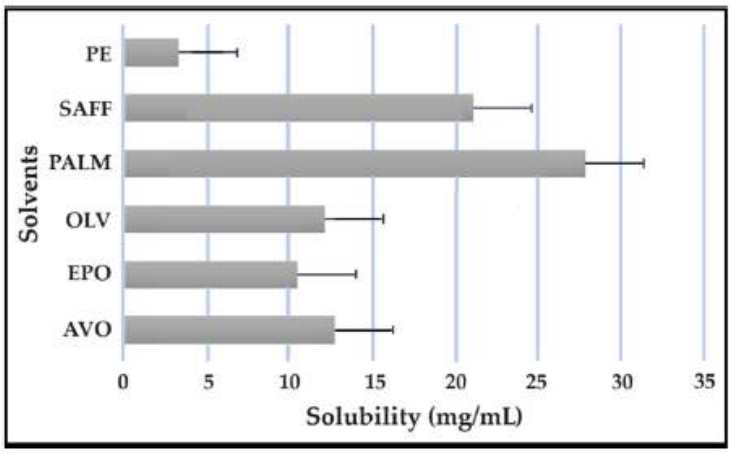

Figure 8. Solubility of CFZ in the solvents.

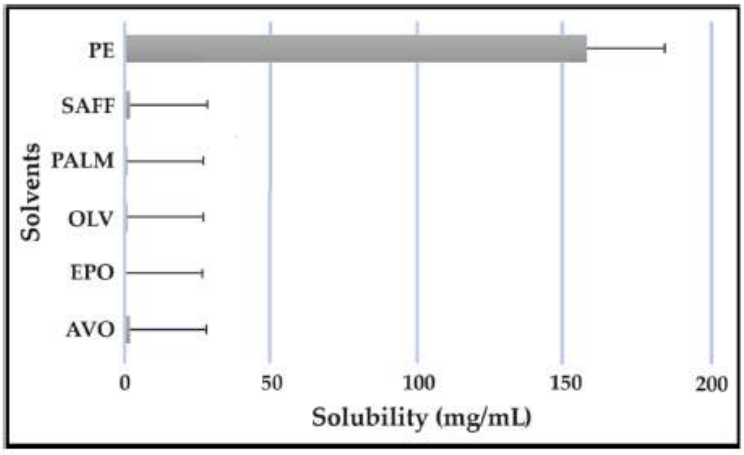

Figure 9. Solubility of INH in the solvents.

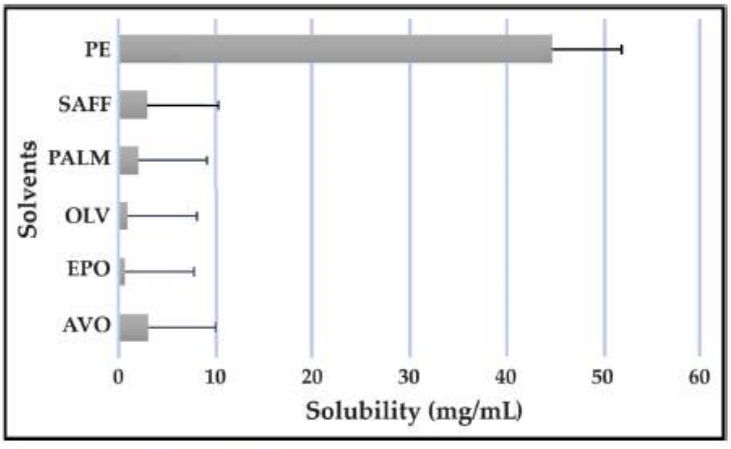

Figure 10. Solubility of PZY in the solvents.

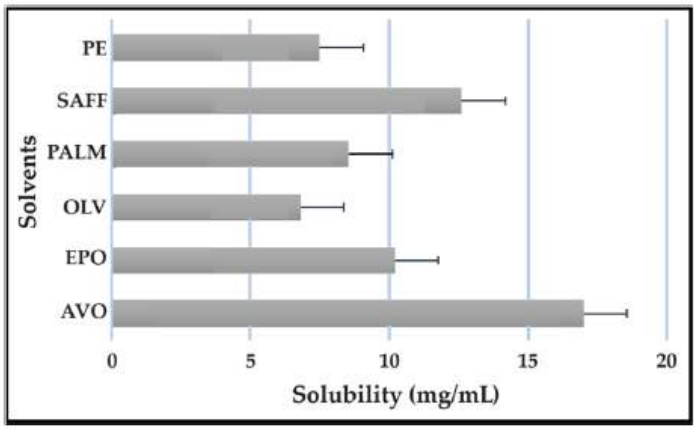

Figure 11. Solubility of PZY in the solvents.

As indicated, the lipophilic drugs, CFZ and RIF, displayed a higher solubility in natural oils compared to the more hydrophilic drugs, INH and PZY. Moreover, INH and PZY exhibited increased solubility in the PE. As expected, the aqueous solubility of individual drugs indicated in Table 1, increased when solubilized in the PE. This is attributed to the addition of Transcutol® and the surfactant phase to the water phase. This addition improved the aqueous solubility of all drugs, since Transcutol® can solubilize both lipophilic and hydrophilic compounds [12]. Similarly, the inclusion of surfactants is also known to improve the solubility of drugs included in emulsions [20]. Clearly, the PE will be effective in solubilizing INH and PZY. However, an external oil phase must be selected to optimize the solubility of both CFZ and RIF.

Table 1. Physicochemical properties of the selected anti-tubercular drugs [6,19,21–31].

Drug	Log P	Molecular Weight (D_a)	Aqueous Solubility	Elimination Half-Life	Metabolized	BCS Classification
CFZ	7.66	473.40	<1 mg/mL	70 days	Hepatic	Class II
INH	0.64	137.14	125 mg/mL	45–110 min [1] 2–4.5 h [2]	Hepatic	Class I/III [3]
PZY	−1.88	123.11	15 mg/mL	3–5 h	Hepatic	Class III
RIF	3.80	822.90	1.51 mg/mL	3–4 h	Hepatic	Class II

[1] rapid metabolizers. [2] slow metabolizers. [3] INH is considered highly water soluble; however, data that reflect its oral absorption and permeability are inconclusive. Therefore, we suggest that INH can be regarded as somewhere between BCS Class I and III.

PALM induced the highest solubilization of CFZ, whereas RIF had the highest solubility in AVO. However, as CFZ causes dose-dependent skin discoloration, the aim is to reduce the concentration of CFZ when it is used together with PZY. It has been found that the dose of CFZ can be substantially reduced from 25 mg/kg to 6.25 mg/kg when administered orally together with PZY due to the synergistic activity achieved when CFZ and PZY are used in combination [27]. Hence, AVO would be the first choice, as adequate solubility of CFZ would be possible if the CFZ was included in reduced concentrations, while providing a vehicle capable to fully solubilizing RIF. Therefore, AVO would be the ideal external oil phase if CFZ, INH, PZY, and RIF, or CFZ, PZY, and RIF were to be included in a SDEDDS. Nonetheless, if it is decided to include CFZ, INH, and RIF, then PALM or SAFF can also be considered, as increased doses of CFZ will have to be included in the absence of PZY. On the other hand, PALM would be best suited for inclusion of CFZ together with PZY and INH. However, when selecting an external oil phase, the skin penetration enhancement properties of the oil should be considered, as this can greatly affect dermal drug delivery [4,6,11]. In addition, the results obtained during the immiscibility studies should be considered, as a higher degree of immiscibility presents a greater opportunity to successfully formulate SDEDDSs [2,11]. Therefore, for the purpose of this work, AVO

was selected as the external oil phase when formulating a SDEDDSs comprising the four antitubercular drugs for microscope examination, due to its favorable solubility properties and the reliable skin penetration enhancement characteristics [6].

2.6. Isothermal Micro Calorimetry Compatibility Studies

Isothermal microcalorimetric studies indicated that differences existed between the slopes and y-intercepts of the measured and theoretical (no interaction) heat flows of certain samples tested during compatibility studies. Additionally, the absolute values of the interactions integral to these samples were also greater than zero. Therefore, potential interactions were observed for the following combinations: OLV and PE (−38.01 J/g); AVO and PE (2.64 J/g); EPO and PE (−29.18 J/g); PALM and PE (26.20 J/g); SAFF and PE (85.90 J/g); PE and PZY (−26.54 J/g); PE and CFZ (36.36 J/g); PE and RIF (39.15 J/g); PE and INH (36.61 J/g); CFZ and INH (2252 kJ/g); as well as INH and RIF (822.89 J/g). However, potential interactions between PE and selected external oil phases were expected because the external oil phases were included due to the clear immiscibility between the internal oil phase and external oil phases during the oil immiscibility studies. Therefore, this expected so-called incompatibility is only due to the inability of the oils to mix, and is considered insignificant, as these excipients were intentionally included as a result of their immiscibility with the internal oil phase in order to enable production of double emulsions.

Remarkably, all of the model drugs exhibited potential incompatibility with the PE. Nonetheless, this may be explained by the increased solubility of the drugs observed in the PE compared to the aqueous solubility reported in the literature. The PE comprises Span®83, Transcutol®, Tween®60 and water, excipients that are frequently included in dermal formulations due to their favorable safety, solubilizing, and emulsion-stabilizing properties [4,6,12]. Therefore, this is deemed a physical incompatibility rather than a chemical incompatibility; therefore, it is still possible to use these specific excipient combinations, as physical incompatibilities do not influence formulation stability [6].

In terms of drug–drug interactions, it is noted that synergism is obtained when CFZ is used in combination with the first-line antitubercular agents INH and RIF [28]. This combination is frequently administered to tuberculosis (TB) patients. It has been well documented that RIF shows significant instability in the presence of dissolved INH in an acidic environment [29]. Hence, the emphasis shifts from incompatibility between drugs to the importance of formulation. Selected controlled-release formulations can retain RIF while releasing INH so as to avoid deleterious drug–drug interactions which may lead to reduced drug absorption and decreased therapeutic efficacy [29]. However, as the pH of the skin is normally between 5.4 and 5.9 [4], this is not considered to be sufficiently acidic to exclude the use of a RIF and INH combination in a SDEDDS.

2.7. Microscope Examination

Evaluation of the PEs by microscopic examination revealed that the PE ratio of 9:9:2 (internal oil phase:water phase:surfactant phase) was the only PE that exhibited droplet formation. This supports the decision to exclusively use this ratio, and only vary the external oil phase according to the antitubercular drugs included in the SDEDDSs. The microscopic image was captured without any drugs present, as shown in Figure 12.

PE 1 (9:9:2) was subjected to droplet size analysis and polydispersity index (PDI) determination. The results are expressed as mean ± SD (n = 3). The droplet size was measured as an average of 173.90 ± 2,30 nm and a PDI value of 0.236 ± 0,11. As shown in Figure 12, the droplet size distribution can be considered homogenous. The accuracy of this ratio is supported by literature comments indicating that the ideal emulsion should contain equal parts water and oil, while being stabilized by appropriate surfactant(s) [30]. However, the presence of PEG as part of the internal oil phase together with Span®83 and Tween®60 included as surfactants may also contribute to the ability of the PE to remain stable, since PEG can be used as a surface-active agent as well as a solvent [11,31]. Next, PE with a ratio of 9:9:2 was prepared, and AVO was used as the external oil phase due

to the favorable solubility properties exhibited by both lipophilic drugs in this vehicle. A checkpoint formulation including a high concentration of the internal oil phase to provide sufficient solubilization of INH and PZY while at the same time providing a moderate amount of the external oil phase, so as to ensure saturated concentrations of the lipophilic components, was selected (Figure 13). This decision is based on the understanding that lipophilic components tend to form a reservoir in the SC or remain in the lipid component of the formulations if the driving force of diffusivity across the SC is not enhanced by formulation techniques such as oversaturation [4,6,11,19].

Figure 12. Microscopic image of PE 1 (9:9:2 ratio) comprising no active ingredients.

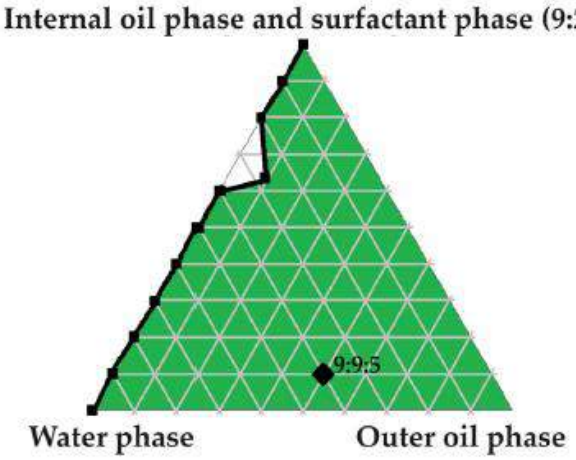

Figure 13. Pseudoternary phase diagram utilized to select a checkpoint formulation (9:9:5) for SDEDDS development.

The checkpoint formulation, namely 9:9:5 (water phase to internal oil and surfactant phase (9:2) to outer oil phase) was prepared. Preparation was commenced by mixing internal oil phase components (Transcutol®:PEG ratio of 9:1) by means of gentle stirring until fully combined. At the same time, the surfactant phase was separately prepared by gently stirring Span®60 and Tween®83 in a ratio of 1:1 until completely blended. Next, surfactant phase and the internal oil phase were combined (9:2 ratio of internal oil phase to surfactant phase) while being subjected to mild stirring for a duration of 30 min. Thereafter,

a predetermined quantity of water, a ratio of 9 in this case, was added in small increments while applying steady agitation. After subjecting this mixture to homogenization for 4 min, in order to generate a PE, the pre-determined quantity of five parts external oil phase was added in a dropwise fashion while gently stirring. After continuous stirring, the mixture was removed from the stirring plate and subjected to sonication until the desired droplet size was achieved. Drugs were included at a concentration of 2% each. Hence, overall composition of SDEDDS can be summarized as follows: 8% drugs (2% of each drug), 36% water, 20% AVO, 29.5% internal oil phase, and 6.5% surfactant phase.

The checkpoint SDEDDS was characterized by measuring droplet size and PDI. The results are expressed as mean ± SD *(n = 3)*. Average droplet size was revealed to be 92.46 ± 5.52 nm, and the PDI value found to be 0.391 ± 0.05. Therefore, the selected PE and SDEDDS were visually examined via transmission electron microscopy (TEM) for the purpose of gaining a reliable visual representation of the droplets that fell into the nano size range. As the highlight of this study, the TEM observation revealed clear small droplet formation inside larger smooth-surfaced droplets, signifying the successful development of a SDEDDS designed to simultaneously contain four drugs. Moreover, the mechanism of spontaneous emulsification was captured as indicated in Figure 14.

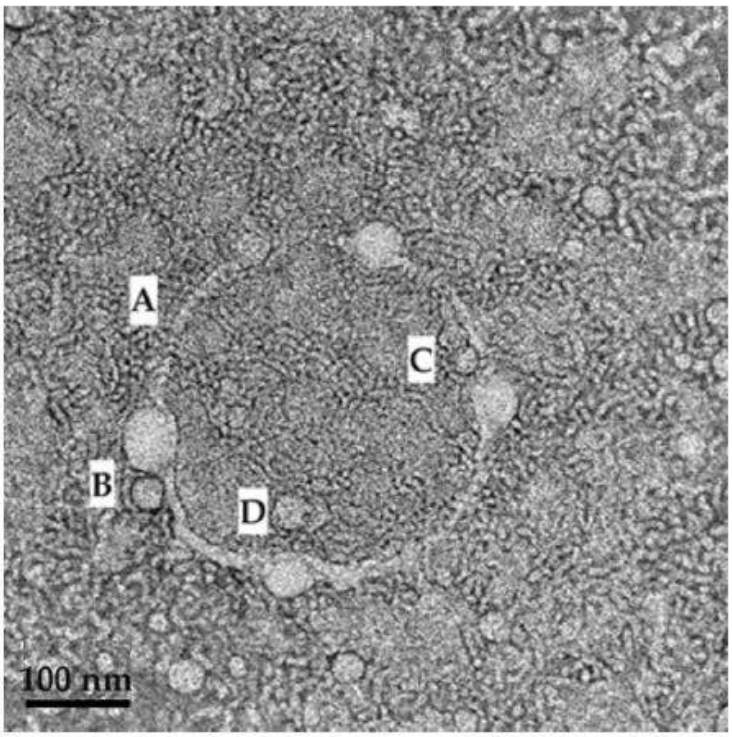

Figure 14. TEM image revealing the mechanism of SDEDDS formation where (**A**) a membrane structure is established by the surfactant phase; (**B**,**C**) and then water is engulfed by the surfactant phase so as to form small droplets inside the internal oil phase. (**D**) indicates where larger droplets harbor smaller droplets inside.

As indicated in Figure 14, clear formation of a small droplet(s) within a larger droplet was achieved. Interestingly, a membrane structure is established by the surfactant phase (A), and then water is engulfed by the surfactant phase, to be captured as small droplets inside the internal oil phase (B and C). However, when considering the scale bar of 100 nm on the TEM image, it is evident that the average droplet size measured during characterization experiments only measured the larger droplets harboring the smaller droplets (D). Hence, this may be considered a novel contribution to the development of dermal SDEDDS, since nano-sized droplets are known to cross the SC with ease compared to larger-sized droplets.

The opportunities of delivering fixed-dose drug combinations via dermal application when included in SDEDDSs instead of SEDDSs can be considered when studying the TEM images of the PE (SEDDS) compared to SDEDDS, displayed in Figure 15a,b.

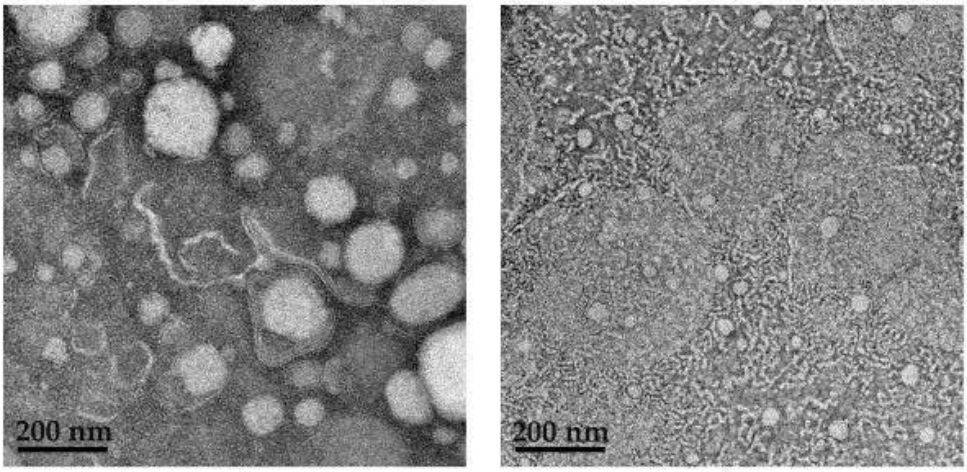

Figure 15. TEM images for (**a**) PE (9:9:2) comprising water to internal oil phase to surfactant phase; (**b**) SDEDDS (9:9:5) containing water to internal oil phase and surfactant phase (9:2) to external oil phase.

As seen in Figure 15a,b, SEDDS is presented as the (a) PE included in (b) SDEDDS. The most noticeable difference is in droplet size, since (a) rendered an average droplet size of 173.90 nm and (b) depicted an average droplet size of 92.46 nm. Interestingly, PDI values signify that (a) is more homogenous compared to (b). However, upon visual examination, (b) seems to be a sample of increased homogeneity compared to (a). This can be attributed to the observation made previously that only large droplets of SDEDDS were measured during droplet size determination due to the incorporation of smaller droplets inside the larger droplets. However, in terms of drug delivery, the TEM images indicate multiple phases presented by SDEDDSs compared to SEDDSs are able to incorporate more than one drug. This is due to the different phases established by water and two immiscible oils than can solubilize polar and non-polar drugs in the same vehicle with potential sustained release characteristics. This aspect requires further examination. TEM images were captured in the absence of drugs, since the red discoloration caused by both CFZ and RIF limits the contrast required for identification of SEDDS and SDEDDS, respectively. Therefore, the importance of preformulation studies during the development of SDEDDSs is emphasized and can be considered the key to success. This is indeed a novel development. To the knowledge of the authors, this represents the first nano-SDEDDS (N-SDEDDS) or W/O/O self-nano-emulsifying drug delivery system (SNEDDS) capable of incorporating four individual drugs that possess strikingly different physicochemical properties.

3. Materials and Methods

3.1. Materials

Combinations of CFZ, INH, PZY, and RIF were utilized as model drugs. CFZ, INH, and RIF were kind gifts from Prof Wilna Liebenberg, head of the Solid-State Pharmaceutical Innovation and Nanotechnology (SPIN) research group at the North-West University (Potchefstroom, South Africa). PZY, Transcutol®, Span®83, and Tween®60 were purchased from Merck (Darmstadt, Germany). AVO, beeswax, EPO, linseed oil, OLV, PALM, SAFF, and shea butter were bought from Nautica Organics Trading (Durban, South Africa). PEG was obtained from DB Fine Chemicals (Sandton, South Africa). Distilled water was acquired through a Rephile Bioscience Ltd. system (Boston, MA, USA).

3.2. Methods for Drug and Excipient Selection

Considering Drug Selection

Antitubercular drugs were selected on the basis of their physicochemical properties and Biopharmaceutical Classification System (BCS) ranking, as indicated in Table 1, to demonstrate the capacity of SDEDDSs to incorporate multiple drugs of different physicochemical natures.

From Table 1, the lipophilicity of drugs may be classified as CFZ >>> RIF >> INH >> PZY. These physicochemical properties were considered in order to obtain an exceptional dermal drug delivery capacity for the SDEDDSs, as the optimal lipophilicity of drugs destined for dermal drug delivery is established at a Log P value of 1–3 and a molecular weight <500 Da [32–34]. None of the selected drugs has a Log P value that falls within the prescribed range for desirable dermal drug delivery. Fortunately, though, INH and PZY have acceptable molecular weights. CFZ, on the other hand, presents a compliable molecular weight. However, this drug is highly lipophilic, as can be concluded by its Log P value of 7.66 and elimination half-life of 70 days. Lastly, the molecular weight of RIF drastically exceeds the prescribed molecular weight, and thus renders it unsuitable for dermal drug delivery. Nevertheless, a recent publication indicated that drugs with a molecular weight that does not exceed 1000 Da can be considered acceptable for dermal drug delivery [35]. Consequently, lipophilicity is considered the determining factor that prohibits the delivery of dermal drugs, instead of the molecular weight of the drugs chosen for this study [35,36].

Developing a topical dosage form containing four drugs to aid in cutaneous tuberculosis (CTB) disease is challenging but necessary due to the daunting nature of *Mycobacterium tuberculosis* [19]. Hence, the requirements for an effective tuberculosis regimen comprise simultaneous administration of various bactericidal and sterilizing anti-tubercular agents that are administered for a sufficient period for the purpose of sustaining anti-microbial effectiveness, while preventing mutations of *Mycobacterium tuberculosis* that might lead to drug resistance [19,37]. Hence, drug combinations are the norm for anti-tubercular treatment regimens [37]. The first-line anti-tubercular drugs utilized during this study included INH, PZY, and RIF [19]. However, resistance against first-line agents is drastically increasing, and there is a need for new anti-tubercular drug combinations as well as new drug entities to assist in the fight against TB [37,38].

CFZ, a riminophenazine antibiotic, is listed by the World Health Organization as a Category B agent for treatment of multidrug-resistant TB (MDR-TB) and extensively drug-resistant TB (XDR-TB) [39]. A meta-analysis study discovered that the overall pooled proportion of treatment success was 61.96% after providing CFZ treatment, compared to a global success rate of 54% for MDR-TB patients and a limited 30% success for XDR-TB patients [39–41]. Additionally, a prospective, randomized, multicenter study concluded that MDR-TB patients treated with the shorter regimen including CFZ had a comparable successful outcome rate when compared to patients treated with the standard regimen [39,42]. Moreover, synergistic effects were observed when utilizing CFZ in combination with first-line anti-tubercular agents in both planktonic and biofilm-forming cultures [43]. Therefore, potential improvements in therapeutic efficacy may be achieved by adding CFZ to standard, readily available anti-tubercular treatment regimens [43]. Thus, CFZ is a worthwhile candidate to consider for treatment of CTB, since CTB is currently treated with first-line anti-tubercular agents [43]. However, SDEDDSs developed during this study can also aid in treatment of non-tuberculosis mycobacteria (NTM) skin infections, since NTM is known for its intrinsic, inducible, and adaptive resistance mechanisms [44]. Therefore, treatment periods of 2–4 months are required for NTM skin and soft tissue infections accompanied by co-administration of multiple antibiotics [44]. CFZ is recommended for treatment of *Mybobacterium fortuitum* and *Mycobacterium abscessus* complex together with other agents such as amikacin, trimethoprim–sulfamethoxazole, linezolid, tetracyclines, quinolones, and gepotidacin (for *Mycobacterium fortuitum* complex) and tigecycline, clarithromycin, omadacycline, and thiostrepton (for *Mycobacterium abcessus* complex) [44]. Unfortunately, the co-administration of antibiotics can lead to challenges such as drug interactions, drug-

related adverse reactions, and high medication costs that have the potential to compromise treatment options as well as patient compliance [44]. However, SDEDDS can be a valuable tool during the development of individualized therapy for conditions such as NTM skin infections and CTB, since multiple drugs as well as different drug combinations can be added to SDEDDSs that are easy to manufacture and upscale if excipient selection is performed correctly [19].

3.3. Preformulation Studies

3.3.1. Oil Immiscibility Studies

It was decided to formulate a W/O/O emulsion for the purpose of providing a water phase to optimally solubilize the hydrophilic drugs, and oil phases capable of ensuring effective solubilization of the lipophilic drugs. Ideally, the external oil phase should possess additional skin penetration enhancement properties to facilitate dermal drug delivery [45]. However, the art of creating an oil-in-oil (O/O) emulsion lies in the mixing of two immiscible lipophilic components to maintain the separation of an internal and external oil phase [2,46]. Therefore, it would be best to employ a 'lipophilic oil' as well as a more 'hydrophilic oil' into a single SDEDDS. Thus, the hydrophilic–lipophilic balance (HLB) values of oils were studied to find oils or excipient mixtures with low and elevated HLB values in order to create and maintain internal and external oil phase separation.

Beeswax (HLB value of 4 or 12), linseed oil (HLB value of 3.23) and Transcutol®:PEG (9:1 ratio), with a combined HLB value of 4.46, were analyzed to find an internal oil phase [47–49]. Shea butter, AVO, OLV, EPO, and PALM were considered as potential external oil phases. Immiscibility experiments were performed by weighing 10 g of a potential internal oil phase and adding it to 10 g of a tested external oil phase [2]. Next, the oil mixtures were subjected to vortexing for 10 min [2]. Thereafter, the vortexed mixtures were poured into separatory funnels and left for 30 min in order to evaluate the degree of separation of the different oil phases [2]. This would enable the determination of the immiscibility percentage in terms of weight differences [2].

3.3.2. Hydrophilic–Lipophilic Balance Consideration

Choosing the correct surface active agent(s) is essential in order to stabilize the emulsions once formulated [50]. The HLB system allows theoretical quantification of the ability of a surfactant and co-surfactant combination to stabilize an emulsion [50–52]. When considering the formulation of multiple emulsions such as SDEDDSs, the inclusion of at least two surface active agents is required to stabilize the formation of both primary and secondary emulsions that as a whole present a multiple emulsion [53]. In the case of W/O/O emulsions, one surface active agent should have a low HLB value to stabilize the formation of a PE at the water-in-oil (W/O) interface, and a second surface active agent with an enhanced HLB value should provide stabilization of the secondary emulsion formation [53]. If surface active agents are selected correctly, the stabilization established by these excipients form the building blocks needed by SDEDDSs to provide protection of the incorporated drugs, the ability to include several drugs due to different emulsion compartments, and to enable sustained release from multi-emulsions [53]. Therefore, the selection of surface active agents for multiple emulsions cannot be based solely on the same concept applied for simplified emulsions. For the latter, a non-ionic, lipid-soluble surfactant with an HLB value ranging between 3–8 will tend to deliver O/O emulsions. A non-ionic surfactant that is preferentially water-soluble, has an HLB value around 8–16, and will tend to stabilize O/W emulsions [54]. Therefore, for the purpose of this study, Span®83 (HLB value of 3.7) and Tween®60 (HLB value of 15) were selected as surfactant and co-surfactant, respectively [55,56]. A fixed ratio of Span®83:Tween®60 (1:1) was maintained throughout the experiments, as this ratio has demonstrated greater stabilization of SEDDSs compared to higher ratios that increase the emulsion range but reduce stability, as indicated by drug precipitation [6,57].

3.3.3. Construction of a Pseudoternary Phase Diagram to Find Potential Primary Emulsions

A water titration method was utilized to construct a pseudoternary phase diagram wherein Transcutol®:PEG (9:1), water and the surfactant phase (Span®83:Tween®60; 1:1) were combined in different ratios to display a triplot. This then is able to indicate the most appropriate combination of additives required to form successful SDEDDSs, as one can establish the self-emulsifying areas and also validate the most effective concentrations and ratios of the included composites. No drugs were used, as it was necessary to establish excipient behavior without the influence of any drugs. The surfactant phase and water were mixed in fixed ratios (10:0, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9, 0:10), while Transcutol®:PEG (9:1) was added in a dropwise manner as a variable component at an ambient temperature. The concentration at which the turbidity of the mixtures is first observed was plotted as the endpoint, employing Triplot software version 4.1.2 [6,58]. Thus, the area of self-emulsification was established as required to find potential PEs.

3.3.4. Evaluation of Primary Emulsions

The PEs selected from the pseudoternary phase diagram were subsequently prepared by adding known quantities of the internal oil phase (Transcutol®:PG; 9:1) to established quantities of the water and surfactant phases. The mixtures were then subjected to homogenization with a Heidolph DIAX600 homogenizer (Schwabach, Germany) for 4 min at 9500 rpm, while being kept at room temperature [2,59]. The PEs were evaluated after a waiting period of 24 h at an ambient temperature in terms of emulsion stability index and self-emulsification performance in order to eliminate poor excipient combinations from further experimentation.

3.3.5. Self-Emulsification Performance

Complete emulsification and emulsification time can be defined as the time required to obtain a macroscopically homogeneous emulsion [60]. To obtain self-emulsification times, PEs were assessed using a Type II Distek 2500 dissolution apparatus (2501049, North Brunswick, NJ, USA). A sample (1 mL) was collected from individual PE formulations and diluted with 100 mL distilled water and maintained at a fixed temperature of 32 ± 0.5 °C with a paddle rotation speed set at 50 rpm to provide gentle agitation. The time it takes for PEs to form a homogeneous mixture upon dilution was noted and classified according to the following grading system [61]:

- Grade A: Fast-forming emulsion (within 1 min) with a clear or bluish color;
- Grade B: Fast-forming (within 1 min) and somewhat less clear emulsion with a bluish-white color;
- Grade C: Fine murky emulsion that forms within 2 min;
- Grade D: Dull, grayish-white emulsion that displays a slightly oily appearance, which indicates slow emulsification (longer than 2 min);
- Grade E: A formulation demonstrating either poor or minimal emulsification with large oil droplets existing on the surface.

Emulsions graded as C or grade D were deemed favorable for dermal drug delivery [4]. For this reason, all emulsions that received a grade other than grade C or D were deemed unsuitable for further investigation.

3.3.6. Emulsion Stability Index (ESI)

The PEs (10 mL) were placed in a water bath set at a temperature of 68 °C for a period of 3 h prior to evaluation [2]. After the 3 h waiting period, the PEs were inspected to determine the variance between the separated layer compared to the initial total volume of the emulsion placed in the water bath [2]. This provides a clear indication of the capacity of an emulsion to retain stability once it has been exposed to increased temperatures for a

short time. The volume of the separated layer was compared with the total volume of the initial emulsion to calculate the ESI using the following equation:

$$ESI = 1 - \left(\frac{Volume\ of\ separated\ layer}{Total\ volume\ of\ emulsions}\right) \times 100\% \quad (1)$$

3.3.7. Construction of Pseudoternary Phase Diagrams for Self-Double-Emulsifying Drug Delivery Systems

The water titration method was again utilized to construct pseudoternary phase diagrams. Drugs were again excluded during pseudoternary phase diagram construction to be able to observe excipient response without the complication of variables introduced by drug inclusion. The internal oil phase was mixed together with the surfactant phase (i.e., PE 1, PE 2 and PE 3, respectively) and water in fixed proportions (9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8 and 1:9), while individual external oil phases were added dropwise as the variable component at an ambient temperature. The concentration at which the turbidity of the mixtures is observed was plotted as the endpoint, employing Triplot software version 4.1.2 [6,58,62,63].

3.3.8. Solubility Studies

An excess amount of individual antitubercular drugs was added to 5 mL of the different vehicles considered during this study. Samples were then vortexed for approximately 2 min. Solubility studies were performed by placing samples in the circular axis (54 rpm) of a rotating solubility bath set at 32 °C (±0.5 °C) for a period of 48 h [6]. The samples were then centrifuged at 3000 rpm for 15 min, followed by the removal of the supernatant from each sample [6]. The supernatant was filtered through a 0.45 µm Millipore® filter and diluted with methanol [6]. A validated high-performance liquid chromatographic method was employed to analyze all samples [6].

3.3.9. Isothermal Micro Calorimetry Compatibility Studies

The compatibility of excipients and drugs was investigated using a previously published method [64]. A 2277 Thermal Activity Monitor, TAM III (TA Instruments, New Castle, DE, USA), equipped with an oil bath with a stability of ±100 µK over 24 h was used. The temperature was maintained at 40 °C and 100 mg samples were tested. The heat flow for each individual component was measured to generate a theoretical response (i.e., baseline), which was accompanied by a comparison of the theoretical response with the calometric output to determine compatibility. If the theoretical response drastically differed from the detected calometric output, interactions between excipients were suspected.

3.3.10. Microscope Examination

All visual examinations of either the PEs or the SDEDDSs were conducted utilizing an Olympus microscope (Tokyo, Japan). TEM measurements were performed on a TECNAI G2 (ACI) instrument operated at an accelerating voltage of 200 kV (Hillsboro, OR, United States of America) [65,66].

3.3.11. Droplet Size and Size Distribution

Droplet size and size distribution were assessed by means of dynamic light scattering performed using a Malvern Zetasizer Nano® ZS (Worcestershire, UK) at 25 °C [6]. All samples were analyzed in triplicate.

4. Conclusions

Future in vitro drug release and permeation studies should be conducted in order to establish the release of the drugs from SDEDDSs. Additionally, nanotoxicology studies should also be performed for the purpose of establishing the extent as well as the safety of the permeation achieved by N-SDEDDSs [67]. However, the final SDEDDS presented an

average droplet size of 92.46 nm and a PDI value of 0.391 which is considered exceptionally small on the basis of literature data [68–70]. Therefore, dermal drug delivery can be considered very likely due to the small droplet size of the SDEDDS [68–70]. This study has clearly demonstrated the potential of SDEDDSs as a dermal drug delivery vehicle by including four model drugs in a single SDEDDS. This was challenging, since the model drugs possess very different physicochemical properties, with individual aqueous solubilities ranging from sparingly soluble to a solubility of 125 mg/mL. Moreover, the selected drugs varied in terms of their BCS classification, namely Class I, II, and III. However, the development of the SDEDDS described here was based on findings from the preformulation studies. Therefore, this work signifies an important step in conducting thorough preformulation experiments that provide a foundation for the formulation and subsequent characterization of SDEDDSs tailored to accommodate selected drugs while being adapted for dermal drug delivery. Furthermore, this paper indicates the importance of carefully constructing drug delivery systems from start to finish—preformulation studies represent the key to success for development of dermal SDEDDSs. To the authors' knowledge, this represents the first published study indicating that SDEDDSs can incorporate four individual drugs successfully into a single dosage form.

Author Contributions: D.v.S., J.M.V. and R.K.H. contributed to the study conceptualization. D.v.S. wrote the original draft preparation. D.v.S., J.M.V. and R.K.H. contributed to writing, review and editing. J.M.V. and R.K.H. were responsible for supervision of the study. D.v.S. conducted laboratory experiments and completed processing of results. J.M.V. handled project administration. J.M.V. and R.K.H. acquired funding acquisition. All authors have read and agreed to the published version of the manuscript.

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Chapter 5

Technical Note submitted to '*Methods and Protocols*'

Chapter 5, titled: '***Development of a HPLC method for analysis of a combination of clofazimine, isoniazid, pyrazinamide, and rifampicin incorporated into a dermal self-double-emulsifying drug delivery system***' is included as a **technical note** submitted for publication in '*Methods and Protocols*' and is under revision (minor changes) at the time when this thesis has been submitted. **Chapter 5** describes analytical method development conducted to successfully analyse the four selected anti-tubercular drugs simultaneously with an accurate, reliable and repeatable method.

methods and protocols

MDPI

Technical Note

Development of a HPLC method for analysis of a combination of clofazimine, isoniazid, pyrazinamide, and rifampicin incorporated into a dermal self-double-emulsifying drug delivery system

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Abstract: We describe the development and validation of a new high performance liquid chromatography (HPLC) method for analysis of a combination of the first-line anti-tubercular drugs isoniazid, pyrazinamide, and rifampicin together with clofazimine. This is a unique challenge since clofazimine and rifampicin are relatively highly lipophilic drugs, whereas isoniazid and pyrazinamide are considerably more hydrophilic. Thus, clear separation of peaks and quantification of four individual drugs can present difficulties during the development of an analytical method. Detection was established at two wavelengths - 254 nm for isoniazid and pyrazinamide and 320 nm for clofazimine and rifampicin. Gradient elution was employed using 0.1% aqueous formic acid (A) and acetonitrile (B); and clear separation of the four drugs was achieved within 10 min. A linear relationship was indicated by a correlation coefficient (r^2) of 0,9999 for each anti-tubercular drug, respectively. The limit of detection (LOD) for the individual drugs was 0.70 µg/mL (isoniazid), 0.30 µg/mL (pyrazinamide), 0.20 µg/mL (rifampicin) and 0.20 µg/mL (clofazimine). Precision experiments rendered a mean recovery percentage of 101.25% (isoniazid), 98.70% (pyrazinamide), 99.68% (rifampicin) and 97.14% (clofazimine). This HPLC method was validated and is reliable, repeatable, and accurate for the purpose of conducting simultaneous HPLC analyses of the four anti-tubercular drugs.

Keywords: Tuberculosis; Clofazimine; High Performance Liquid Chromatography; Isoniazid; Pyrazinamide; Rifampicin.

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1. Introduction

Tuberculosis (TB) is a significant threat to public health, as 20 – 40% of the global population is affected by this deadly disease [1,2]. Moreover, TB is still the leading cause of death caused by a single infectious disease despite the End TB Strategy of the World Health Organization (WHO) [3]. A potentially interesting approach that may aid the WHO strategy was recently reported that entails the introduction of clofazimine, which is mainly used for the treatment of leprosy, into first-line TB regimens in order to combat drug resistance, potentially shorten treatment times, and improve therapeutic outcomes [4]. Combining clofazimine with first-line TB drugs, in particular rifampicin, resulted in synergistic suppression and elimination of the growth of actively replicating bacteria [4]. Additionally, clofazimine exhibited improved efficacy when used in combination with

isoniazid and rifampicin against slowly replicating mycobacteria, including those within biofilm-forming cultures [4]. These findings signify the potential advantage of adding clofazimine to first-line regimens due to its enhanced efficacy in eliminating slowly replicating bacteria [4]. Furthermore, synergism between clofazimine and pyrazinamide was demonstrated through oral administration of this drug combination, which substantially reduced the effective amount of clofazimine required, from 25 mg/kg to 6.25 mg/kg [5].

This synergism will be of particular advantage for developing a topical treatment for cutaneous TB, as clofazimine is generally avoided due to dose-dependent skin discoloration [6,7]. This notorious dose-dependent skin discoloration is accompanied by other adverse effects such as severe gastrointestinal reactions (nausea, vomiting, abdominal pain, diarrhea), conjunctival deposition, and dark-brown pigmentation of fingernails. This is attributed to the accumulation of clofazimine in adipose cells in the heart, liver, breasts, adrenal glands, pancreas, spleen, bone marrow, and the lamina propria of the jejunum. The notably long half-life of clofazimine, ranging from 65 to 70 days, can be linked to the highly lipophilic nature of this drug. Overall, topical delivery of clofazimine will drastically decrease serious adverse effects since systemic absorption is bypassed [8,9]. Furthermore, the use of decreased clofazimine concentrations will assist in the formulation of dosage regimens. Clofazimine has poor aqueous solubility and incorporating sufficient clofazimine into a solubilized dosage form is challenging [10,11].

The structures of the four anti-tubercular drugs, isoniazid, pyrazinamide, clofazimine and rifampicin, discussed here are illustrated in Figure 1 together with their log P values and aqueous solubilities [1,8,11–22]. Moreover, rifampicin is amphoteric with a pKa of 1.7 due to the 4-hydroxyl group and a pK_{a2} of 7.9 due to the *N*-methyl piperazine nitrogen atom, with an isoelectric point at pH 4.8 in an aqueous solution [23].

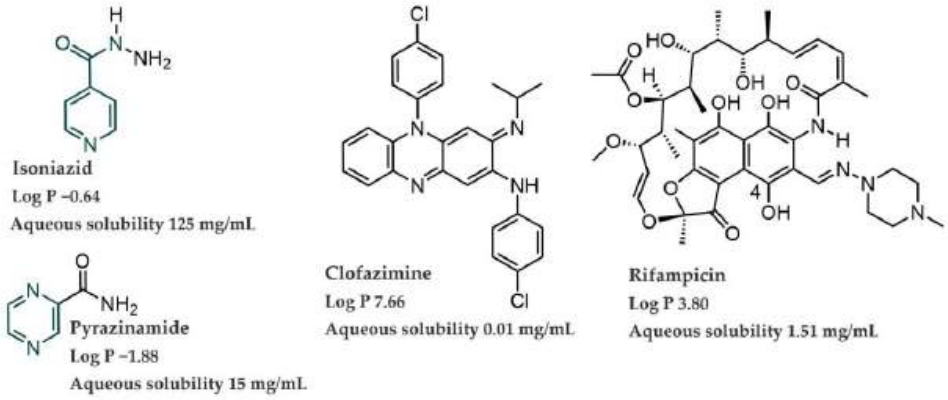

Figure 1. Structures of isoniazid, pyrazinamide, clofazimine, and rifampicin with their respective log P values and aqueous solubilities.

High performance liquid chromatographic (HPLC) analysis of a combination of these four compounds with their large differences in polarity has not been reported. However, an HPLC method for the simultaneous analysis of isoniazid, pyrazinamide, rifampicin, and ethambutol has been published [24]. This technique employed gradient elution with a mobile phase comprising 20 mM monobasic sodium phosphate buffer with 0.2% triethylamine (pH 7.0) and acetonitrile, at a flow rate of 1.5 mL/min [24]. In this study though, we have to deal with the highly lipophilic clofazimine, which has a log P value of 7.66 that is substantially more lipophilic than ethambutol with a log P value of −0.14 [1,25].

Our objective was to develop and validate a rapid and reliable HPLC method to accurately quantify isoniazid, pyrazinamide, clofazimine, and rifampicin in combined formulations. This was required to evaluate the effects of different routes of administration and formulations, such as involving solid oral dosage forms and transdermal, or topical formulations. As is now described, method development and validation were conducted

according to the International Conference on Harmonization (ICH) and Good Manufacturing Practice requirements and specifications [1,26]. Therefore, in the following sections, the materials utilized, the procedures followed to prepare the standard solutions, the gradient elusion applied for analysis, formulation of the spontaneous emulsions, and method validation are described and discussed. Certain conclusions could be drawn with regard to the accuracy, reliability, and repeatability of the method according to the ICH guidelines.

2. Materials and Methods

All chemicals were of analytical reagent grade. Deionized water, obtained from a Milli-Q system, was used for all experiments. Clofazimine, isoniazid, and rifampicin were gifts from Prof. Wilna Liebenberg, head of the Solid-state Pharmaceutical Innovation and Nanotechnology (SPIN) research group at the North-West University, Potchefstroom, South Africa. Pyrazinamide was purchased from Merck (Darmstadt, Germany). Acetonitrile and formic acid were purchased from Merck (Darmstadt, Germany) and Sigma-Aldrich (Johannesburg, South Africa), respectively. For HPLC analyses, a Nexera-i® model:LC-2040C 3D Plus, a Phenomenex Luna® 5 µm C18(2) 100A CC column 150x4.6 mm (Torrance, USA), photodiode array detector, and an injector module, and LabSolutions software were used for analysis and data acquisition (Kyoto, Japan).

Stock standard solutions were individually prepared for each drug. Rifampicin and clofazimine standard solutions were prepared with acetonitrile, and standard solutions of isoniazid and pyrazinamide were prepared in water. All standard solutions were initially prepared as 1000 µg/mL solutions prior to being serially diluted with a mixture of deionized water:acetonitrile (1:1) to yield 7 drug concentrations. Gradient elution was carried out with mobile phase A comprising 0.1% aqueous formic acid; and mobile phase B consisting of acetonitrile. A flow rate of 1.0 mL/min was used with the initial mobile phase ratio at 7% B for 2.5 min, after which it was increased to 55% B at 6 min when a gradient shift of 0/100% [(A)/(B)] was maintained until 8 min. Hereafter, the gradient was readjusted to the initial gradient of 93/7% (A)/(B) until the end of the 10 min run time.

The generation of self-double-emulsifying drug delivery systems (SDEDDSs) involved the preparation of a primary emulsion comprising a mixture of Transcutol® and polyethylene glycol (PEG) (9:1) as the internal oil phase, together with water and a surfactant phase containing Span®83 and Tween®60 (1:1). For clarity, the primary emulsion excipient ratios are expressed as 9:9:2, representing the internal oil phase:water:surfactant phase. After preparation of the primary emulsion, a predetermined quantity of the external oil phase (avocado oil) was added to the primary emulsion in small increments while stirring without heat application in order to achieve the formation of a homogeneous SDEDDS. The four drugs were included at a concentration of 2% of each individual drug, respectively. All samples were kept at an ambient temperature and all samples comprising rifampicin were stored in amber bottles, flasks, and vials to avoid photodegradation of the rifampicin. The SDEDDS was extracted by diluting 1 mL of SDEDDS up to 100 mL with methanol and was subsequently sonicated for 5 min. Samples were filtered with a 0.45 µm syringe filter into HPLC vials for analysis.

Validation of the method was performed by incorporating linearity, accuracy, precision, limit of detection (LOD), limit of quantification (LOQ), system stability, and repeatability. Linearity was calculated by constructing a regression plot of drug concentration vs. peak area response that enabled the generation of a regression equation for each antitubercular drug.

3. Results and Discussion

The individual regression equations are given in Table 1. Next, the correlation coefficient (r^2) was calculated on the basis that a strong linear relationship is signified by a correlation coefficient of 1 [1,26]. Accuracy results are also displayed in Table 1. Accuracy

parameters were in concordance through rendering recovery percentages between 98% and 102 % (%RSD < 15 %).

Table 1. Validation parameters obtained for anti-tubercular drugs.

	Isoniazid	Pyrazinamide	Rifampicin	Clofazimine
Concentration range (μg/mL)	5.29-520.00	5.19-500.00	6.01-385.00	5.21-335.00
Regression equation	y = 924.51x + 259.47	y = 1154.40x – 1589.60	y = 4209.90x – 7194.10	y = 2431.60x – 624.93
r^2 (> 0.99) [1]	0.9999	0.9999	0.9999	0.9999
Accuracy mean recovery (%) (%RSD) [2] (100 ± 2%)	100.04 (±0.06)	101.28 (±0.70)	100.15 (±0.80)	99.74 (±0.28)
Stability (%RSD) (< 15%)	1.84	0.78	0.69	1.06
System repeatability (±SD) [3]	1.46 (±0.18)	3.11 (±0.04)	5.71 (±0.16)	5.96 (±0.34)
Retention time (min) %RSD	0.34	0.94	0.88	0.23

[1] r^2 = Correlation coefficient; [2] %RSD = Percentage relative standard deviation; [3] SD = Standard deviation.

The stability of the drugs was evaluated over a period of 24 h to ensure that no significant decomposition occurred during this time frame (Table 1). During the stability evaluation, no drug displayed concentrations that deviated more than 15% from the starting concentration. Therefore, all selected drugs were considered stable for a period of at least 24 h. System repeatability was verified by evaluating the peak area as well as the retention times of the drugs during six consecutive injections. All %RSD (percentage relative standard deviation) values were less than 2% (Table 1) and as a result, the method was found to be suitable for the analysis of these drugs. Figure 2 illustrates the HPLC chromatograms for isoniazid, pyrazinamide, rifampicin, and clofazimine, respectively.

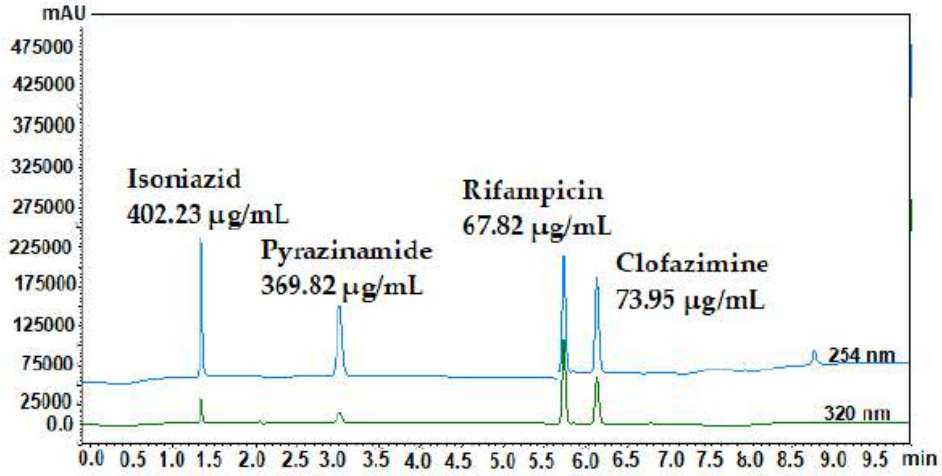

Figure 2. HPLC chromatographs of sample concentrations for isoniazid, pyrazinamide, rifampicin, and clofazimine, respectively. The top chromatogram was obtained using the photo-diode array detector at 254 nm (blue line) and the bottom chromatogram at 320 nm (green line).

It was decided to analyze rifampicin and clofazimine at a wavelength of 320 nm (Figure 1) as it allowed easier quantification when injecting decreased drug concentrations. The limit of quantification (LOQ) is the lowest concentration of drug that can be quantitatively ascertained, above which analysis is possible with the specified degree of accuracy and precision [1,26]. LOQ is used particularly for determining impurities and/or degradation products [1,26]. The LOD, on the other hand, can be determined according to several methods, although the individual LOD concentrations were experimentally determined for the purpose of this study. The determined LOD was 0.70 µg/mL for isoniazid, 0.30 µg/mL for pyrazinamide, 0.20 µg/mL for rifampicin, and 0.20 µg/mL for clofazimine. Subsequently, the LOQ was determined as 2.31 µg/mL (isoniazid), 0.99 µg/mL (pyrazinamide), 0.66 µg/mL (rifampicin), and 0.66 µg/mL (clofazimine).

This method was applied to analyze the concentrations of the drugs incorporated into the SDEDDSs. Here, excipients that are frequently and safely utilized during formulations of dermal emulsions were considered, namely Transcutol®, PEG, avocado oil, water, Span®83, and Tween®60. The results obtained from the precision analyses of the SDEDDSs are given in Table 2.

Table 2. Precision data obtained for anti-tubercular drugs (n=9). 172

		Isoniazid	Pyrazinamide	Rifampicin	Clofazimine
Concentration range (μg/mL)		250-515	240-490	180-380	90-170
Repeatability (intra-day)	%RSD	0.03	0.02	0.01	0.03
	Mean recovery (%)	100.30	100.95	99.91	98.91
Intermediate precision (inter-day)	%RSD (day 2)	0.07	0.04	0.04	0.75
	Mean recovery percentage (day 2)	99.56	99.70	98.89	98.45
	%RSD (day 3)	0.02	0.01	0.17	0.05
	Mean recovery percentage (day 3)	101.25	98.70	99.68	97.14

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As indicated in Table 2, the precision experiments involved measuring drug concentrations at three different concentration levels for each drug. The three different concentration levels for isoniazid were 250 μg/mL, 400 μg/mL, and 515 μg/mL; for pyrazinamide, 240 μg/mL, 370 μg/mL, and 490 μg/mL; for rifampicin, 180 μg/mL, 290 μg/mL, and 380 μg/mL; and for clofazimine, 90 μg/mL, 140 μg/mL, and 170 μg/mL. As the method complied with all predefined parameters, this overall method was established as precise and accurate. In Figure 3 the HPLC chromatograms with clearly defined peaks for the four anti-tubercular drugs are shown; these appear at the predetermined retention times (*cf.* Fig. 2), with no interference from the different excipients used for preparation of the dermal SDEDDS formulations. In terms of inter-day precision, one concentration of each drug was analyzed each day for a three-day period. As the method complied with all predefined parameters, this overall method was established as precise and accurate. 174 175 176 177 178 179 180 181 182 183 184 185

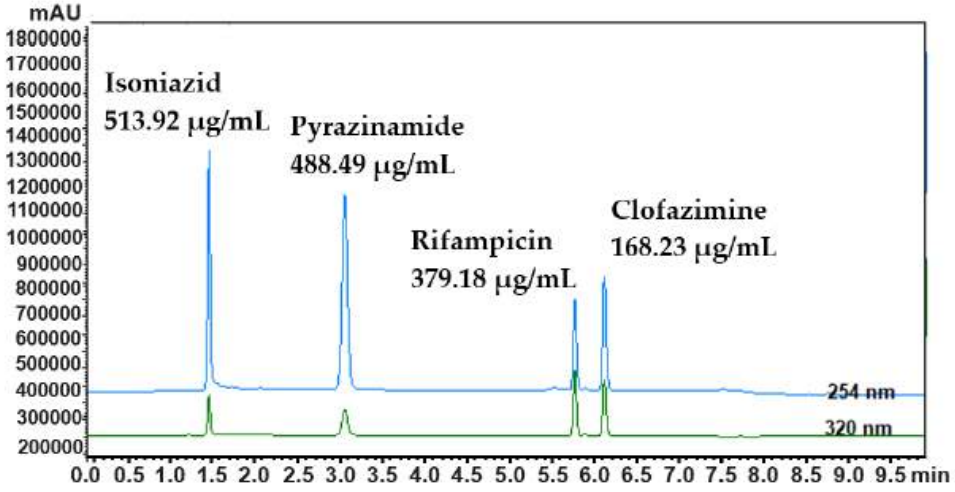

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Figure 3. HPLC chromatographs of isoniazid, pyrazinamide, rifampicin and clofazimine formulated into the transdermal/topical self-double emulsifying drug delivery systems. The top chromatogram was obtained using the photo-diode array detector at 254 nm and the bottom chromatogram at 320 nm. 187 188 189 190

As is evident from Table 2 and Figure 3, it was possible to achieve simultaneous quantification of isoniazid, pyrazinamide, rifampicin, and clofazimine. This method is therefore considered reliable and sensitive and complies with the ICH guidelines for method validation.

4. Conclusions

Validation parameters such as linearity, LOD and LOQ, accuracy, precision, sample stability, and system suitability were established. This method was also used to determine the sensitivity of the method when analyzing transdermal/topical SDEDDSs. The method was confirmed to be reliable for quantifying the four selected anti-tubercular drugs, with no interference from excipients generally used for the formulation of topical and/or transdermal products. Future work will include adding more anti-tubercular drugs that can be analyzed with a single method since it is now established that multiple drug treatment regimens must be used for treatment of TB in order to suppress the emergence of drug resistance and maintain therapeutic efficacy. The proposed work will also be extended to include other excipients that are frequently utilized during the development of oral dosage forms of the individual drugs.

Author Contributions: DVS, JMV, RKH and FVDK contributed to the study conceptualization. DVS wrote the original draft preparation. DVS, JMV, RKH and FVDK contributed to writing, review and editing. JMV and RKH were responsible for supervision of the study. DVS conducted laboratory experiments and completed processing of results. JMV handled project administration. JMV and RKH acquired funding.

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Chapter 6

Conclusion and Future Prospects

6.1. Final Conclusions

Treatment of mycobacterial infections remains a daunting challenge due to its ability to present itself in an asymptomatic form, as well as an opportunistic clinical disease (Klever *et al*., 2023). Moreover, the complexity of the treatment is intensified by the intrinsically resistant structure of mycobacteria (Zheng & Lupoli, 2023). Inherent mechanisms of resistance can be listed as: (1) mycobacterial cell wall impermeability, (2) latency (intrinsic mechanism of drug tolerability), (3) reduced drug permeability through porin alteration, (3) efflux of antibiotics through active transport, (4) target modification, (5) inactivation of drugs through enzyme degradation or modification, and (6) activation of a transcriptional regulator (Peterson *et al*., 2023; Zheng & Lupoli, 2023; Nasiri *et al*., 2017). Furthermore, mycobacteria can acquire molecular resistance mechanisms against administered antibiotics (Finin *et al*., 2023; Zheng & Lupoli, 2023). These mechanisms of resistance differ from most other bacteria in which acquired drug resistance is achieved via horizontal transfer of mobile genetic elements. Mycobacterial species spontaneously mutate in their chromosomal genes (Shea *et al*., 2023; Nasiri *et al*., 2017).

However, mycobacterial infections are not only limited to tuberculosis (TB), which on a global scale can be deemed a leading cause of death, but also include pathogenic mycobacteria equally referred to as non-tuberculosis mycobacteria (NTM) (Wang *et al*., 2023; Zheng & Lupoli, 2023). Furthermore, infections caused by NTM are considered a growing concern in the clinical setting, since these bacteria are now resistant to many generally used antibiotics (Zheng & Lupoli, 2023). Hence, when reflecting on the intrinsic and acquired resistance capacities of mycobacteria, there is an intensified need to use existing antibiotics as adjuvants during treatment to increase the efficacy of existing antimicrobial agents (Zheng & Lupoli, 2023). Therefore, for the purpose of this study, clofazimine was introduced as an adjuvant agent to first-line anti-tubercular agents (i.e., isoniazid, pyrazinamide, and rifampicin) (Mashele *et al*., 2022).

Mycobacteria is the causative organism of numerous skin infections (Wang *et al*., 2023). The incidence of NTM dermal infections and cutaneous tuberculosis (CTB) is increasing drastically as a consequence of antimicrobial resistance and negligence in vaccinating, diagnosing, and treating TB during the Corona Virus Disease of 2019 (COVID-19) pandemic (Wang *et al*., 2023;

Williams *et al*., 2023; Zheng & Lupoli, 2023). Therefore, this study provided a dermal drug delivery vehicle that can be considered exceptional if one considers the ease of upscaling, as well as the high probability of utilising this vehicle for individualised therapy according to the resistance profiles of individual patients (Fang *et al*., 2024; Baquero-Artigao *et al*., 2023; Chang *et al*., 2021). Individualised therapy might be an idealistic goal, but in fact, it is a predominantly logical solution to treating patients experiencing treatment failure due to antimicrobial drug resistance (Fang *et al*., 2024; Baquero-Artigao *et al*., 2023; Chang *et al*., 2021). Moreover, individualised therapy can restrict the occurrence of antimicrobial drug resistance when treating patients with antibiotics where the causative bacteria are responsive to the treatment instead of blindly providing broad-spectrum antibiotic treatment that potentially contributes to global antimicrobial drug resistance (Flynn & Guarner, 2023; Zakhour *et al*., 2023; Zhong *et al*., 2023). Therefore, adapting clofazimine for topical delivery while incorporated into a self-double emulsifying drug delivery system (SDEDDS) as a novel contribution to fixed-dose anti-tubercular drug delivery (van Staden *et al*., 2022) was researched.

The first review article focused on the potential of using SDEDDSs as topical drug delivery systems for the dermal route of administration to aid in the treatment of CTB and other mycobacterial infections as a prelude to the evaluation of related systems for a more effective treatment of CTB and other mycobacterial infections at large. As a starting point, the possibility of considering the adaptation of the highly lipophilic riminophenazine clofazimine, with its potential for the treatment of multidrug resistant TB, for this purpose, was researched. Additionally, recently reported synergism achieved by adding clofazimine to first-line TB regimens signifies the need to consider clofazimine. Therefore, the biological effects and pharmacology of clofazimine were reviewed. The potential of plant-based oils acting as skin penetration enhancers as well as these materials behaving as anti-microbial components for transporting the incorporated drug, were also discussed. This review article provided a literature background to the reader to confirm the relevancy of this study and thoroughly introduced clofazimine as an adjuvant drug to first-line anti-tubercular drugs, considering both its advantages as an adjuvant and the challenges of adapting clofazimine for dermal drug delivery. This work was published as a review article in *'Antibiotics' (*van Staden *et al*., 2022*).*

Hereafter, a second review article was presented to discuss the reasoning when selecting excipients for dermal self-emulsifying drug delivery systems (SEDDSs)/SDEDDSs. The ease of formulation generated by the spontaneous emulsification technique itself is intriguing because of the simplified production procedure and unlimited upscaling possibilities. However, spontaneous emulsification relies solely on selecting excipients that complement each other in order to create a vehicle aimed at optimising drug delivery. If the excipients are not compatible or are unable to spontaneously transpire into emulsions once they are exposed to mild agitation, no self-

emulsification will be achieved. Therefore, the generalised view of excipients as inert bystanders facilitating delivery of an active compound cannot be accepted when selecting excipients needed to produce SEDDSs and/or SDEDDSs. Consequently, a unique challenge to select excipients capable of solubilising incorporated drug(s) was provided, while also considering excipient-excipient compatibility, drug-excipient compatibility, and dermal drug delivery properties such as skin penetration enhancement characteristics and skin tolerability of excipients. Hence, during this study a 'Formulation Recommendation System' (FRS) was developed to equip the reader to optimally select excipients during development of spontaneous dermal emulsions based on the biopharmaceutical classification of drugs intended for incorporation into SEDDSs/SDEDDSs. This is a novel contribution to the development of spontaneous dermal emulsions since the usefulness of considering auxiliary excipients such as emollients, humectants, preservatives, antioxidants, and cosolvents are discussed. Additionally, it can be considered extremely specialised since the process of selecting general excipients capable of generating spontaneous emulsions, when utilised in combination, is already challenging. This work was published as a review article in *'Pharmaceutics'* (van Staden *et al*., 2023a).

Chapter 4, included as a research article, where SDEDDSs are considered remarkable lipid-based systems that are most probably superior to other lipid-based oral drug delivery systems in terms of providing drug protection against the gastrointestinal (GI) environment, inhibition of drug efflux as mediated by P-glycoprotein, improved lymphatic drug uptake, improved control over plasma drug concentration profiles of drugs, enhanced stability, and drug loading efficiency. Moreover, the increasing interest in spontaneous dermal emulsions is considered given their ability to overcome different biological barriers. These systems have been reported to deliver drugs across mucus membranes as well as the outermost layer of the skin into the underlying layers. This article provides background and insight into the development of a double spontaneous emulsion with the aim of incorporating four anti-tubercular drugs, clofazimine, isoniazid, pyrazinamide, and rifampicin into one double emulsion. Methods involved studies of oil immiscibility; construction of pseudoternary phase diagrams; determination of self-emulsification performance and establishing the emulsion stability index for primary emulsions; solubility; isothermal micro-calorimetric compatibility studies, and examination of emulsions by light microscopy as well as transmission electron microscopy (TEM). Overall, this work demonstrated the potential of SDEDDSs as a dermal drug delivery vehicle. The key to success here is performing preformulation studies to enable the development of dermal SDEDDSs. To the best of our knowledge, this work represents the first successful example of the production of SDEDDSs capable of incorporating four individual drugs. Additionally, the droplet size of the final SDEDDS was measured as an average of 92,46 ± 5,52 nm and the polydispersity index (PDI) value was concluded as 0,391 ± 0,05, indicating the successful formulation of a SDEDDS in the nano-range.

Furthermore, upon TEM observation, the images obtained revealed that the small droplets contained within the larger droplets of the SDEDDS were smaller than 92,46 nm since droplet size measurement only allowed detection of the larger droplets. Again, to the best of our knowledge, this is the smallest droplet size of a SDEDDS yet reported in literature. Moreover, the surfactant phase concentration of 6,5% m/m, included during formulation of this SDEDDS, can also be considered the lowest concentration of surfactant phase reported that was able to establish self-double emulsification and exceptionally spontaneous double emulsification of nano-sized droplets. This work was published in '*Pharmaceuticals*' (van Staden *et al*., 2023b).

Finally, Chapter 5 is introduced as a technical note submitted to '*Methods and Protocols*' during September 2023. Here, the need for the development of accurate and reliable analytical methods for the simultaneous analysis of combinations of TB drugs, which have divergent physicochemical properties, is discussed. The development and validation of a novel high performance liquid chromatographic (HPLC) method for the analysis of a combination of the first-line anti-tubercular drugs isoniazid, pyrazinamide, and rifampicin together with clofazimine is described. This is a unique challenge, since clofazimine and rifampicin are relatively highly lipophilic drugs, whereas isoniazid and pyrazinamide are considerably more hydrophilic. Thus, clear separation of peaks and quantification of four individual drugs can present difficulties during the development of the analytical method. Detection was established at two wavelengths, namely 254 nm for isoniazid and pyrazinamide, and 320 nm for clofazimine and rifampicin. Gradient elution was employed using 0.1% aqueous formic acid (A) and acetonitrile (B); and clear separation of the four drugs was achieved within 10 min. A linear relationship was indicated by a correlation coefficient of 0,9999 for each anti-tubercular drug, respectively. The limit of detection for the individual drugs was 0,12 µg/mL (isoniazid), 1,69 µg/mL (pyrazinamide), 1,74 µg/mL (rifampicin) and 0,41 µg/mL (clofazimine), individually. Precision experiments yielded a mean recovery percentage of 101,25% (isoniazid), 98,70% (pyrazinamide), 99,68% (rifampicin), and 97,14% (clofazimine). This HPLC analysis method was validated and is reliable, repeatable, and accurate for the purpose of conducting simultaneous HPLC analyses of the four anti-tubercular drugs.

In conclusion, the current contribution to pharmaceutical sciences rendered:

- Development of the first SDEDDS capable of incorporating a fixed-dose anti-tuberculosis drug combination.
- The first and only SDEDDS designed to incorporate four drugs with contrasting physicochemical properties. Without exception, only SDEDDS comprising one drug (Bhattacharjee *et al.*, 2023, Wang *et al*., 2021; Bhattacharjee *et al.*, 2017; Hu *et al.*, 2016; Wang *et al*., 2015) or SEDDS capable of incorporating one or two drugs are reported in

the literature (Claus *et al*., 2023; Damasio *et al*., 2023; Lu *et al*., 2023; Sanaei Oskouei *et al*., 2023; Zewail *et al*., 2021).

- Establishing double self-emulsification with a surfactant phase concentration of 6,5% m/m (Span®83:Tween®60 included in a 1:1 ratio), while the lowest effective surfactant concentration for a double emulsion, produced by traditional formulation techniques, to our knowledge, was reported as a single amphiphile Brij®93 surfactant component at 8% m/m or using a mixture of Span®80 and Span®85 10% m/m to stabilise double emulsions (Ding *et al*., 2019; Tenorio-Garcia *et al*., 2022). In terms of dermal drug delivery, the lowest surfactant concentration employed during formulation of SDEDDSs ranged from 8% m/m polyglycerol polyricinoleat (Hu *et al*., 2016) to 13,6% m/m (comprising 10% Tween®60, 3% polyglycerol polyricinoleate, and 0,6% bentonite clay) (Wang *et al*., 2021). This is considered a crucial requirement during formulation of dermal dosage forms, as surfactants are known to cause skin irritation after prolonged use (Lechuga *etal*., 2023; Hu *et al*., 2016).
- The production of a SDEDDS with the smallest droplet size reported yet in literature. The smallest droplet size to our knowledge for dermal SDEDDS was reported as 4.63 ± 0.46 µm (Wang *et al*., 2021).
- Demonstrating the mechanism of spontaneous double emulsification at 100 nm by utilising TEM images. Another article recently reported a mechanism of spontaneous double emulsification visualised by microscopy for SDEDDS generated within the micro range and intended for oral administration of gentamicin (Bhattacharjee *et al*., 2023).
- Development and validation of a simplified and shortened HPLC method for simultaneous analyses of clofazimine, isoniazid, pyrazinamide, and rifampicin.
- Providing guidelines that are based on the Biopharmaceutical Classification System via an FRS to enable simplified decision-making during formulation of spontaneous emulsions.

6.2. Future Prospects

Some recommendations to broaden knowledge on the development of topical SDEDDS produced by this study include:

- Conducting *in vitro* release and permeation studies. SDEDDSs have been reported to facilitate controlled release of drugs when administered orally (Bhattacharjee *et al*., 2023). Therefore, it may be worthwhile to investigate the release of SDEDDSs in terms of topical/transdermal drug delivery.
- Once *in vitro* release and permeation from SDEDDS are established, the inclusion of different auxiliary excipients can be explored for the purpose of refining controlled release

from topical/transdermal SDEDDS (Bhattacharjee *et al*., 2023; Wadhwa *et al*., 2022; Wang *et al*., 2021; Hu *et al*., 2016).

- Establish the physical and chemical stability of optimised SDEDDS by evaluating the formulation for three months while stored at 4 ± 2 ºC/65 ± 3% relative humidity (RH) and 25 ± 2 ºC/65 ± 3% RH. Samples should be assessed at 0, 1, 2, and 3 months for appearance, droplet size, and drug loading; and all tests should be performed in triplicate (Wang *et al*., 2021). This is of particular importance due to the reduced surfactant phase concentration, since the reduced concentration might not be able to efficiently stabilise SDEDDS in the long term.
- Determine the efficacy of SDEDDS comprising the combination of clofazimine, isoniazid, pyrazinamide, and rifampicin against TB, leprosy, and NTM infections. This is considered important due to the global burden of antimicrobial drug resistance (Flynn & Guarner, 2023). This burden can be reduced by introducing adjuvant agents, such as clofazimine, to other generally used antibiotic regimens as a simplified treatment option compared to synthesising new drug entities, which is an expensive and time-consuming process (Zheng & Lupoli, 2023).
- After the efficacy of SDEDDS comprising the combination of clofazimine, isoniazid, pyrazinamide, and rifampicin is established, the dosage required to successfully treat divergent mycobacterial skin infections together with the frequency of dermal application must be determined. This is significant since the reason for including clofazimine and pyrazinamide was based on the finding that the dosage of orally administered clofazimine can be substantially reduced from 25 mg/kg to 6.25 mg/kg when utilised in combination with pyrazinamide (Ammerman *et al*., 2018). This is deemed promising information to improve patient adherence, as clofazimine is known for its dose-dependent skin discoloration (Stadler *et al*., 2023).
- Perform cytotoxicity assessment via cell impedance by utilising methylthiazol tetrazolium-assay and neutral red assay on 2 cell lines: human immortalized keratinocytes and BJ-5ta (fibroblasts) cells. (Olivier *et al*., 2023; Magogotya *et al*., 2022). This is also crucial to establish the safety and tolerability of SDEDDS, especially upon prolonged use, as would be the case during the treatment of mycobacterial skin infections (Wang *et al*., 2023; Zheng & Lupoli, 2023; Brito *et al*., 2022).

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Annexure A

Analytical Method Development and Validation

A.1. Introduction

For HPLC analyses, a Nexera-i® mod-el:LC-2040C 3D Plus, a Phenomenex Luna® 5 µm C18(2) 100A CC column 150x4.6 mm (Torrance, USA), photodiode array detector, and an injector module, and LabSolutions software were used for analysis and data acquisition (Kyoto, Japan). Stock standard solutions were individually prepared for each anti-tubercular drug. Rifampicin and clofazimine standard solutions were prepared with acetonitrile, and standard solutions of isoniazid and pyrazinamide were prepared in water. All standard solutions were initially prepared as 1000 µg/mL solutions prior to being serially diluted with a mixture of deionized water: acetonitrile (1:1) to yield 7 drug concentrations. Gradient elution was carried out with mobile phase A comprising 0.1% aqueous formic acid and mobile phase B consisting of acetonitrile. A flow rate of 1.0 mL/min was used with the initial mobile phase ratio at 7% B for 2.5 min, after which it was increased to 55% B at 6 min when a gradient shift of 0/100% [(A)/(B)] was maintained until 8 min. Hereafter, the gradient was readjusted to the initial gradient of 93/7% (A)/(B) until the end of the 10 min run time.

Table A1: *Linearity data obtained for CFZ*

Concentration (µg/ml)	Peak area 1	Peak area 2	Mean peak area
5.21	12865	13885	13375
10.42	26531	27477	27004
20.83	50001	54441	52221
41.67	99381	98701	99041
83.33	196958	200602	198780
166.67	401939	400439	401189
333.33	794416	830378	812397
666.66	1625964	1623664	1624814
R^2		**0.9999**	
Slope		**2431.60**	
Intercept		**624.93**	

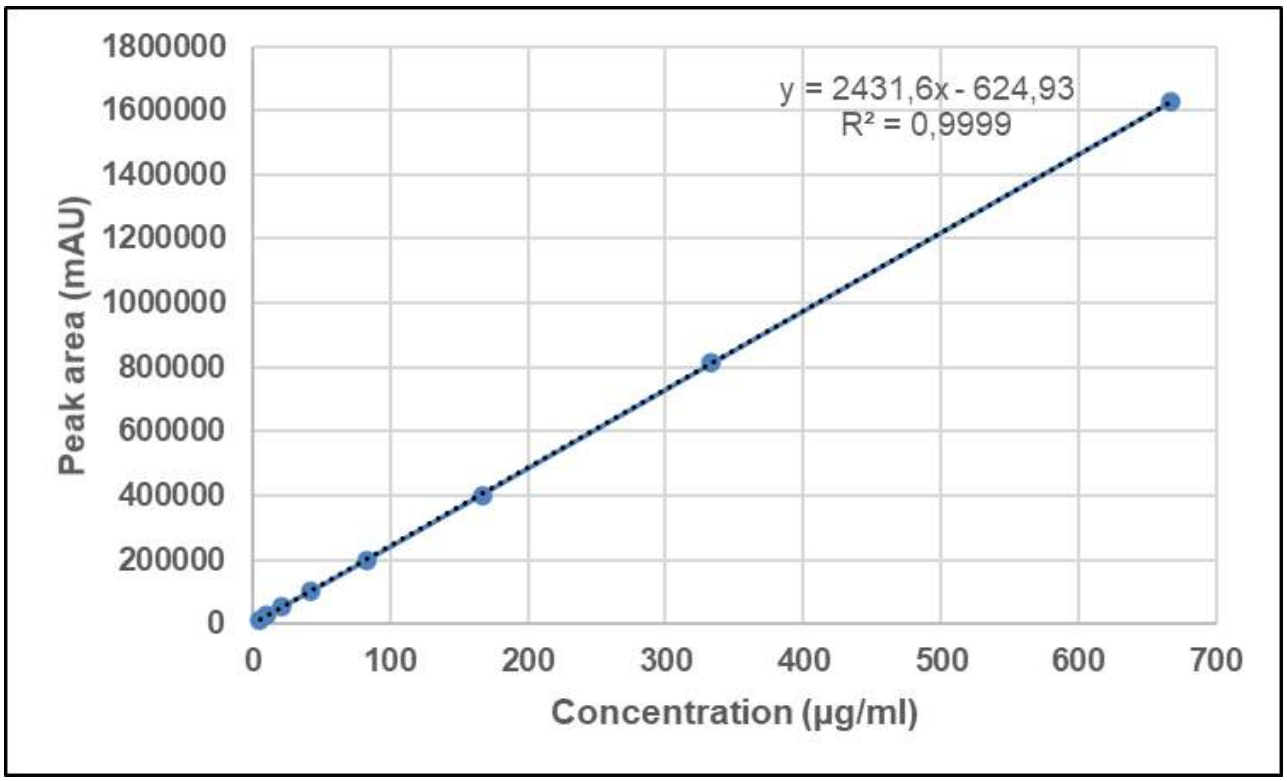


Figure A1: Linear regression curve for CFZ

Table A2: *Linearity data obtained for INH*

Concentration (µg/ml)	Peak area 1	Peak area 2	Mean peak area
5.19	5695	5685	5690
10.39	11291	11106	11198.50
20.77	22413	22776	22594.50
41.54	45599	45779	45689
83.03	91409	92003	91706
166.17	189869	191852	190860.50
332.33	385463	379350	382406.50
665.32	758672	770972	764822
R^2		**0.9999**	
Slope		**924.51**	
Intercept		**259.47**	

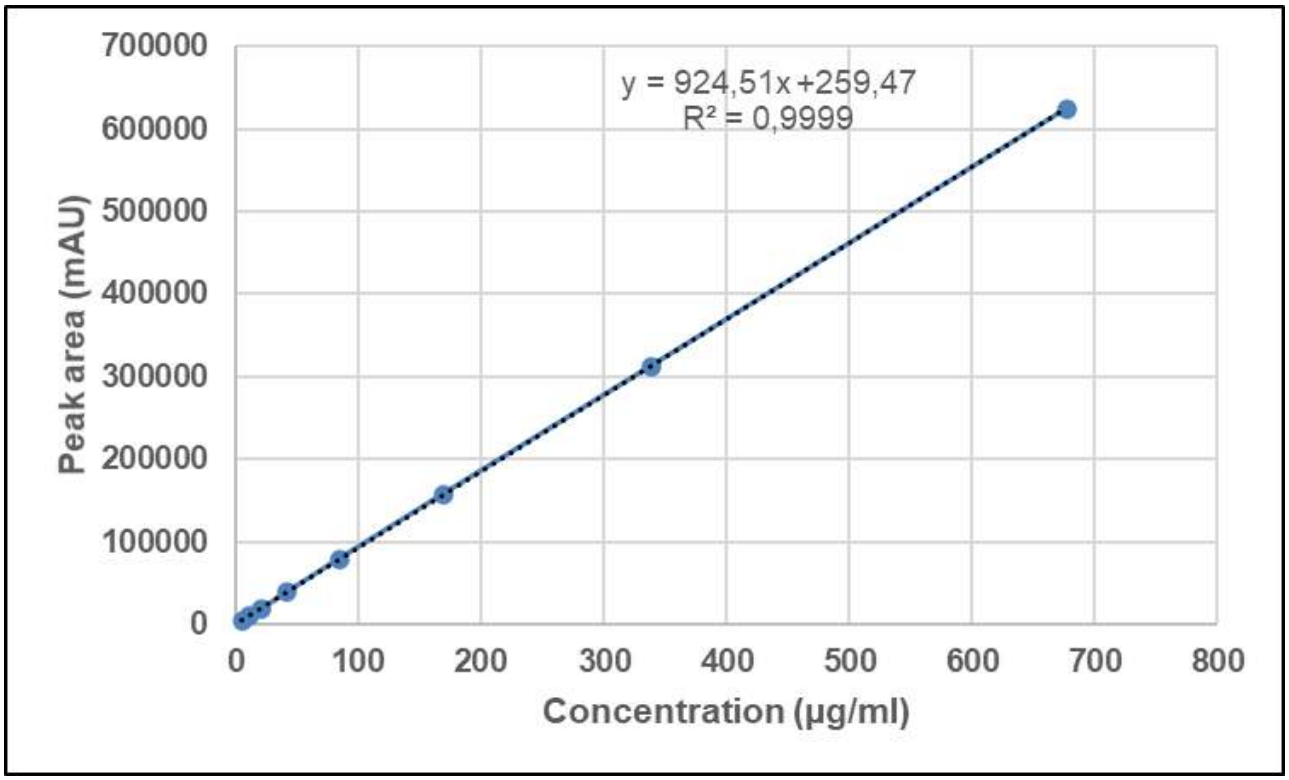


Figure A2: *Linear regression curve for INH*

Table A3: *Linearity data obtained for PZY*

Concentration (µg/ml)	Peak area 1	Peak area 2	Mean peak area
5.19	5593	5787	5690
10.39	11150	11247	11198.50
20.77	22463	22726	22594.50
41.54	45391	45987	45689
83.03	91624	91788	91706
166.17	189913	191808	190860.50
332.33	381004	383809	382406.50
665.32	762608	767036	764822
R^2		**0.9999**	
Slope		**1154.40**	
Intercept		**1589.60**	

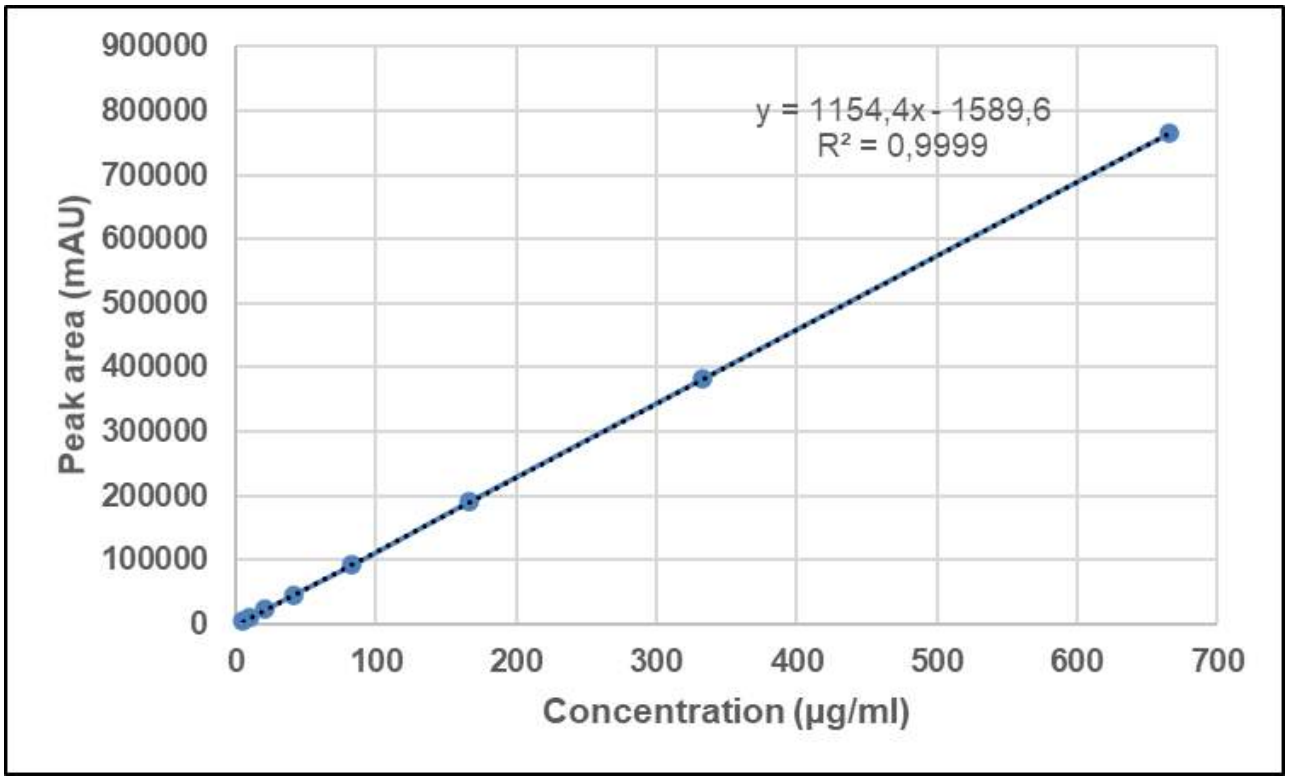


Figure A3: *Linear regression curve for PZY*

Table A4: *Linearity data obtained for RIF*

Concentration (µg/ml)	Peak area 1	Peak area 2	Mean peak area
6.01	20386	22532	21459
12.02	45003	44687	44845
24.04	91269	90323	90796
48.08	190066	185766	187916
96.17	390197	407761	398979
192.33	812764	807170	809967
384.67	1627986	1590388	1609187
R^2		**0.9999**	
Slope		**4209.90**	
Intercept		**7194.40**	

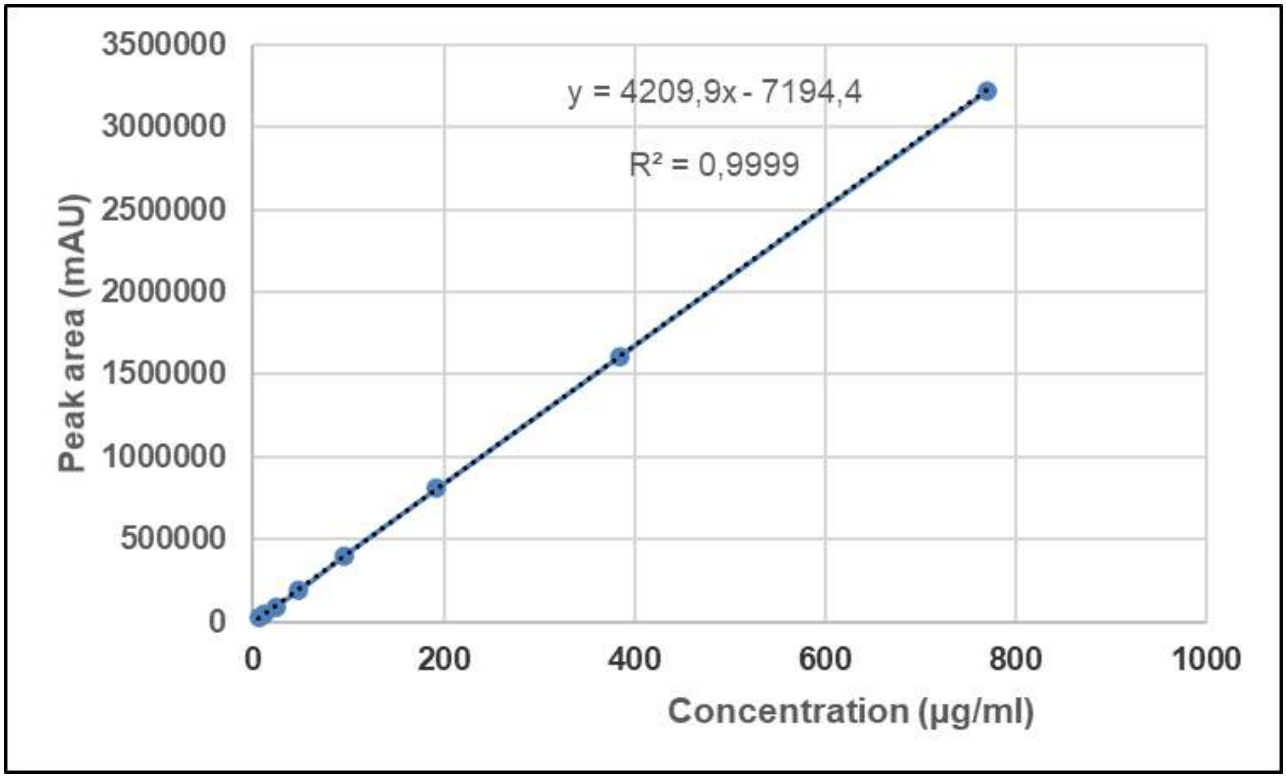


Figure A4: *Linear regression curve for RIF*

Table A5: *Repeatability results for CFZ, INH, PZY and RIF*

	Isoniazid		Pyrazinamide		Rifampicin		Clofazimine	
Injection number	**Peak area (mAU)**	**Retention time (min)**	**Peak area (mAU)**	**Retention time (min)**	**Peak area (mAU)**	**Retention time (min)**	**Peak area (mAU)**	**Retention time (min)**
1	533064	1.46	592709	3.11	355301	5.70	634194	5.92
2	532540	1.46	607233	3.11	361187	5.71	634635	5.94
3	534183	1.46	598757	3.11	362510	5.72	632022	5.97
4	532238	1.46	602279	3.11	362473	5.72	636890	5.97
5	536836	1.46	590490	3.11	363508	5.72	634792	5.97
6	531249	1.46	597960	3.11	365523	5.72	635338	5.97
Mean	**533351.70**	**1.46**	**598238**	**3.11**	**361750.30**	**5.71**	**634645.20**	**5.96**
SD	**1790.47**	**0.003**	**5600.41**	**0.001**	**3171.04**	**0.009**	**1450.60**	**0.020**
%RSD	**0.34**	**0.18**	**0.94**	**0.04**	**0.88**	**0.16**	**0.23**	**0.34**

Table A6: *Stability results for CFZ, INH, PZY and RIF over a period of 24*

	Isoniazid		Pyrazinamide		Rifampicin		Clofazimine	
Time (h)	Peak area (mAU)	% Recovery	Peak area (mAU)	% Recovery	Peak area (mAU)	% Recovery	Peak area (mAU)	% Recovery
0	265402	100.00	893201	100.00	990722	100.00	4976424	100.00
1	276913	104.30	908576	101.70	1013193	102.30	5078964	102.10
2	274745	103.50	893222	100.00	1000874	101.00	5054928	101.60
3	274374	103.40	891639	99.80	993495	100.30	4948672	99.40
4	261545	98.50	893238	100.00	1001046	101.00	5097520	102.40
5	262600	98.90	898559	100.60	991267	100.10	4919879	98.90
6	258652	97.50	895146	100.20	986722	99.60	4986722	100.20
7	269404	10.50	878840	98.40	990243	100.00	4935242	99.20
8	263695	99.40	888765	99.50	982068	99.10	4992401	100.30
9	265402	100.00	883996	99.00	995326	100.50	4992701	100.30
10	263120	99.10	889785	99.60	993007	100.20	4949552	99.50
11	263552	99.30	896108	100.30	983387	99.30	4970846	99.90
12	262747	99.00	887056	99.30	987796	99.70	4934676	99.20
13	268003	101.00	886828	99.30	984661	99.40	4933981	99.10
14	261613	98.60	880828	98.60	999555	100.90	4922351	98.90
15	271578	102.30	898980	100.60	999125	100.80	5010734	100.70
16	272451	102.70	889122	99.50	999129	100.80	5049422	101.50
17	265895	100.20	883810	98.90	994017	100.30	4930242	99.10
18	270980	102.10	896110	100.30	990552	100.00	5002631	100.50
19	261561	98.60	878896	98.40	990981	100.00	5102698	102.50
20	265422	100.00	889360	99.60	1000745	101.00	4949856	99.50

Table A6: *Stability results for CFZ, INH, PZY and RIF over a period of 24*

21	259996	98.00	889466	99.60	991269	100.10	4988638	100.20
22	268963	101.30	887963	99.40	996581	100.60	4965238	99.80
23	271398	102.30	899611	100.70	997745	100.70	4997551	100.40
24	265603	100.10	898770	100.60	1001055	101.00	5012896	100.70
Mean	**266624.60**	**100.50**	**891115**	**99.80**	**994182.40**	**100.30**	**4988190.60**	**100.20**
SD	**4894.02**	**1.84**	**6940.71**	**0.78**	**6815.56**	**0.69**	**52694.86**	**1.06**
%RSD	**1.84**	**1.84**	**0.78**	**0.78**	**0.69**	**0.69**	**1.06**	**1.06**

Table A7: *Accuracy results for CFZ*

Concentration spiked (µg/ml)	Peak area 1 (mAU)	Peak area 2 (mAU)	Mean peak area (mAU)	Recovery	
				(µg/ml)	%
86.16	208714	208903	208808.50	86.13	99.96
85.73	208024	208005	208014.5	85.80	100.08
86.08	207995	207901	207948	85.78	99.65
114.00	276044	276870	276457	113.95	99.96
113.89	275038	275099	275068.50	113.38	99.55
113.79	273336	273842	273589	112.77	99.10
170.19	412864	412289	412576.50	169.93	99.85
170.52	412971	412718	412844.50	170.04	99.72
170.19	412119	412395	412257	169.80	99.77
				Mean	**99.74**
				SD	**0.27**
				%RSD	**0.28**

Table A8: *Accuracy results for INH*

Concentration spiked (µg/ml)	Peak area 1 (mAU)	Peak area 2 (mAU)	Mean peak area (mAU)	Recovery	
				(µg/ml)	%
169.33	157010	157021	157015.50	169.56	100.14
166.00	153995	153945	153970	166.26	100.16
167.00	154892	154539	154715.50	167.07	100.04
222.44	205910	205920	205915	222.45	100.00
222.56	206200	206010	206105	222.65	100.04
221.78	205310	205350	205330	221.82	100.01
338.33	313048	313049	313033.50	338.31	99.99
335.00	309971	309977	309974	335.00	100.00
333.67	308744	308759	308751.50	333.68	100.00
				Mean	**100.04**
				SD	**0.06**
				%RSD	**0.06**

Table A9: *Accuracy results for PZY*

Concentration spiked (µg/ml)	Peak area 1 (mAU)	Peak area 2 (mAU)	Mean peak area (mAU)	Recovery	
				(µg/ml)	%
166.17	190566	190897	190731.50	166.60	100.26
166.33	191033	190964	190998.50	166.83	100.30
168.33	193711	193796	193753.50	169.22	100.53
221.56	258429	259647	259038	225.77	102.16
222.00	258938	259463	259200.50	225.91	101.76
222.67	256915	259941	258428	225.24	101.46
332.33	388963	389715	389339	338.64	101.90
333.33	389618	391952	390785	339.89	101.97
332.00	387612	384894	386253	335.97	101.20
				Mean	**101.28**
				SD	**0.70**
				%RSD	**0.70**

Table A10: *Accuracy results for RIF*

Concentration spiked (µg/ml)	Peak area 1 (mAU)	Peak area 2 (mAU)	Mean peak area (mAU)	Recovery	
				(µg/ml)	%
54.19	238689	237988	238338.50	54.45	100.47
58.25	239138	241008	240073	58.75	100.85
59.24	242976	241339	242157.50	60.36	101.89
77.61	319994	318116	319055	77.51	99.86
77.10	316855	316991	316923	77.01	99.88
77.18	314399	313316	313857.50	76.28	98.84
115.54	476637	479521	478079	115.29	99.78
116.04	479961	480061	480011	115.75	99.75
115.65	479418	479637	479527.50	115.64	99.99
				Mean	**100.15**
				SD	**0.80**
				%RSD	**0.80**

Table A11: *Intra-day precision for CFZ*

Concentration spiked (µg/mL)	Peak area 1 (mAU)	Peak area 2 (mAU)	Mean peak area (mAU)	Recovery	
				µg/mL	%
170	408823	408082	408452.50	168.23	98.96
170	407068	406649	406858.50	167.58	98.58
170	408722	410063	409392.50	168.62	99.19
140	336984	336984	336984	138.84	99.17
140	342686	328983	335834.50	138.37	98.84
140	330778	330669	330723.50	136.27	97.34
85	211356	212297	211826.50	87.37	97.08
90	210671	213990	212330.50	87.58	97.31
90	212226	211167	211696.50	87.32	97.02
				Mean	**98.17**
				SD	**0.95**
				%RSD	**0.01**

Table A12: *Intra-day precision for INH*

Concentration spiked (µg/mL)	Peak area 1 (mAU)	Peak area 2 (mAU)	Mean peak area (mAU)	Recovery	
				µg/mL	%
515	466618	469546	468082	506.02	98.26
515	469246	481518	475382	513.92	99.79
515	487159	492724	489941.50	529.67	102.85
400	373795	365077	369436	393.32	98.33
400	373930	371957	372943.50	403.12	100.78
400	363829	373008	368418.50	398.22	99.56
250	233645	235184	234414.50	253.27	101.31
250	235993	233197	234595	253.47	101.39
250	232122	235578	233850	252.66	101.06
				Mean	**100.37**
				SD	**1.51**
				%RSD	**0.02**

Table A13: *Intra-day precision for PZY*

Concentration spiked (µg/mL)	Peak area 1 (mAU)	Peak area 2 (mAU)	Mean peak area (mAU)	Recovery	
				µg/mL	%
490	550886	573769	562327.50	488.49	99.69
490	572381	573566	572973.50	497.72	101.58
490	572227	573920	573073.50	497.80	101.59
370	424012	424012	424012	368.68	99.64
370	425368	422982	424175	368.82	99.68
370	424283	424994	424638.50	369.22	99.79
240	272111	272206	272158.50	237.13	98.80
240	271064	272129	271596.50	236.65	98.60
240	272162	271571	271866.50	236.88	98.70
				Mean	**99.79**
				SD	**1.12**
				%RSD	**0.01**

Table A14: *Intra-day precision for RIF*

Concentration spiked (µg/mL)	Peak area 1 (mAU)	Peak area 2 (mAU)	Mean peak area (mAU)	Recovery	
				µg/mL	%
380	1585827	1592424	1589126	379.18	99.78
380	1590529	1595053	1592791	380.05	100.01
380	1592845	1590184	1591515	379.75	99.93
290	1233839	1193724	1213782	290.02	100.00
290	1196889	1189913	1193401	285.18	98.34
290	1192105	1194580	1193343	285.17	98.33
180	749482	748330	748906	179.60	99.78
180	746735	747147	746941	179.13	99.52
180	748310	748990	748650	179.54	99.74
				Mean	**99.49**
				SD	**0.67**
				%RSD	**0.01**

Table A15: *Inter-day precision for CFZ*

Parameters (%Recovery)	Day 1	Day 2	Day 3	Between days
	1459042	146019	1686090	
	1519367	1496369	1616793	
	1433148	1599663	1515589	
Mean	**1470519**	**1080684**	**1606157**	**1385787**
SD	**44240.48**	**811089.40**	**85746.64**	**313692.20**
%RSD	**0.03**	**0.75**	**0.05**	**0.28**

Table A16: *Inter-day precision for INH*

Parameters (%Recovery)	Day 1	Day 2	Day 3	Between days
	822928	690508	756632	
	779132	768542	778971	
	800834	672652	746643	
Mean	**808107.67**	**710567.33**	**760748.67**	**759807.90**
SD	**25095.87**	**50995.15**	**16552.50**	**30881.17**
%RSD	**0.03**	**0.07**	**0.02**	**0.04**

Table A17: *Inter-day precision for PZY*

Parameters (%Recovery)	Day 1	Day 2	Day 3	Between days
	1518281	1320723	1489961	
	1473858	1239408	1488932	
	1527587	1215513	1524436	
Mean	**1506575**	**1258548**	**1501110**	**1422078**
SD	**28713.56**	**55154.70**	**20207.75**	**34692**
%RSD	**0.02**	**0.04**	**0.01**	**0.03**

Table A18: *Inter-day precision for RIF*

Parameters (%Recovery)	Day 1	Day 2	Day 3	Between days
	600495	818752	797745	
	594260	761161	599630	
	590614	755494	600017	
Mean	**595123**	**778469**	**665797.30**	**679796.40**
SD	**4996.71**	**35000.98**	**114270.20**	**51422.63**
%RSD	**0.01**	**0.04**	**0.17**	**0.07**

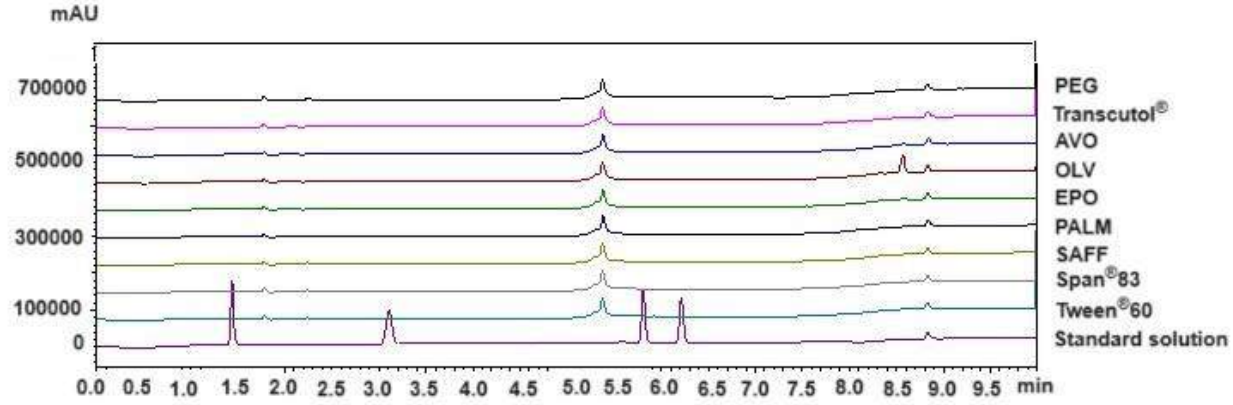


Figure A5: *Chromatogram of CFZ, INH, PZY and RIF standard solution and excipients considered for inclusion in SDEDDS displayed at 254 nm*

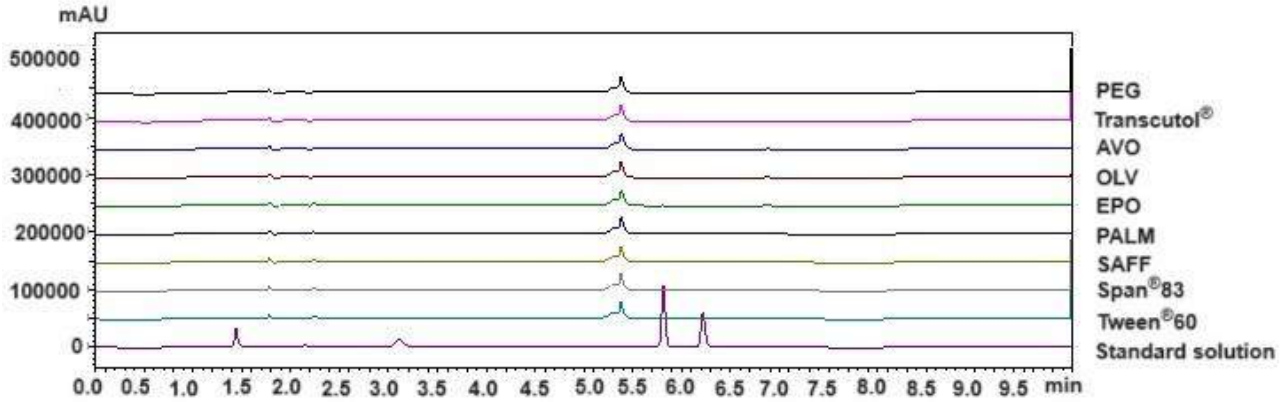


Figure A6: *Chromatogram of CFZ, INH, PZY and RIF standard solution and excipients considered for inclusion in SDEDDS displayed at 320 nm*

Annexure B

Immiscibility Studies

B.1. Introduction

Immiscibility experiments were performed by weighing 10 g of a potential internal oil phase and adding it to 10 g of a considered external oil phase. Next, the oil mixtures were subjected to vortexing for 10 min, whereafter the vortexed mixtures were poured into separatory funnels and left to investigate the degree of separation after a period of 30 min. The separatory funnel was then used to distinguish different oil phases upon separation as to determine the immiscibility percentage in terms of weight differences.

Table B1: *Immiscibility results obtained for mixtures of Transcutol®:PEG (9:1) and potential external oil phases (n=3)*

External oil phase	**AVO**	**EPO**	**OLV**	**PALM**	**SAFF**
Round 1 percentage immiscibility (%)	75.89	84.50	77.13	63.50	60.69
Round 2 percentage immiscibility (%)	72.40	82.76	83.76	71.83	61.18
Round 3 percentage immiscibility (%)	80.78	88.37	79.18	65.72	59.74
Mean Percentage immiscibility (%)	75.36	85.21	80.02	67.90	60.54
SD	421	2.87	3.39	4.31	0.73
%RSD	5.51	3.37	4.24	6.44	1.20

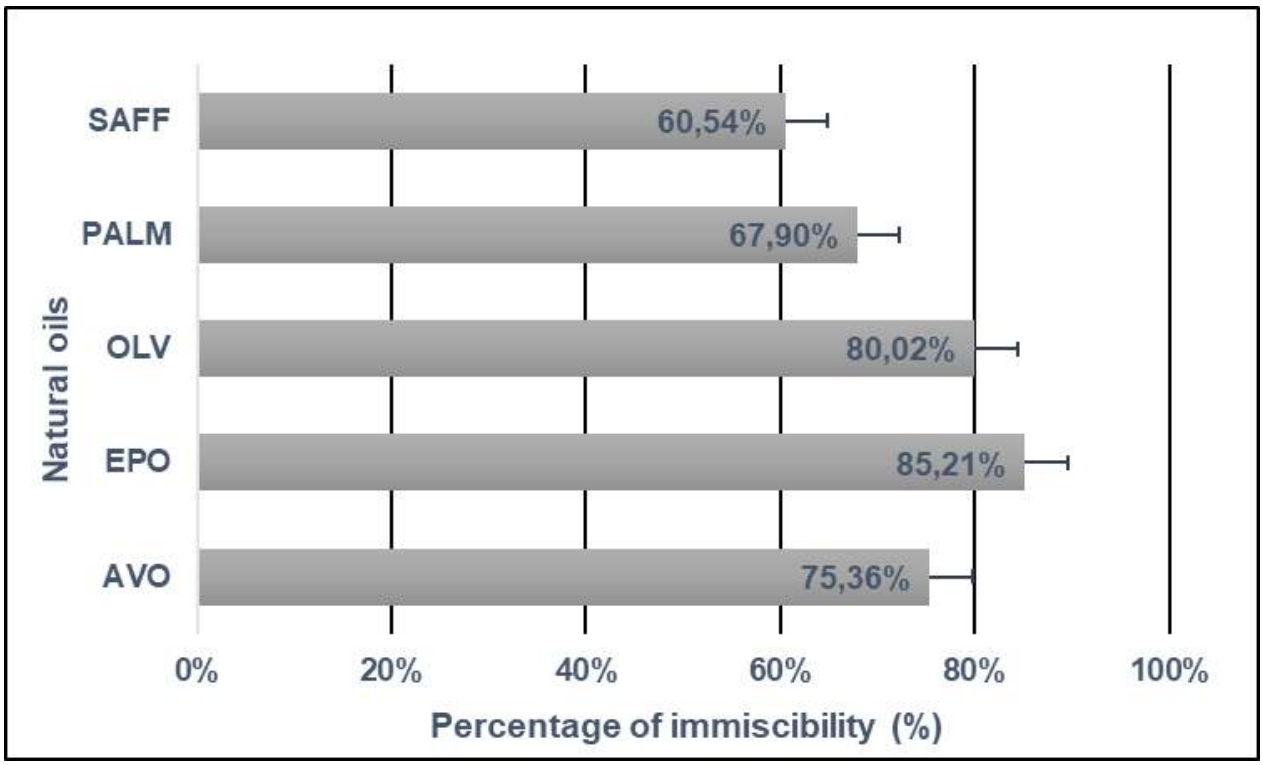


Figure B1: *Percentage immiscibility for mixtures of Transcutol®:PEG (9:1) and potential external oil phases (n=3)*

Annexure c

Isothermal Microcalorimetry Studies

C.1. Introduction

A 2277 Thermal Activity Monitor, TAM III (TA Instruments, New Castle, Delaware (DE), USA), equipped with an oil bath with a stability of ± 100 µK over 24 h was used. The temperature was maintained at 40°C and 100 mg samples tested. The heat flow for each individual component was measured to generate a theoretical response (i.e., base-line), which was accompanied by a comparison of the theoretical response with the detected calometric output to determine compatibility. If the theoretical response drastically differed from the detected calometric output, interactions between excipients were suspected.

Table C1: *Summary of compatibility for different ingredient combinations as evaluated by isothermal microcalorimetry*

Sample number	Combinations tested	Difference between slopes and y-intercepts of heat flows (J/g or kJ/g)	Reason for potential incompatibility
1	**PE + CFZ**	**Difference observed (36.36 J/g)**	**Potential physical incompatibility**
2	**PE + INH**	**Difference observed (36.61 J/g)**	**Potential physical incompatibility**
3	**PE + PZY**	**Difference observed (−26.54 J/g)**	**Potential physical incompatibility**
4	**PE + RIF**	**Difference observed (39.15 J/g)**	**Potential physical incompatibility**
5	**PE + AVO**	**Difference observed (2.64 J/g)**	**Immiscibility of liquids**
6	**PE + EPO**	**Difference observed (−29.18 J/g)**	**Immiscibility of liquids**

Table C1: *Summary of compatibility for different ingredient combinations as evaluated by isothermal microcalorimetry (continued)*

Sample number	Combinations tested	Difference between slopes and y-intercepts of heat flows (J/g or kJ/g)	Reason for potential incompatibility
7	**PE + OLV**	**Difference observed (−38.01 J/g)**	**Immiscibility of liquids**
8	**PE + PALM**	**Difference observed (26.20 J/g)**	**Immiscibility of liquids**
9	**PE + SAFF**	**Difference observed (85.90 J/g)**	**Immiscibility of liquids**
10	**PE + BENTONITE CLAY**	**Difference observed (1.5843 J/g)**	**Potential physical incompatibility**
11	**AVO + CFZ**	No difference observed	Compatible
12	**AVO + INH**	No difference observed	Compatible
13	**AVO + PZY**	No difference observed	Compatible
14	**AVO + RIF**	No difference observed	Compatible
15	**AVO + BENTONITE CLAY**	No difference observed	Compatible
16	**EPO + CFZ**	No difference observed	Compatible
17	**EPO + INH**	No difference observed	Compatible
18	**EPO + PZY**	No difference observed	Compatible
19	**EPO + RIF**	No difference observed	Compatible
20	**EPO + BENTONITE CLAY**	No difference observed	Compatible

Table C1: *Summary of compatibility for different ingredient combinations as evaluated by isothermal microcalorimetry (continued)*

Sample number	Combinations tested	Difference between slopes and y-intercepts of heat flows (J/g or kJ/g)	Reason for potential incompatibility
21	**OLV + CFZ**	No difference observed	Compatible
22	**OLV + INH**	No difference observed	Compatible
23	**OLV + PZY**	No difference observed	Compatible
24	**OLV + RIF**	No difference observed	Compatible
25	**OLV + BENTONITE CLAY**	No difference observed	Compatible
26	**PALM + CFZ**	No difference observed	Compatible
27	**PALM + INH**	No difference observed	Compatible
28	**PALM + PZY**	No difference observed	Compatible
29	**PALM + RIF**	No difference observed	Compatible
30	**PALM + BENTONITE CLAY**	No difference observed	Compatible
31	**SAFF + CFZ**	No difference observed	Compatible
32	**AVO + INH**	No difference observed	Compatible
33	**AVO + PZY**	No difference observed	Compatible
34	**SAFF + RIF**	No difference observed	Compatible

Table C1: *Summary of compatibility for different ingredient combinations as evaluated by isothermal microcalorimetry (continued)*

Sample number	Combinations tested	Difference between slopes and y-intercepts of heat flows (J/g or kJ/g)	Reason for potential incompatibility
35	**CFZ + BENTONITE CLAY**	No difference observed	Compatible
36	**INH + BENTONITE CLAY**	No difference observed	Compatible
37	**PZY + BENTONITE CLAY**	No difference observed	Compatible
38	**RIF + BENTONITE CLAY**	No difference observed	Compatible
39	**CFZ + INH**	**Difference observed (2252 kJ/g)**	**Synergism**
40	**CFZ + PZY**	No difference observed	Compatible
41	**CFZ + RIF**	No difference observed	Compatible
42	**INH + PZY**	No difference observed	Compatible
43	**INH + RIF**	**Difference observed (822.89 J/g).**	**RIF shows significant instability in the presence of dissolved INH in an acidic environment**
44	**PZY +RIF**	No difference observed	Compatible

Compatibility Report
Ampoule (6-6-22).rslt

Summary

Name:	*Ampoule (6-6-22).rslt*
Report created:	Jul 14, 2023 14:13:03
Start time:	Jun 06, 2022 10:15:38
End time:	Jun 08, 2022 13:14:14
Operator:	MG
Results file path:	Ampoule (6-6-22).rslt

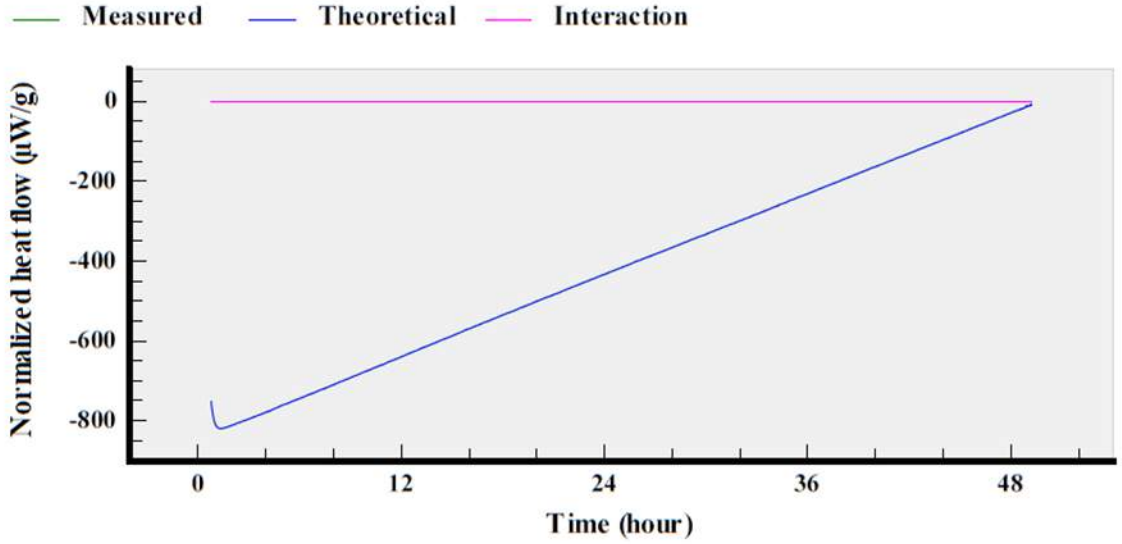
General Experiment Info

Bath temperature:	40 °C

TAM Assistant Analysis Report
Compatibility

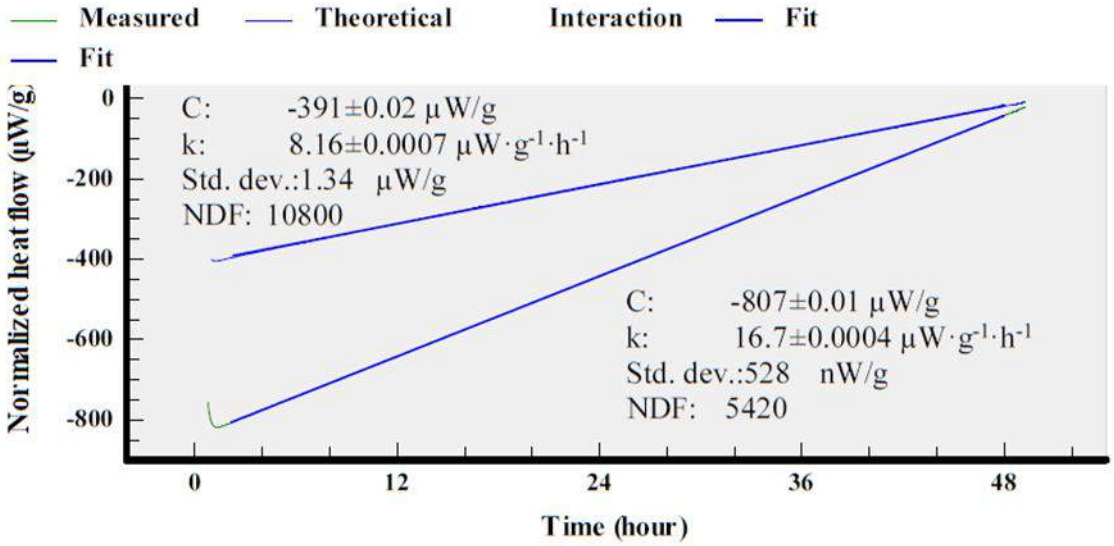
Primere emulsie (Primere emulsie)

Mass, Primere emulsie:	109 mg
Measurement signal:	Data series.Signal, Ch 4:1 [Heat flow] *Ampoule (5-18-22).rslt*
Reaction start:	May 18, 2022 11:23:40
Interaction integral:	0 J/g
Interaction integral time:	2 days, 0 hrs, 27 min, 2 sec



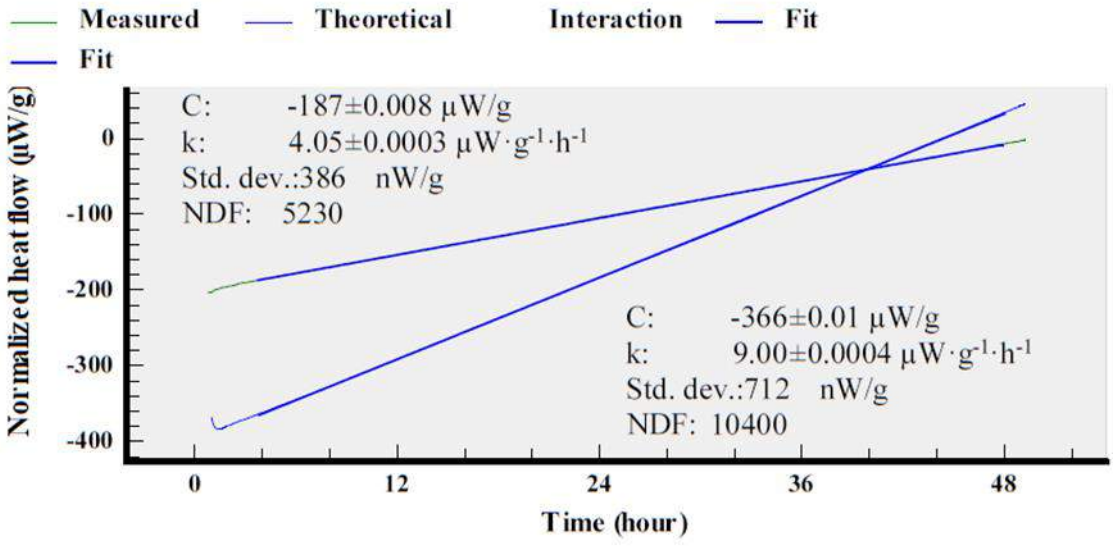
Primere emulsie+Olyfolie (Primere emulsie+Olyfolie)

Mass, Primere emulsie:	52.2 mg
Mass, Olyfolie:	56.3 mg
Measurement signal:	Data series.Signal, Ch 4:2 [Heat flow] *Ampoule (5-18-22).rslt*
Reaction start:	May 18, 2022 11:23:40
Interaction integral:	-38.009 J/g
Interaction integral time:	2 days, 0 hrs, 27 min, 2 sec



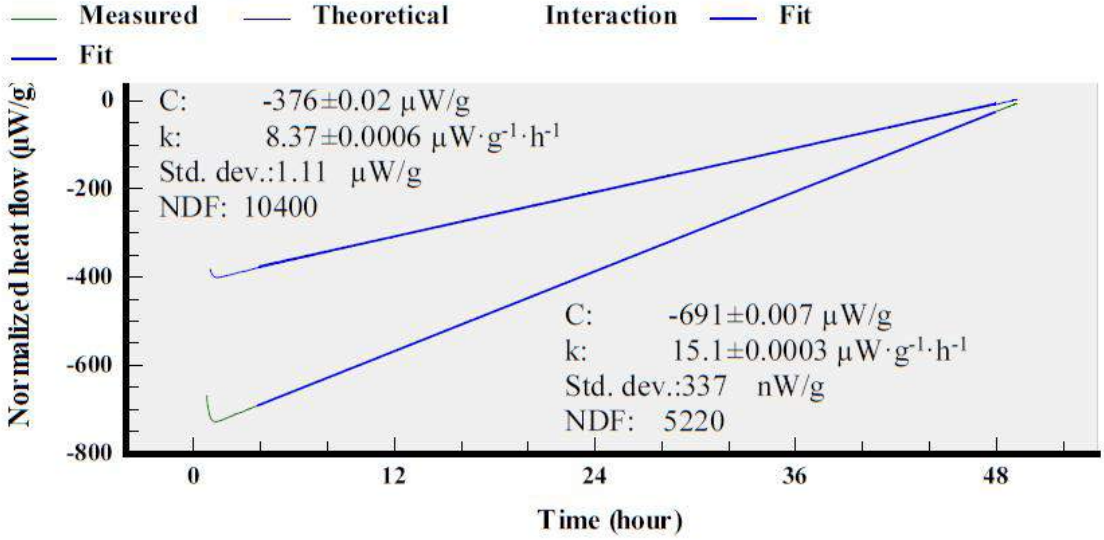
Primere emulsie+Avo olie (Primere emulsie+Avo olie)

Mass, Primere emulsie:	52 mg
Mass, Avo olie:	53 mg
Measurement signal:	Data series.Signal, Ch 4:3 [Heat flow] *Ampoule (5-18-22).rslt*
Reaction start:	May 18, 2022 11:23:40
Interaction integral:	12.643 J/g
Interaction integral time:	2 days, 0 hrs, 27 min, 2 sec



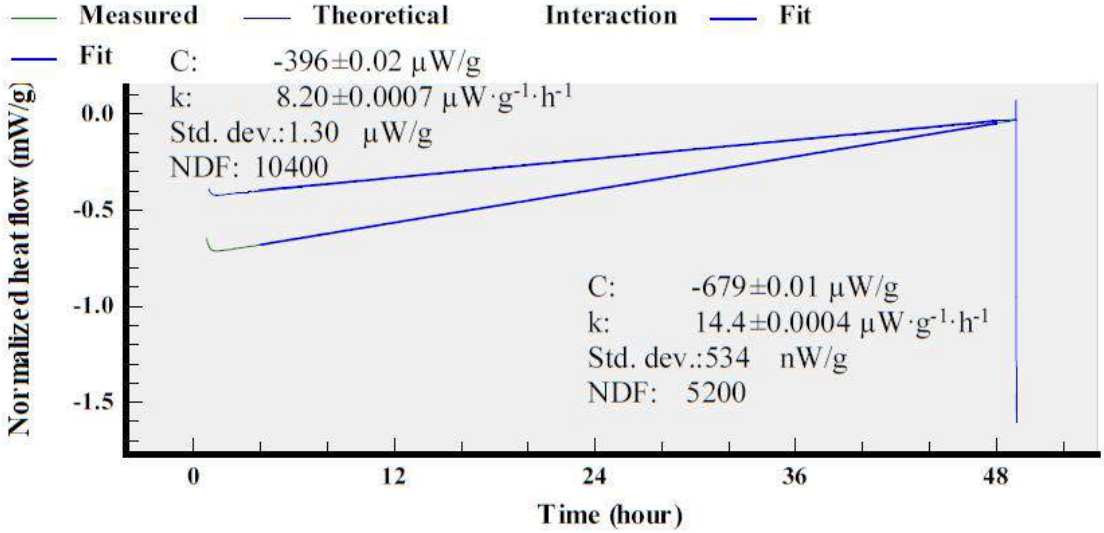
Primere emulsie+EPO (Primere emulsie+EPO)

Mass, Primere emulsie:	53 mg
Mass, EPO:	53 mg
Measurement signal:	Data series.Signal, Ch 4:4 [Heat flow]
	Ampoule (5-18-22).rslt
Reaction start:	May 18, 2022 11:23:40
Interaction integral:	-29.817 J/g
Interaction integral time:	2 days, 0 hrs, 27 min, 2 sec



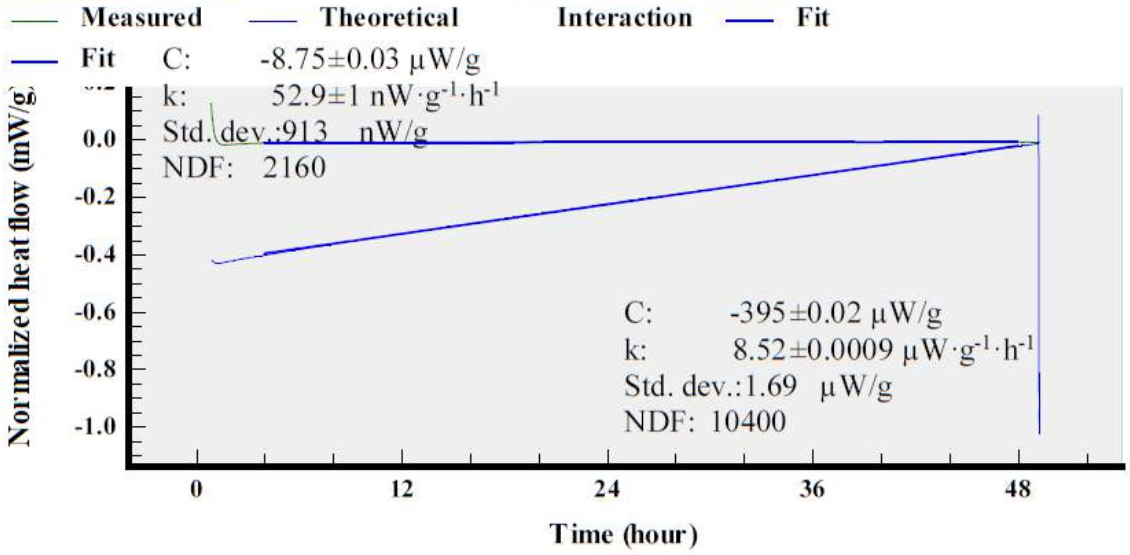
Primere emulsie+PZY (Primere emulsie+PZY)

Mass, Primere emulsie:	52 mg
Mass, PZY:	52 mg
Measurement signal:	Data series.Signal, Ch 4:5 [Heat flow]
	Ampoule (5-18-22).rslt
Reaction start:	May 18, 2022 11:23:40
Interaction integral:	-26.542 J/g
Interaction integral time:	2 days, 0 hrs, 27 min, 2 sec



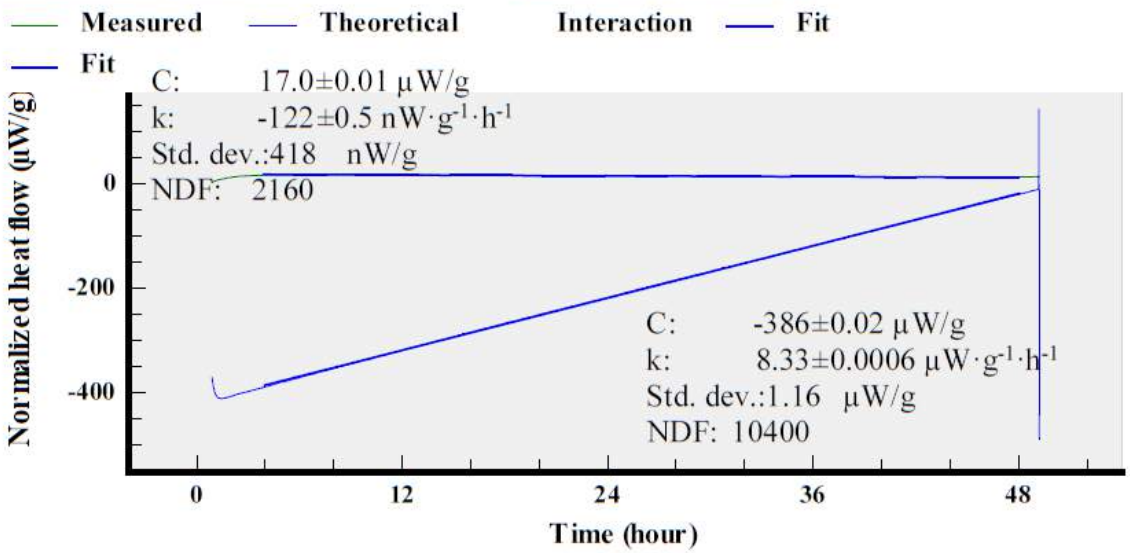
Primere emulsie+CFZ (Primere emulsie+Primere emulsie+CFZ)

Mass, Primere emulsie:	49.2 mg
Mass, Primere emulsie+CFZ:	49.3 mg
Measurement signal:	Data series.Signal, Ch 4:1 [Heat flow] *Ampoule (5-25-22).rslt*
Reaction start:	May 25, 2022 10:15:31
Interaction integral:	36.335 J/g
Interaction integral time:	2 days, 0 hrs, 23 min, 13 sec



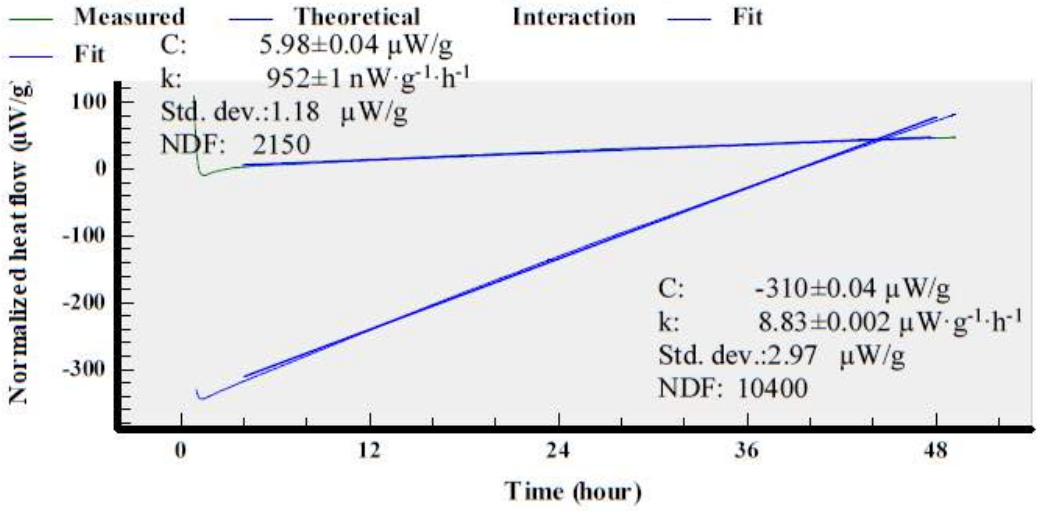
Primere emulsie+RIF (Primere emulsie+RIF)

Mass, Primere emulsie:	48.8 mg
Mass, RIF:	49.9 mg
Measurement signal:	Data series.Signal, Ch 4:3 [Heat flow] *Ampoule (5-25-22).rslt*
Reaction start:	May 25, 2022 10:15:31
Interaction integral:	39.151 J/g
Interaction integral time:	2 days, 0 hrs, 23 min, 13 sec



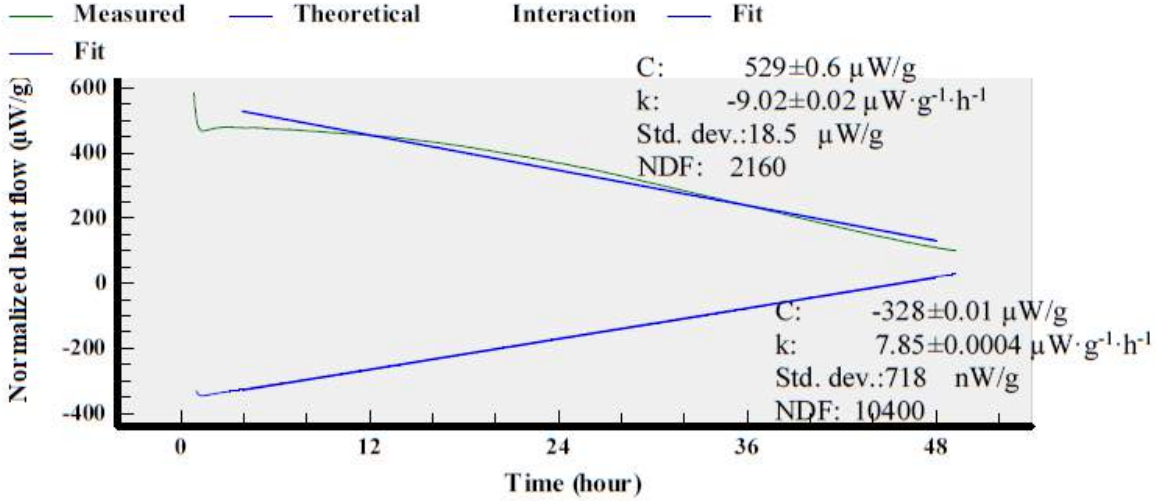
Primere emulsie+Palm-olie (Primere emulsie+Palm-olie)

Mass, Primere emulsie:	55.3 mg
Mass, Palm-olie:	50.8 mg
Measurement signal:	Data series.Signal, Ch 4:4 [Heat flow] *Ampoule (5-25-22).rslt*
Reaction start:	May 25, 2022 10:15:31
Interaction integral:	26.203 J/g
Interaction integral time:	2 days, 0 hrs, 23 min, 13 sec



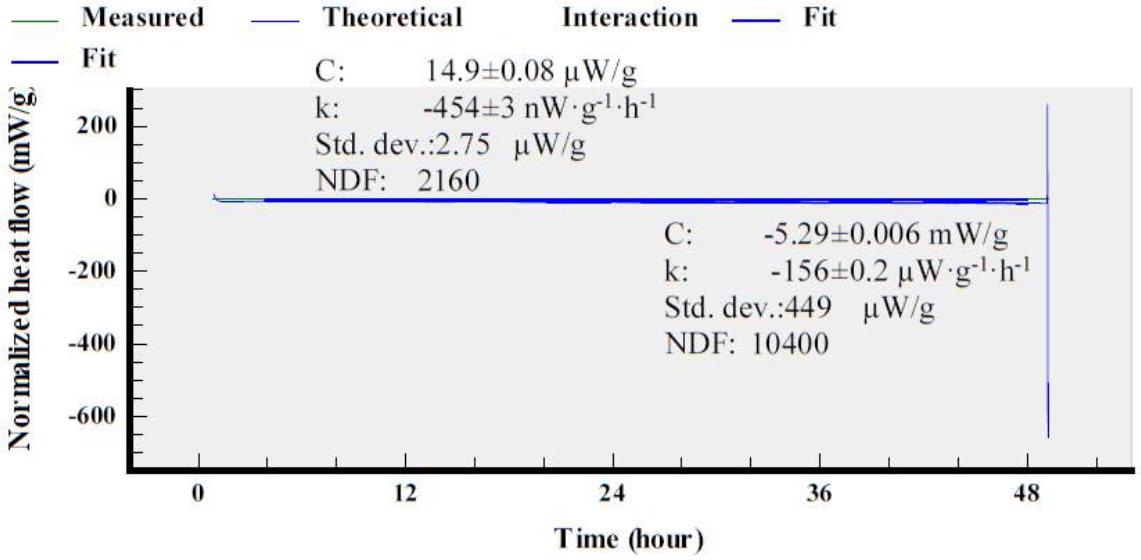
Primere emulsie+Sunflower oil (Primere emulsie+Sunflower oil)

Mass, Primere emulsie:	51.6 mg
Mass, Sunflower oil:	51.5 mg
Measurement signal:	Data series.Signal, Ch 4:5 [Heat flow] *Ampoule (5-25-22).rslt*
Reaction start:	May 25, 2022 10:15:31
Interaction integral:	85.901 J/g
Interaction integral time:	2 days, 0 hrs, 23 min, 13 sec



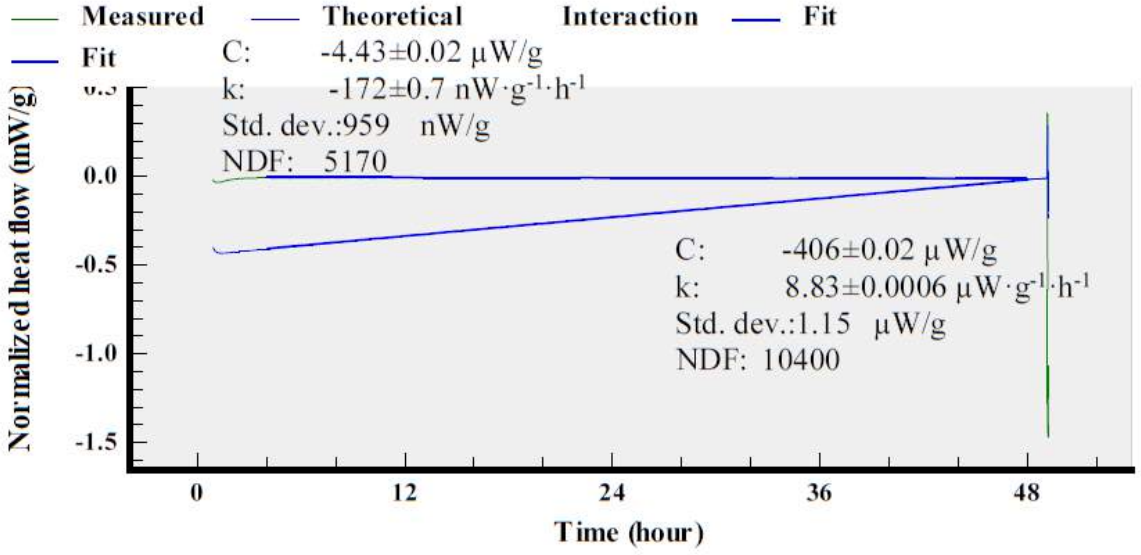
Primere emulsie+Bentonite clay (Primere emulsie+Bentonite clay)

Mass, Primere emulsie:	50.5 mg
Mass, Bentonite clay:	50.5 g
Measurement signal:	Data series.Signal, Ch 4:6 [Heat flow] *Ampoule (5-25-22).rslt*
Reaction start:	May 25, 2022 10:15:31
Interaction integral:	1.5843 kJ/g
Interaction integral time:	2 days, 0 hrs, 23 min, 13 sec



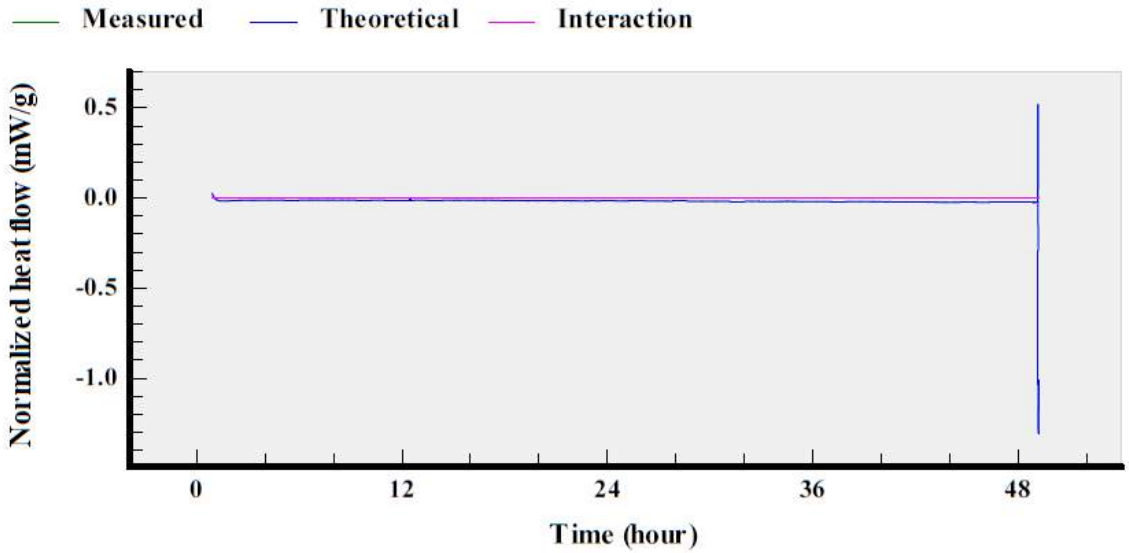
Primere emulsie+INH (Primere emulsie+INH)

Mass, Primere emulsie:	55 mg
Mass, INH:	51 mg
Measurement signal:	Data series.Signal, Ch 4:1 [Heat flow] *Ampoule (5-31-22).rslt*
Reaction start:	May 31, 2022 11:30:00
Interaction integral:	36.605 J/g
Interaction integral time:	2 days, 0 hrs, 19 min, 45 sec



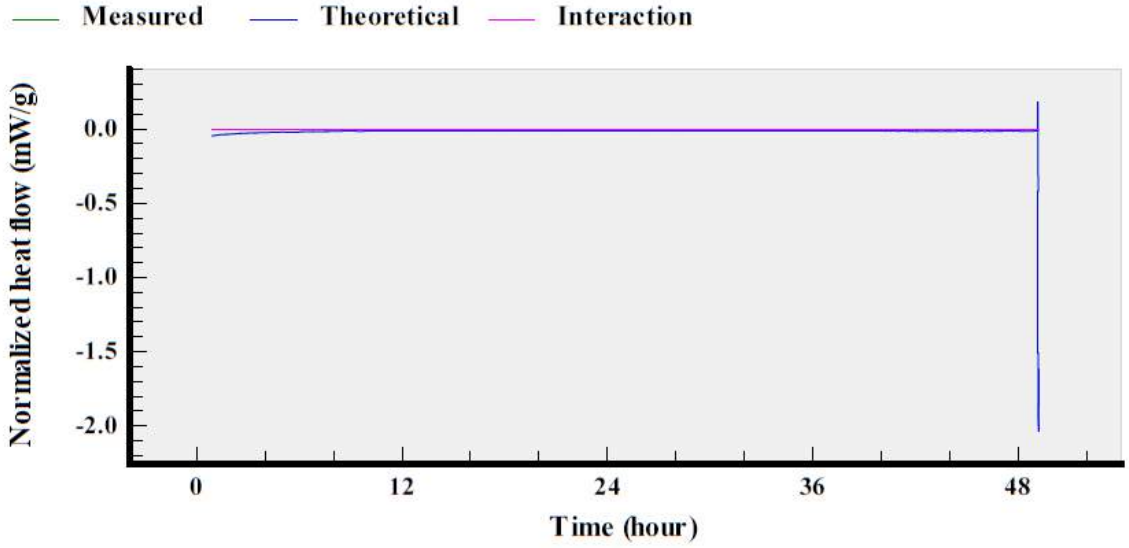
Bentonite Clay (Bentonite Clay)

Mass, Bentonite Clay:	102 mg
Measurement signal:	Data series.Signal, Ch 4:2 [Heat flow] *Ampoule (5-31-22).rslt*
Reaction start:	May 31, 2022 11:30:00
Interaction integral:	0 J/g
Interaction integral time:	2 days, 0 hrs, 19 min, 45 sec



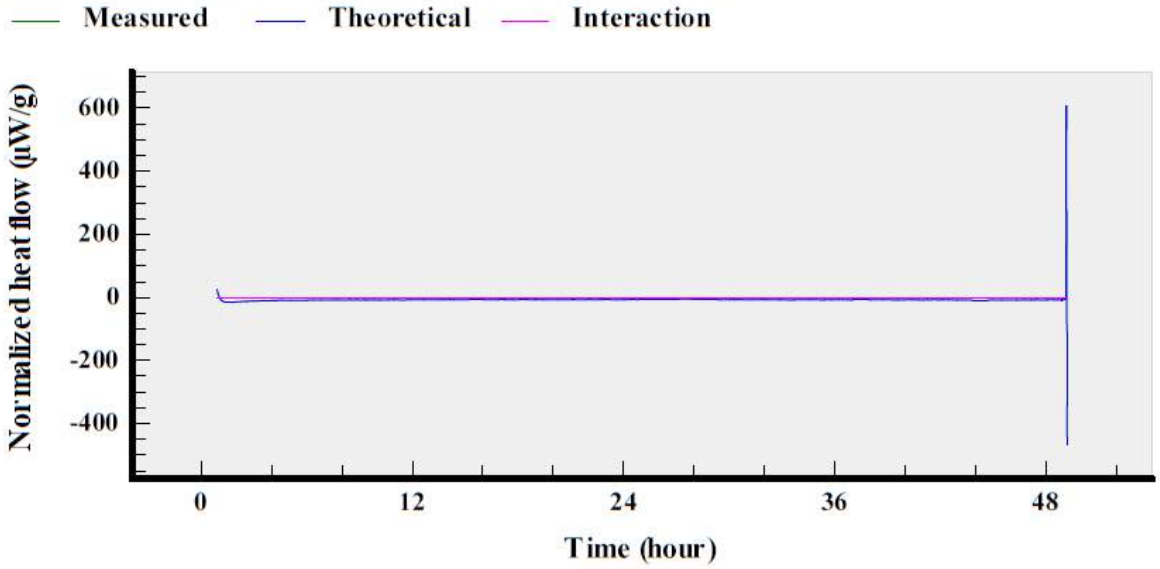
CFZ (CFZ)

Mass, CFZ:	103 mg
Measurement signal:	Data series.Signal, Ch 4:3 [Heat flow] *Ampoule (5-31-22).rslt*
Reaction start:	May 31, 2022 11:30:00
Interaction integral:	0 J/g
Interaction integral time:	2 days, 0 hrs, 19 min, 45 sec



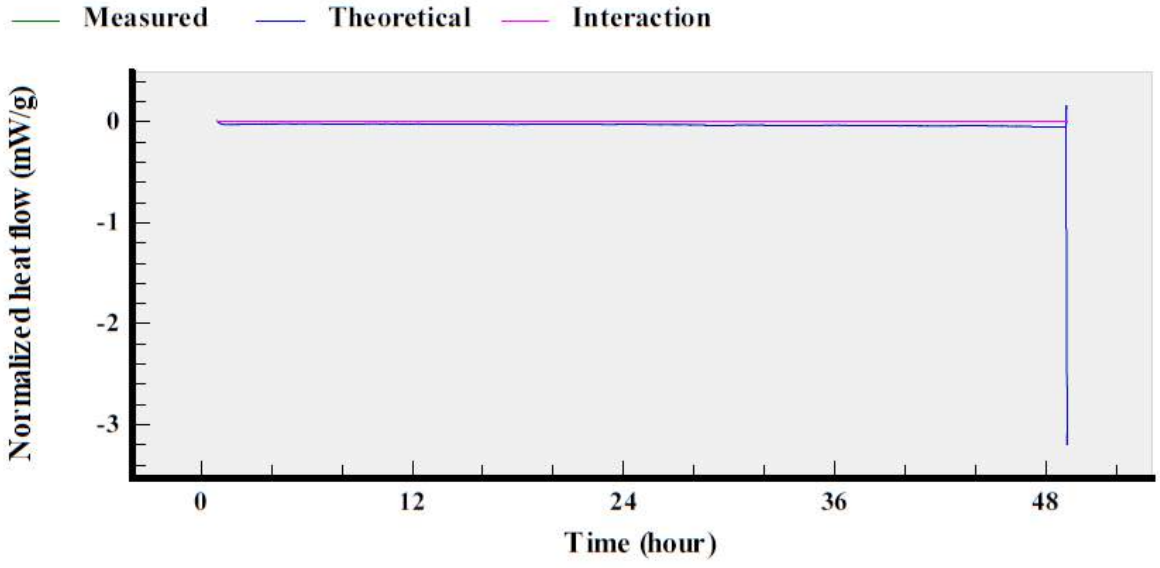
INH (INH)

Mass, INH:	104 mg
Measurement signal:	Data series.Signal, Ch 4:4 [Heat flow] *Ampoule (5-31-22).rslt*
Reaction start:	May 31, 2022 11:30:00
Interaction integral:	0 J/g
Interaction integral time:	2 days, 0 hrs, 19 min, 45 sec



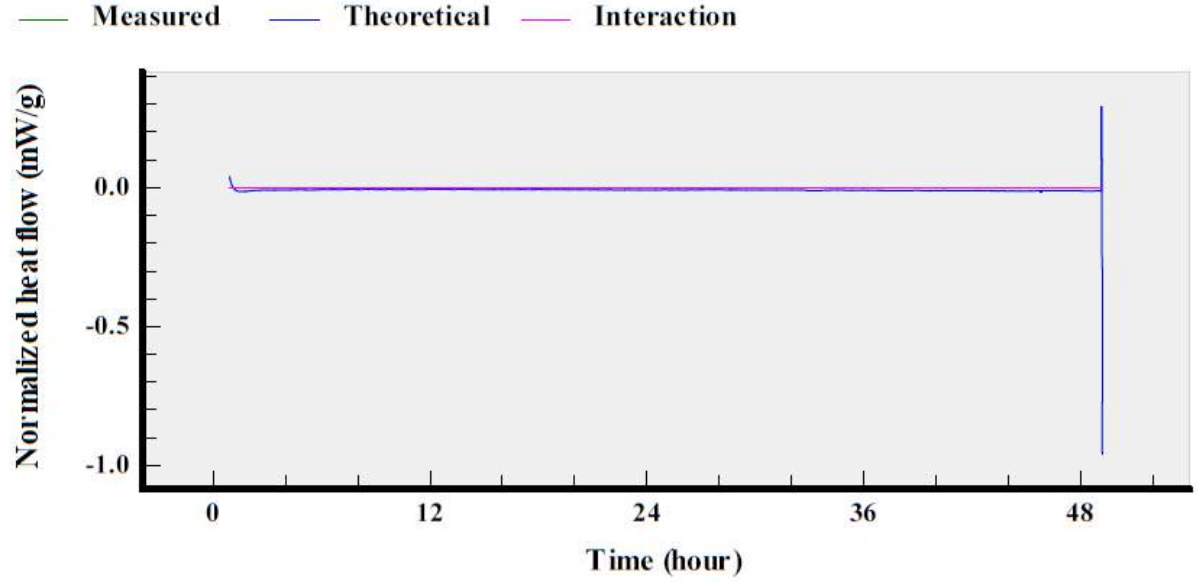
PZY (PZY)

Mass, PZY:	105 mg
Measurement signal:	Data series.Signal, Ch 4:5 [Heat flow] *Ampoule (5-31-22).rslt*
Reaction start:	May 31, 2022 11:30:00
Interaction integral:	0 J/g
Interaction integral time:	2 days, 0 hrs, 19 min, 45 sec



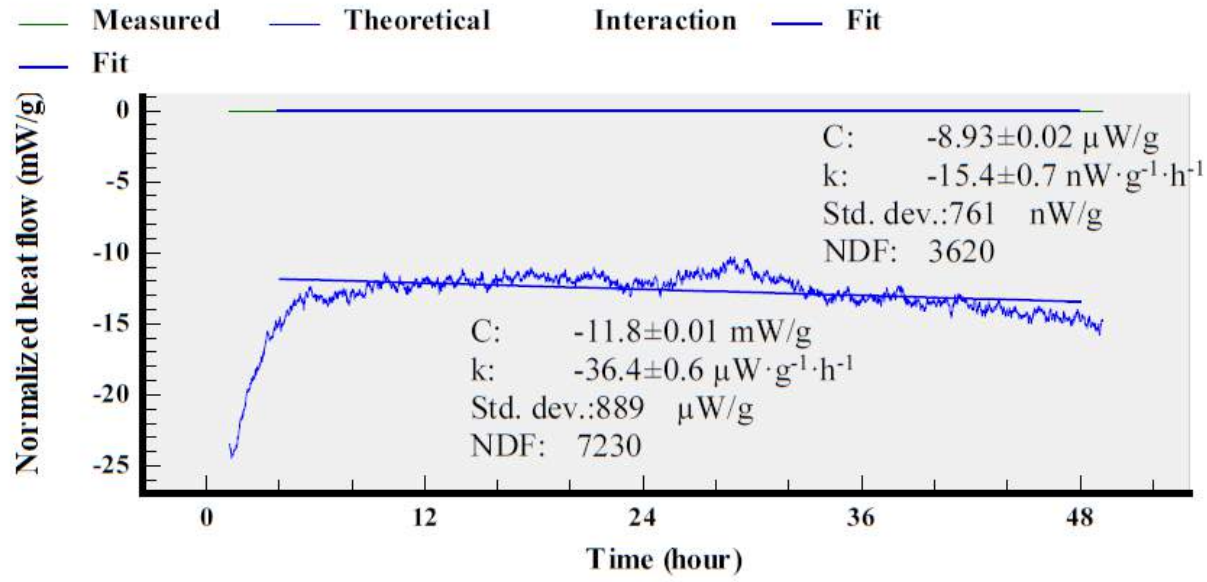
RIF (RIF)

Mass, RIF:	106 mg
Measurement signal:	Data series.Signal, Ch 4:6 [Heat flow] *Ampoule (5-31-22).rslt*
Reaction start:	May 31, 2022 11:30:00
Interaction integral:	0 J/g
Interaction integral time:	2 days, 0 hrs, 19 min, 45 sec



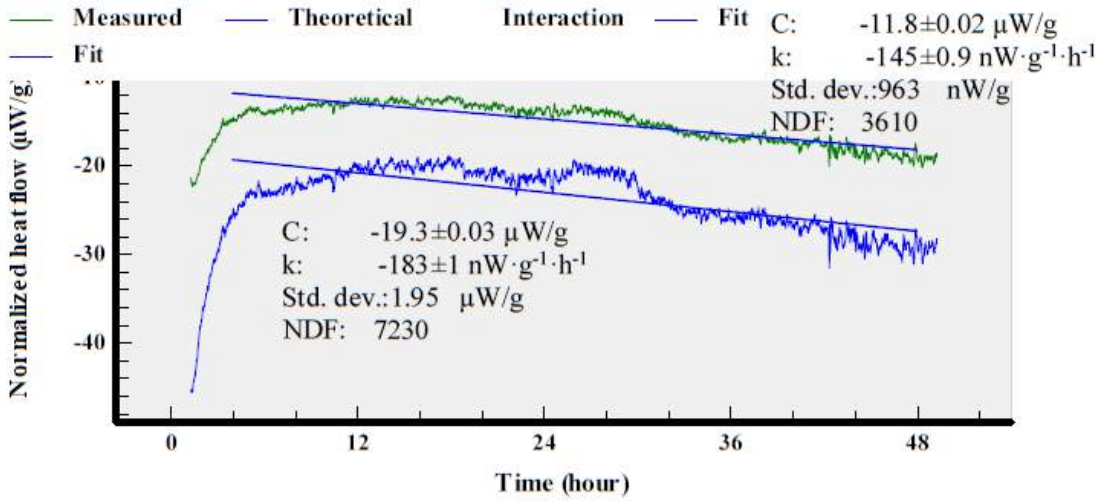
CFZ+INH (CFZ+INH)

Mass, CFZ:	54.7 mg
Mass, INH:	58.6 g
Measurement signal:	Data series.Signal, Ch 4:1 [Heat flow] *Ampoule (6-3-22).rslt*
Reaction start:	Jun 03, 2022 9:15:53
Interaction integral:	2.2523 kJ/g
Interaction integral time:	1 days, 23 hrs, 57 min, 38 sec



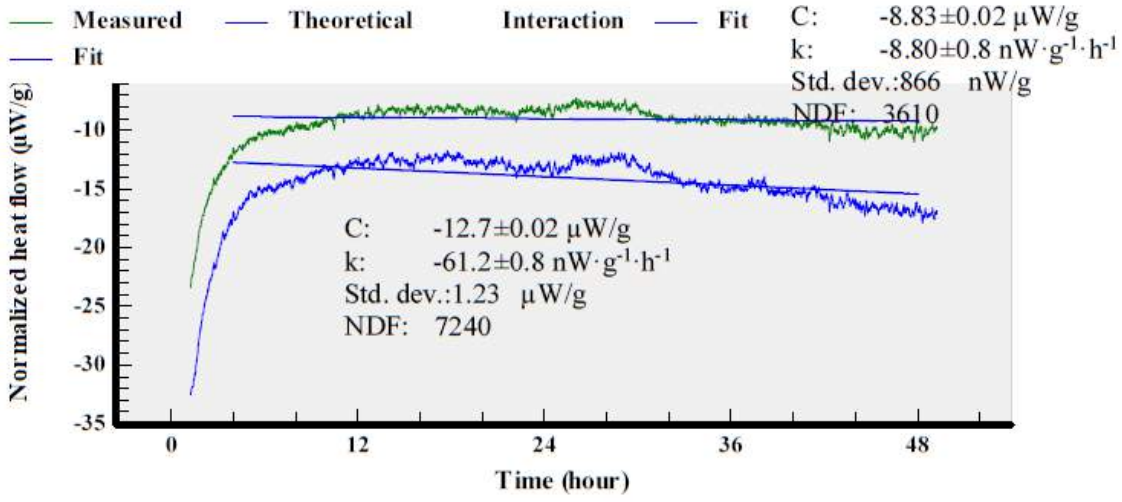
CFZ+PZY (CFZ+PZY)

Mass, CFZ:	54.7 mg
Mass, PZY:	59.4 mg
Measurement signal:	Data series.Signal, Ch 4:2 [Heat flow] *Ampoule (6-3-22).rslt*
Reaction start:	Jun 03, 2022 9:15:53
Interaction integral:	1.5166 J/g
Interaction integral time:	1 days, 23 hrs, 57 min, 38 sec



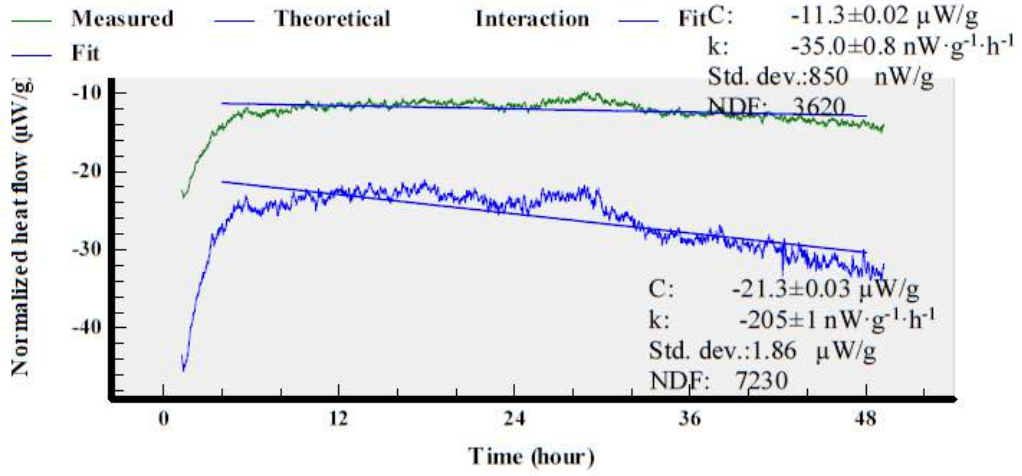
CFZ+RIF (CFZ+RIF)

Mass, CFZ:	47.5 mg
Mass, RIF:	53 mg
Measurement signal:	Data series.Signal, Ch 4:3 [Heat flow] *Ampoule (6-3-22).rslt*
Reaction start:	Jun 03, 2022 9:15:53
Interaction integral:	902.4 mJ/g
Interaction integral time:	1 days, 23 hrs, 57 min, 38 sec



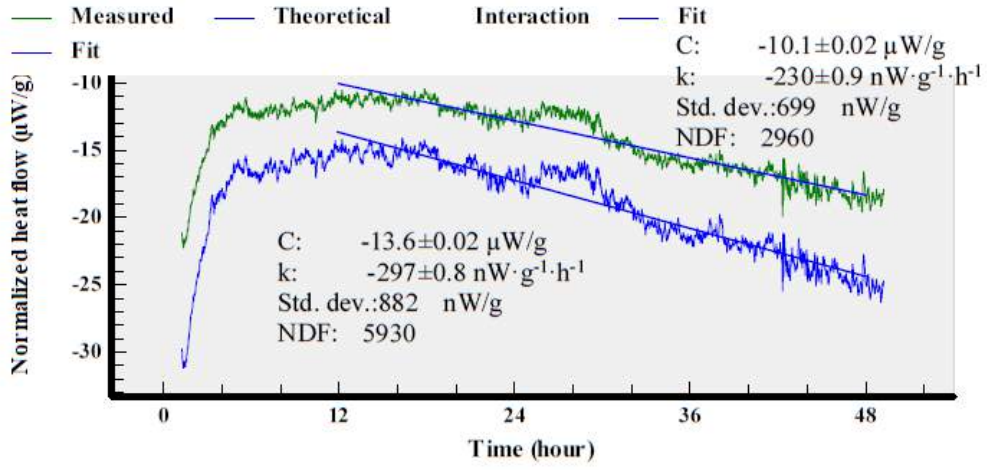
PZY+INH (PZY+INH)

Mass, PZY:	49.5 mg
Mass, INH:	48.4 mg
Measurement signal:	Data series.Signal, Ch 4:4 [Heat flow] *Ampoule (6-3-22).rslt*
Reaction start:	Jun 03, 2022 9:15:53
Interaction integral:	2.4238 J/g
Interaction integral time:	1 days, 23 hrs, 57 min, 38 sec



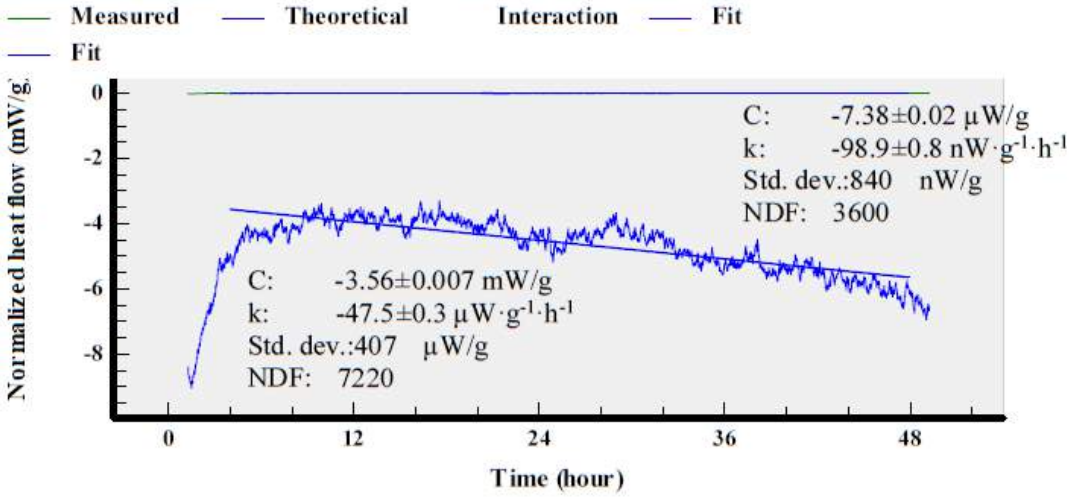
PZY+RIF (PZY+RIF)

Mass, PZY:	51.3 mg
Mass, RIF:	50 mg
Measurement signal:	Data series.Signal, Ch 4:5 [Heat flow] *Ampoule (6-3-22).rslt*
Reaction start:	Jun 03, 2022 9:15:53
Interaction integral:	844.3 mJ/g
Interaction integral time:	1 days, 23 hrs, 57 min, 38 sec



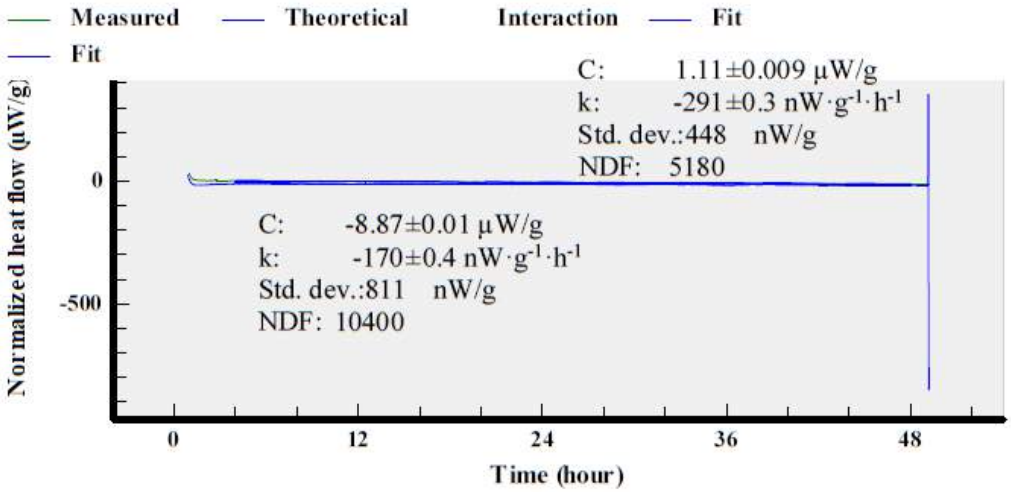
INH+RIF (INH+RIF)

Mass, INH:	53 mg
Mass, RIF:	50 g
Measurement signal:	Data series.Signal, Ch 4:6 [Heat flow] *Ampoule (6-3-22).rslt*
Reaction start:	Jun 03, 2022 9:15:53
Interaction integral:	822.89 J/g
Interaction integral time:	1 days, 23 hrs, 57 min, 38 sec



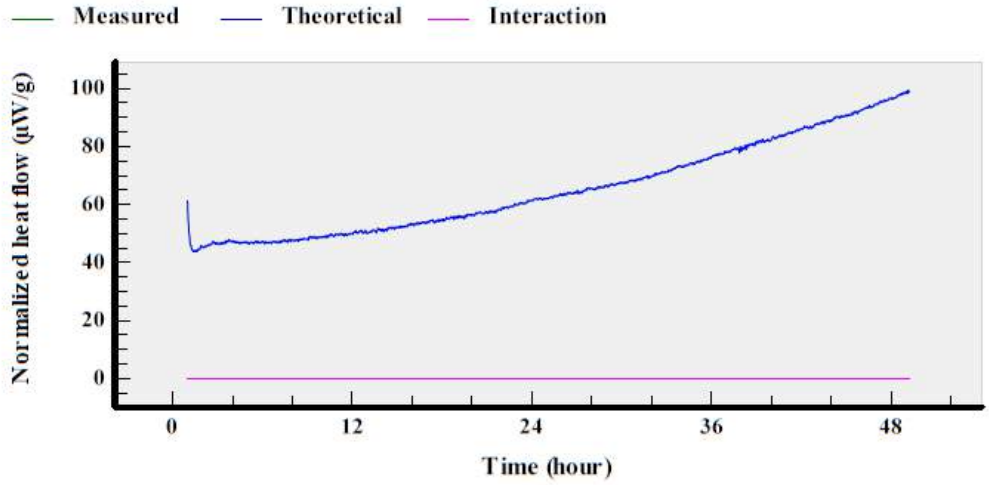
INH+Bentonite Clay (INH+Bentonite Clay)

Mass, INH:	51 mg
Mass, Bentonite Clay:	56 mg
Measurement signal:	Data series.Signal, Ch 4:1 [Heat flow] *Ampoule (6-6-22).rslt*
Reaction start:	Jun 06, 2022 11:11:22
Interaction integral:	1.4295 J/g
Interaction integral time:	2 days, 0 hrs, 14 min, 19 sec



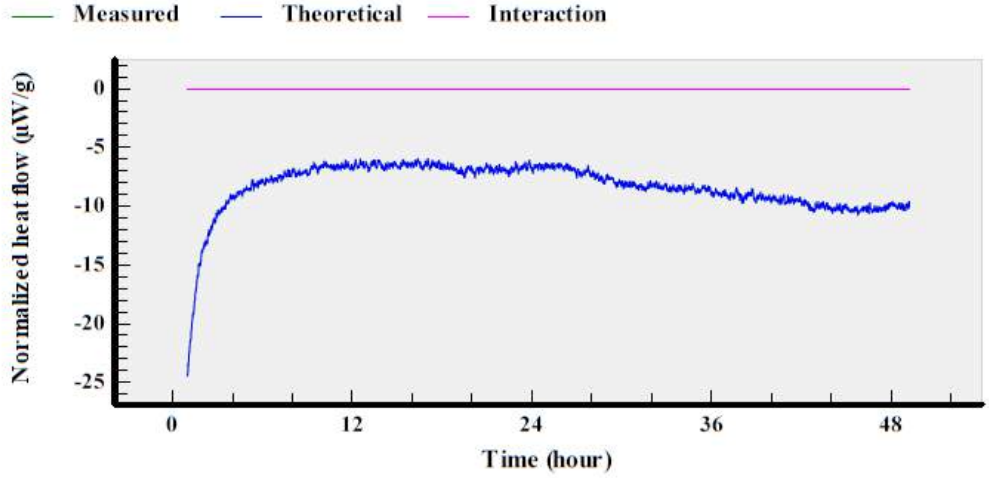
Avo olie (Avo olie)

Mass, Avo olie:	108 mg
Measurement signal:	Data series.Signal, Ch 4:2 [Heat flow] *Ampoule (6-6-22).rslt*
Reaction start:	Jun 06, 2022 11:11:22
Interaction integral:	0 J/g
Interaction integral time:	2 days, 0 hrs, 14 min, 19 sec



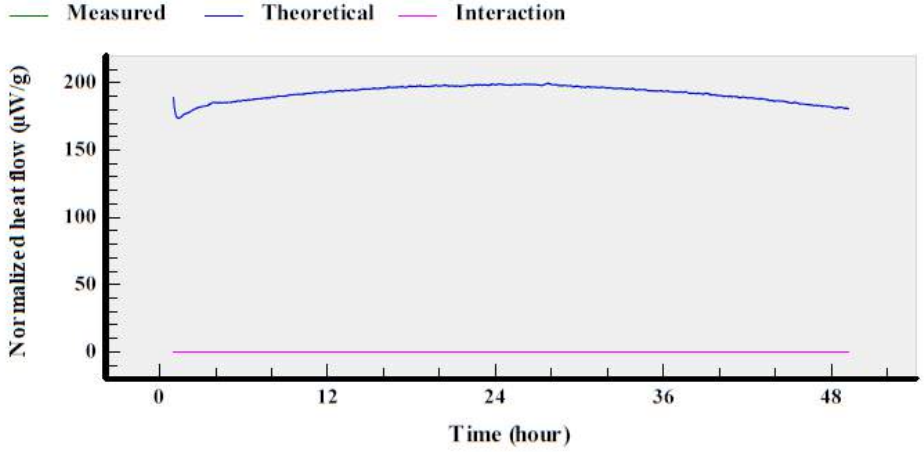
Olyf-olie (Olyf-olie)

Mass, Olyf-olie:	105 mg
Measurement signal:	Data series.Signal, Ch 4:3 [Heat flow] *Ampoule (6-6-22).rslt*
Reaction start:	Jun 06, 2022 11:11:22
Interaction integral:	0 J/g
Interaction integral time:	2 days, 0 hrs, 14 min, 19 sec



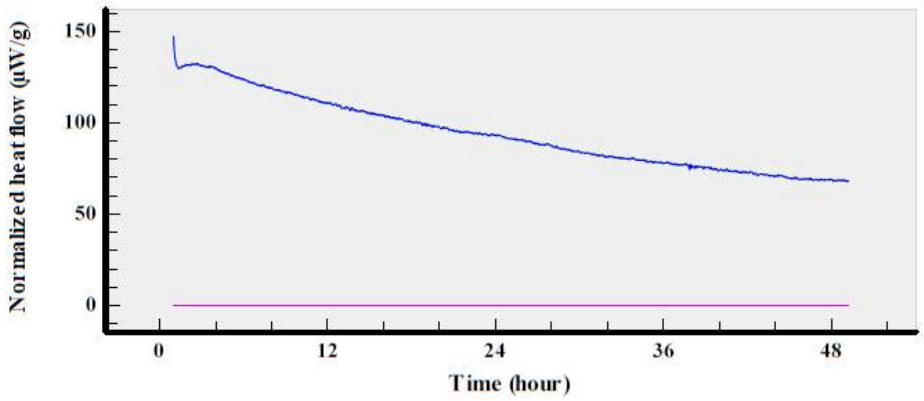
Palm-olie (Palm-olie)

Mass, Palm-olie:	103 mg
Measurement signal:	Data series.Signal, Ch 4:4 [Heat flow] *Ampoule (6-6-22).rslt*
Reaction start:	Jun 06, 2022 11:11:22
Interaction integral:	0 J/g
Interaction integral time:	2 days, 0 hrs, 14 min, 19 sec



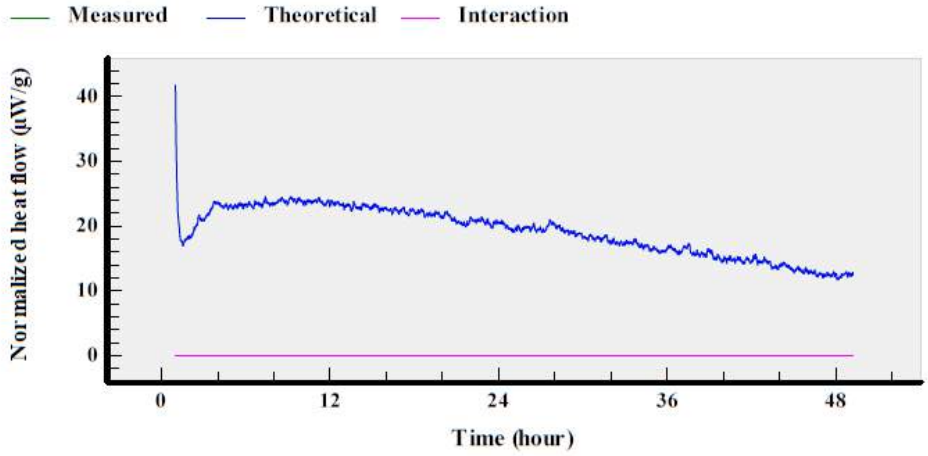
Saff-olie (Saff-olie)

Mass, Saff-olie:	100 mg
Measurement signal:	Data series.Signal, Ch 4:5 [Heat flow] *Ampoule (6-6-22).rslt*
Reaction start:	Jun 06, 2022 11:11:22
Interaction integral:	0 J/g
Interaction integral time:	2 days, 0 hrs, 14 min, 19 sec



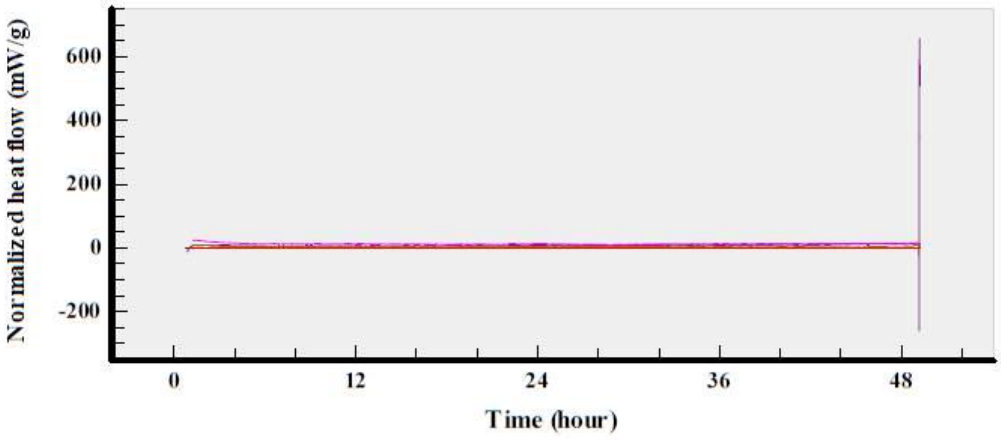
EPO (EPO)

Mass, EPO:	50 g
Measurement signal:	Data series.Signal, Ch 4:6 [Heat flow] *Ampoule (6-6-22).rslt*
Reaction start:	Jun 06, 2022 11:11:22
Interaction integral:	0 J/g
Interaction integral time:	2 days, 0 hrs, 14 min, 19 sec



Interaction graph

- Primere emulsie (Primere emulsie)
- Primere emulsie+Olyfolie (Primere emulsie+Olyfolie)
- Primere emulsie+Avo olie (Primere emulsie+Avo olie)
- Primere emulsie+EPO (Primere emulsie+EPO)
- Primere emulsie+PZY (Primere emulsie+PZY)
- Primere emulsie+CFZ (Primere emulsie+Primere emulsie+CFZ)
- Primere emulsie+RIF (Primere emulsie+RIF)
- Primere emulsie+Palm-olie (Primere emulsie+Palm-olie)
- Primere emulsie+Sunflower oil (Primere emulsie+Sunflower oil)
- Primere emulsie+Bentonite clay (Primere emulsie+Bentonite clay)
- Primere emulsie+INH (Primere emulsie+INH)
- Bentonite Clay (Bentonite Clay)
- CFZ (CFZ)
- INH (INH)
- PZY (PZY)
- RIF (RIF)
- CFZ+INH (CFZ+INH)
- CFZ+PZY (CFZ+PZY)
- CFZ+RIF (CFZ+RIF)
- PZY+INH (PZY+INH)
- PZY+RIF (PZY+RIF)
- INH+RIF (INH+RIF)
- INH+Bentonite Clay (INH+Bentonite Clay)
- Avo olie (Avo olie)
- Olyf-olie (Olyf-olie)
- Palm-olie (Palm-olie)
- Saff-olie (Saff-olie)
- EPO (EPO)



Components

Primere emulsie in Primere emulsie:

Mass:	109 mg
Measurement signal:	Data series.Signal, Ch 4:1 [Heat flow] *Ampoule (5-18-22).rslt*
Reaction start:	May 18, 2022 11:23:40

Olyfolie:

Mass:	105 mg
Measurement signal:	Data series.Signal, Ch 4:3 [Heat flow] *Ampoule (6-6-22).rslt*
Reaction start:	Jun 06, 2022 11:11:22

Avo olie:

Mass:	108 mg
Measurement signal:	Data series.Signal, Ch 4:2 [Heat flow] *Ampoule (6-6-22).rslt*
Reaction start:	Jun 06, 2022 11:11:22

EPO:

Mass:	109 mg
Measurement signal:	Data series.Signal, Ch 4:6 [Heat flow] *Ampoule (6-6-22).rslt*
Reaction start:	Jun 06, 2022 11:11:22

PZY:

Mass:	105 mg
Measurement signal:	Data series.Signal, Ch 4:5 [Heat flow] *Ampoule (5-31-22).rslt*
Reaction start:	May 31, 2022 11:30:00

Primere emulsie+CFZ:

Mass:	103 mg
Measurement signal:	Data series.Signal, Ch 4:3 [Heat flow] *Ampoule (5-31-22).rslt*
Reaction start:	May 31, 2022 11:30:00

RIF:

Mass:	106 mg
Measurement signal:	Data series.Signal, Ch 4:6 [Heat flow] *Ampoule (5-31-22).rslt*
Reaction start:	May 31, 2022 11:30:00

Palm-olie:

Mass:	103 mg
Measurement signal:	Data series.Signal, Ch 4:4 [Heat flow] *Ampoule (6-6-22).rslt*
Reaction start:	Jun 06, 2022 11:11:22

Sunflower oil:

Mass:	100 mg
Measurement signal:	Data series.Signal, Ch 4:5 [Heat flow] *Ampoule (6-6-22).rslt*
Reaction start:	Jun 06, 2022 11:11:22

Bentonite clay:

Mass:	102 mg
Measurement signal:	Data series.Signal, Ch 4:2 [Heat flow] *Ampoule (5-31-22).rslt*
Reaction start:	May 31, 2022 11:30:00

INH in INH:

Mass:	104 mg
Measurement signal:	Data series.Signal, Ch 4:4 [Heat flow] *Ampoule (5-31-22).rslt*
Reaction start:	May 31, 2022 11:30:00

Bentonite Clay in Bentonite Clay:

Mass:	102 mg
Measurement signal:	Data series.Signal, Ch 4:2 [Heat flow] *Ampoule (5-31-22).rslt*
Reaction start:	May 31, 2022 11:30:00

CFZ in CFZ:

Mass:	103 mg
Measurement signal:	Data series.Signal, Ch 4:3 [Heat flow] *Ampoule (5-31-22).rslt*
Reaction start:	May 31, 2022 11:30:00

CFZ:

Mass:	47.5 mg
Measurement signal:	Data series.Signal, Ch 4:3 [Heat flow] *Ampoule (6-3-22).rslt*
Reaction start:	Jun 03, 2022 9:15:53

INH:

Mass:	48.4 mg
Measurement signal:	Data series.Signal, Ch 4:4 [Heat flow] *Ampoule (6-3-22).rslt*
Reaction start:	Jun 03, 2022 9:15:53

PZY:

Mass:	51.3 mg
Measurement signal:	Data series.Signal, Ch 4:5 [Heat flow] *Ampoule (6-3-22).rslt*
Reaction start:	Jun 03, 2022 9:15:53

RIF:

Mass:	104 mg
Measurement signal:	Data series.Signal, Ch 4:6 [Heat flow] *Ampoule (6-3-22).rslt*
Reaction start:	Jun 03, 2022 9:15:53

Olyf-olie in Olyf-olie:

Mass:	105 mg
Measurement signal:	Data series.Signal, Ch 4:3 [Heat flow] *Ampoule (6-6-22).rslt*
Reaction start:	Jun 06, 2022 11:11:22

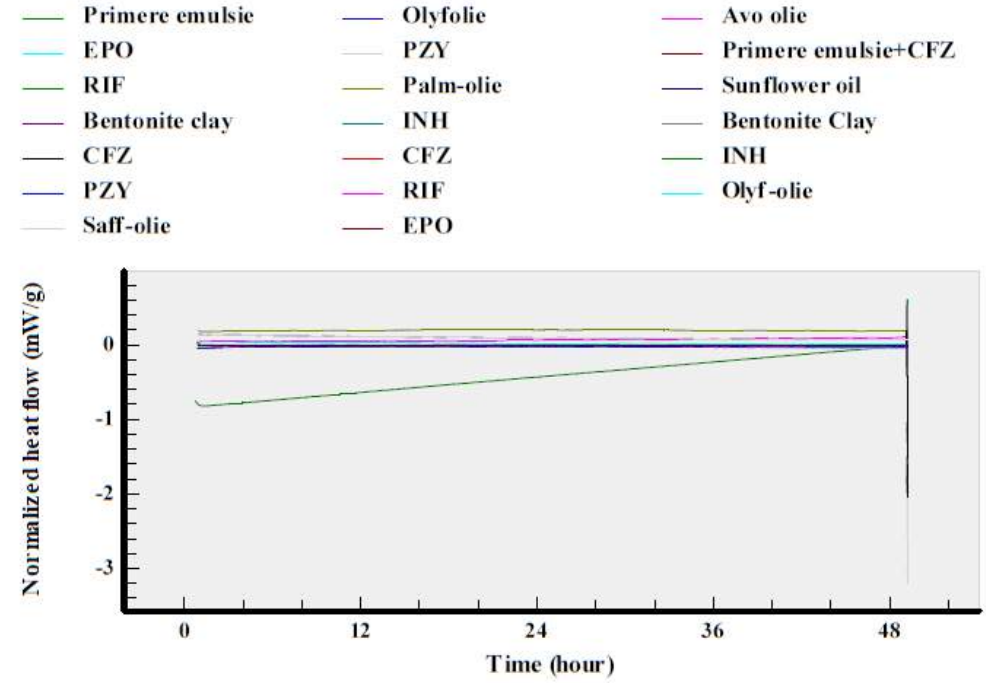
Saff-olie in Saff-olie:

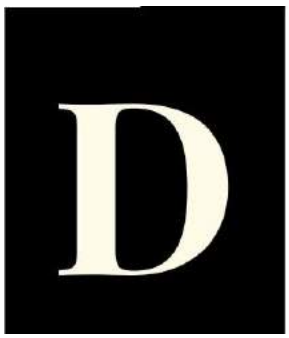
Mass:	100 mg
Measurement signal:	Data series.Signal, Ch 4:5 [Heat flow] *Ampoule (6-6-22).rslt*
Reaction start:	Jun 06, 2022 11:11:22

EPO in EPO:

Mass:	50 g
Measurement signal:	Data series.Signal, Ch 4:6 [Heat flow] *Ampoule (6-6-22).rslt*
Reaction start:	Jun 06, 2022 11:11:22

Components Graph





Annexure D

Development of a Primary Emulsion

D.1. Introduction

The water titration method was utilised to construct a pseudoternary phase diagram where Transcutol®:PEG (9:1), water and the surfactant phase (Span®83:Tween®60; 1:1) were combined in different ratios to display a triplot. This illustration is then able to indicate the most appropriate combination of additives to form successful SDEDDSs, as one can establish the self-emulsifying areas and also validate the most effective concentrations and ratios of the included composites. No drugs were included to establish excipient behaviour without the influence of any drugs. The surfactant phase and water were mixed in fixed ratios (10:0, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9, 0:10) while Transcutol®:PEG (9:1) was added in a dropwise manner as a variable component at an ambient temperature. The concentration at which the turbidity of the mixtures is first observed was plotted as the endpoint employing Triplot software version 4.1.2. Thus, the area of self-emulsification was established as required to find potential primary emulsions.

The primary emulsions selected from the constructed pseudoternary phase diagram were subsequently prepared by adding known quantities of the internal oil phase (Transcutol®:PG; 9:1) to established quantities of the water and surfactant phases. The mixtures were then subjected to homogenization with a Heidolph DIAX600 homogenizer (Schwabach, Germany) for 4 min at 9500 rpm while kept at room temperature. The primary emulsions were evaluated after a waiting period of 24 h at an ambient temperature in terms of emulsion stability index and self-emulsification performance to eliminate poor excipient combinations from further experimentation.

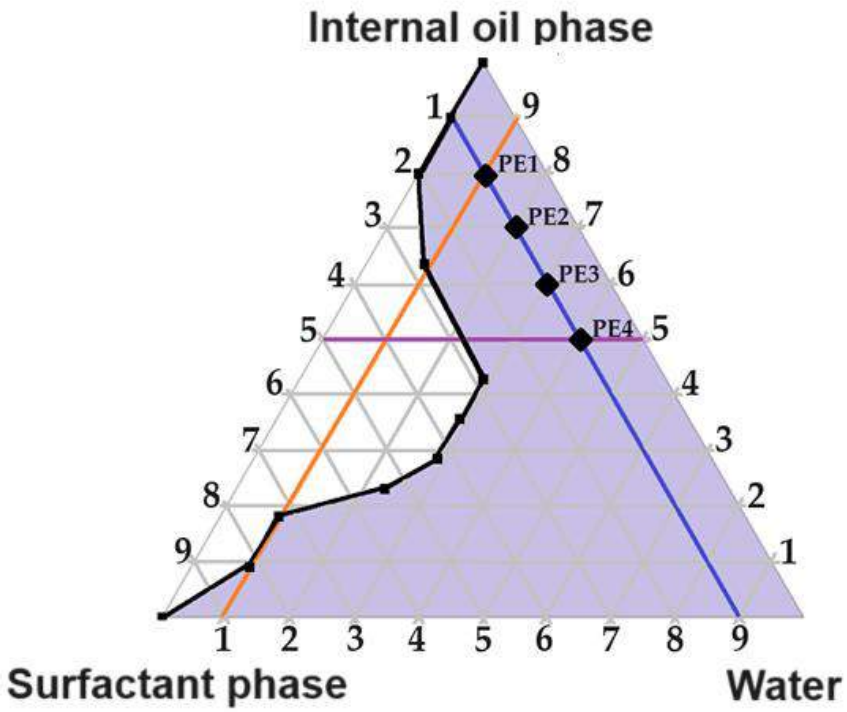


Figure D1: *Pseudoternary phase diagram of water, surfactant phase and internal oil phase. The numbering represents the given parts of the different phases included in a ratio at a specific point on the pseudoternary phase diagram*

Table D1: *Evaluation of primary emulsions*

	PE1	**PE2**	**PE3**	**PE4**
Self-emulsification time	3 min	4 min, 30 s	5 min, 10 s	5 min, 50 s
Self-emulsification grading	D	D	D	E
Emulsion Stability Index (%)	100%	100%	100%	80%

Annexure E

Pseudoternary Phase Diagrams constructed to consider Self-Double-Emulsification capacity of External Oil Phases

E.1. Introduction

The water titration method was again utilised to construct pseudoternary phase diagrams. Drugs were again excluded during pseudoternary phase diagram construction to be able to observe excipient response without adding variables that can be accompanied by drug inclusion. The internal oil phase was mixed together with the surfactant phase (i.e., PE 1, PE 2 and PE 3, as seen in Annexure D) and water in fixed proportions (9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8 and 1:9) while individual external oil phases were added dropwise as the varying component at an ambient temperature. The concentration at which the turbidity of the mixtures is observed was plotted as the endpoint employing Triplot software, version 4.1.2.

Primary emulsion with the ratio, 9:9:2, was prepared and it was decided to add avocado oil as the external oil phase due to the favourable solubility properties exhibited by both lipophilic drugs in this vehicle. A checkpoint formulation was selected, as seen in Figure E6, with the idea of including a high concentration of the internal oil phase to provide sufficient solubilisation of isoniazid and pyrazinamide while selecting a checkpoint formulation that provides a moderate amount of the external oil phase so as to ensure saturated concentrations of the included lipophilic components (i.e., clofazimine and rifampicin).

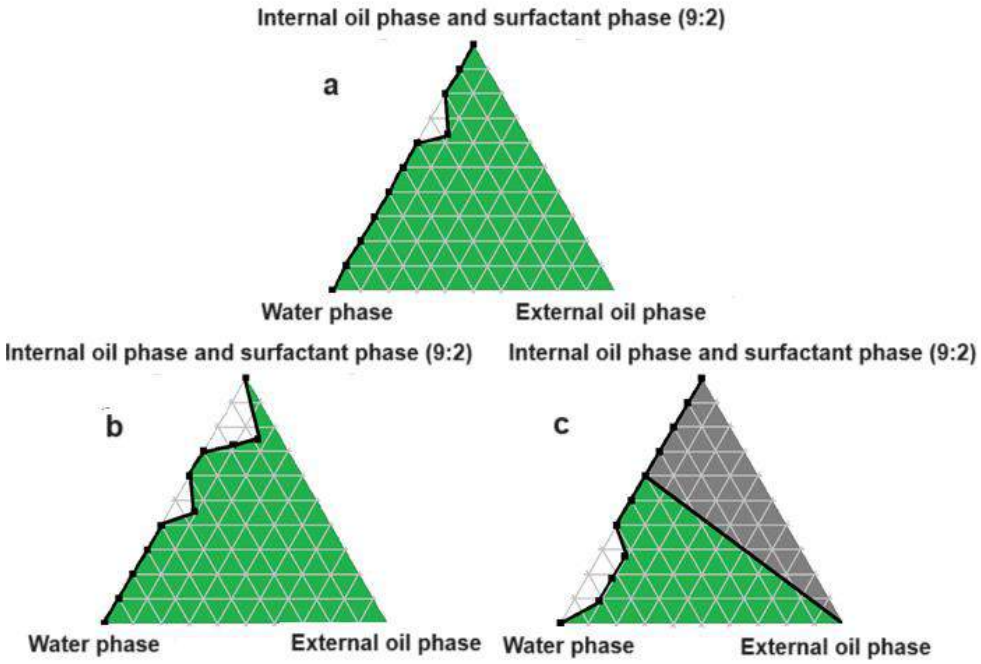


Figure E1: *Pseudoternary phase diagrams of water, avocado oil, and fixed proportions of the surfactant and internal oil phases. In (a) the internal oil and surfactant phases are combined in a ratio of 9:2; in (b) the internal oil and surfactant phases are blended in an 8:3 ratio; and in (c) the internal oil and surfactant phases are included as a 7:4 ratio*

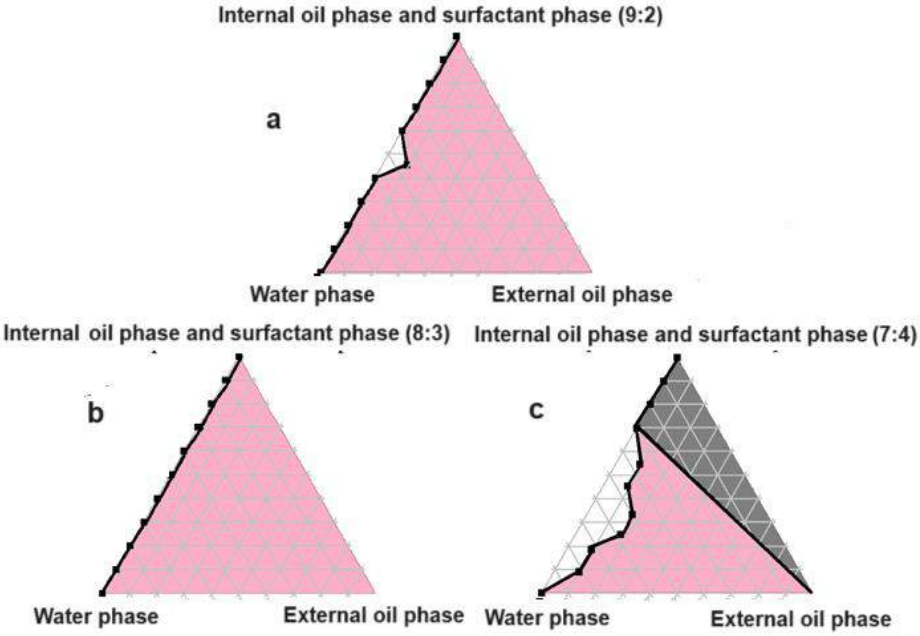


Figure E2: *Pseudoternary phase diagrams of water, evening primrose oil, and fixed proportions of the surfactant and internal oil phases. In (a) the internal oil and surfactant phases are combined in a ratio of 9:2; in (b) the internal oil and surfactant phases are blended in an 8:3 ratio; and in (c) the internal oil and surfactant phases are included as a 7:4 ratio*

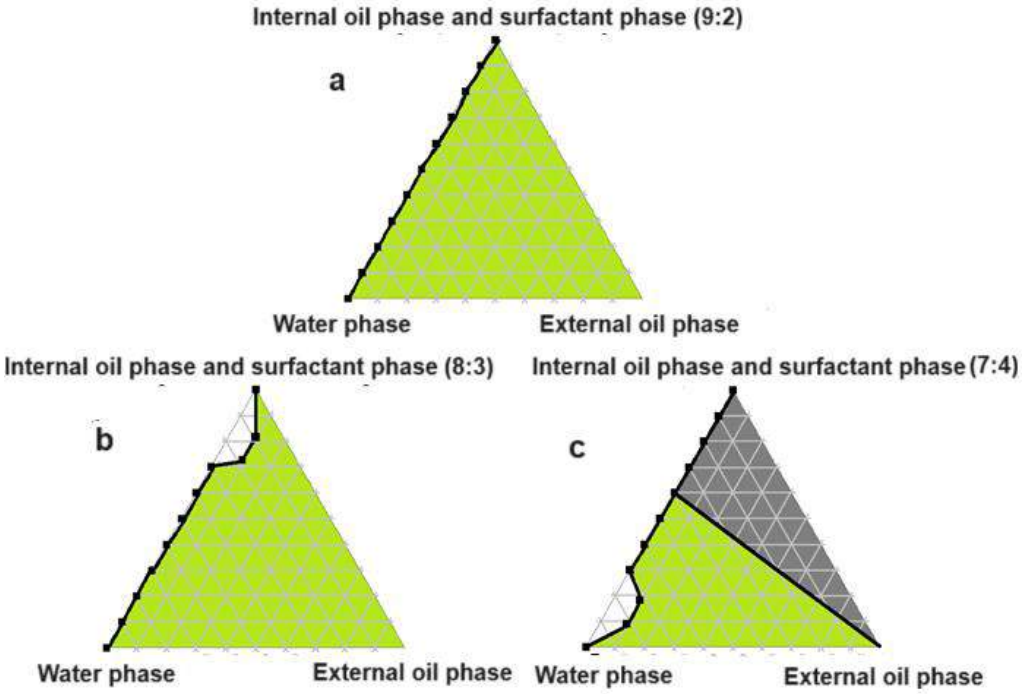


Figure E3: *Pseudoternary phase diagrams of water, olive oil, and fixed proportions of the surfactant and internal oil phases. In (a) the internal oil and surfactant phases are combined in a ratio of 9:2; in (b) the internal oil and surfactant phases are blended in an 8:3 ratio; and in (c) the internal oil and surfactant phases are included as a 7:4 ratio*

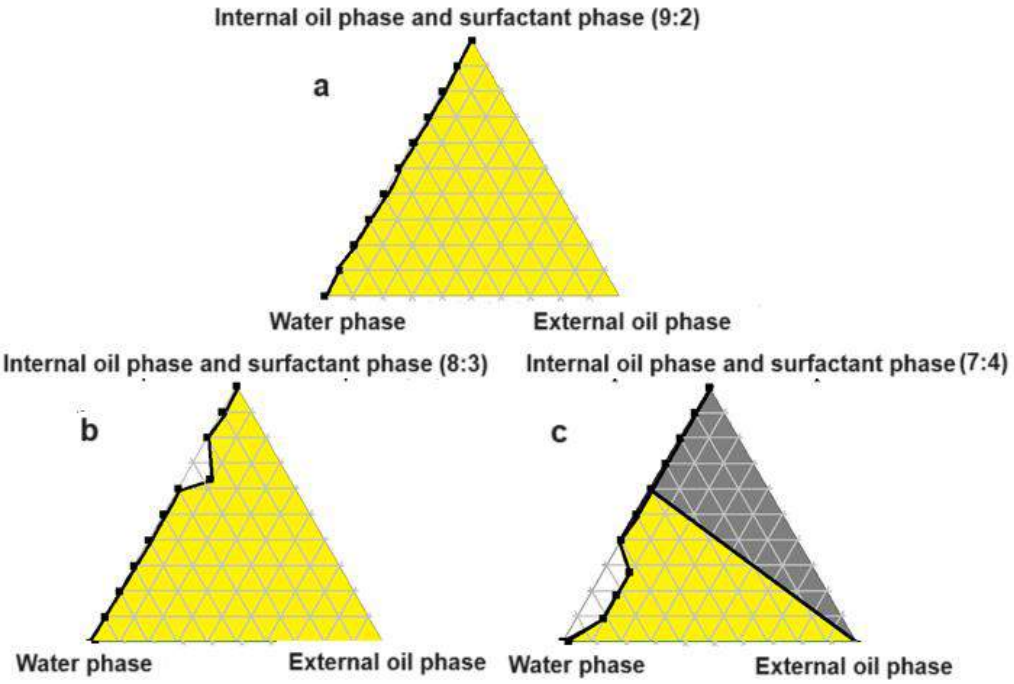


Figure E4: *Pseudoternary phase diagrams of water, palm oil, and fixed proportions of the surfactant and internal oil phases. In (a) the internal oil and surfactant phases are combined in a ratio of 9:2; in (b) the internal oil and surfactant phases are blended in an 8:3 ratio; and in (c) the internal oil and surfactant phases are included as a 7:4 ratio*

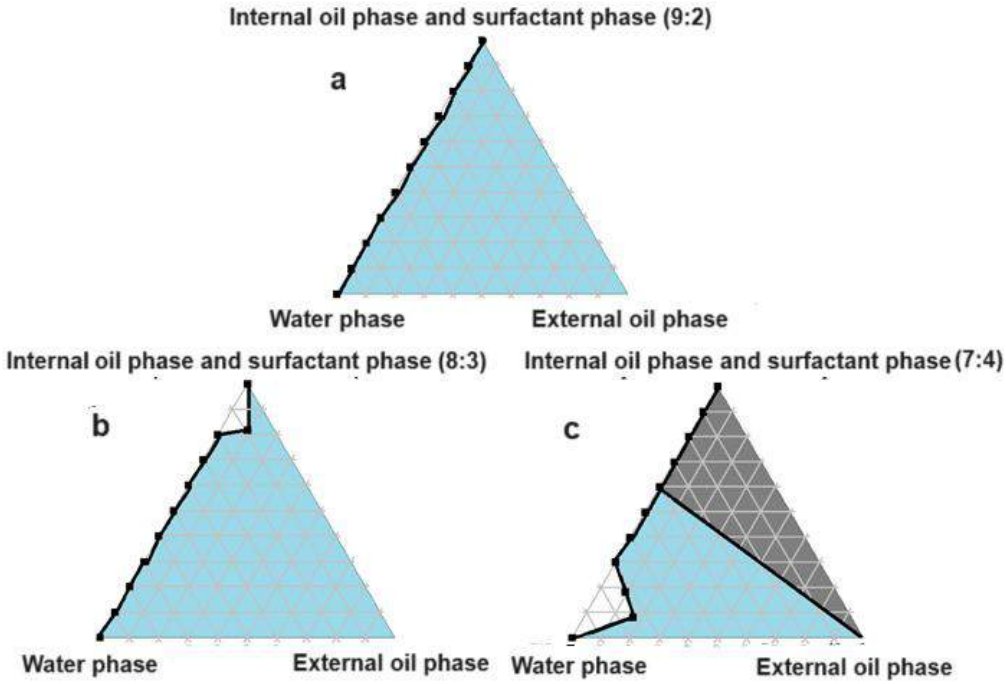


Figure E5: *Pseudoternary phase diagrams of water, safflower oil, and fixed proportions of the surfactant and internal oil phases. In (a) the internal oil and surfactant phases are combined in a ratio of 9:2; in (b) the internal oil and surfactant phases are blended in an 8:3 ratio; and in (c) the internal oil and surfactant phases are included as a 7:4 ratio*

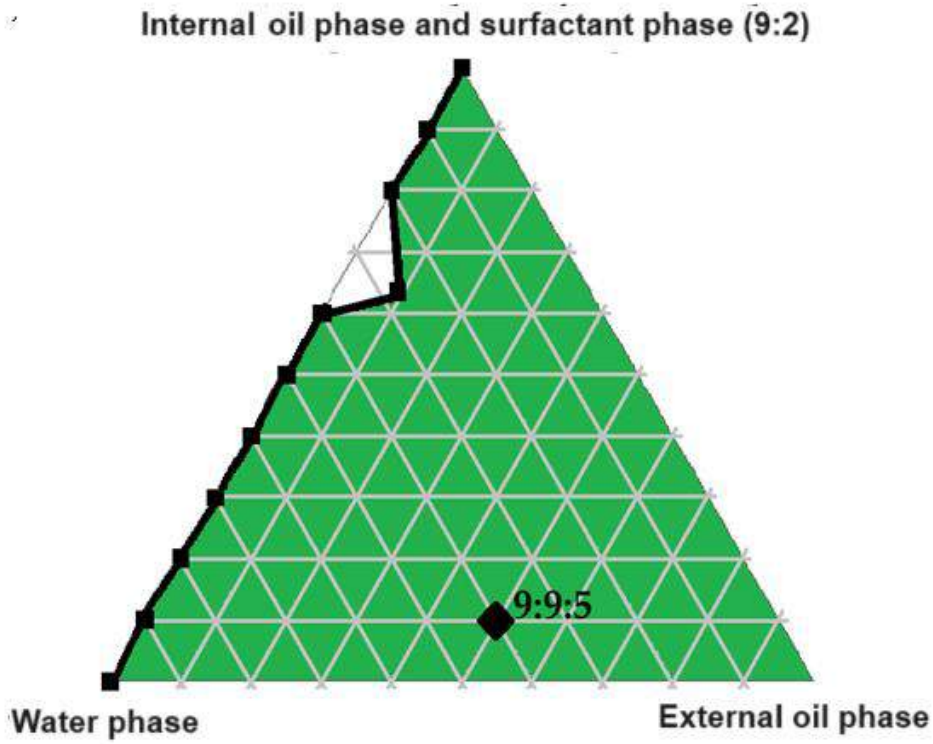


Figure E6: *Pseudoternary phase diagram utilised to select a checkpoint formulation (9:9:5) for SDEDDS development*

Annexure F

Solubility Studies

F.1. Introduction

An excess amount of individual anti-tubercular drugs was added to 5 mL of the different vehicles considered during this study. Hereafter, samples were vortexed for approximately 2 min. Solubility studies were performed by placing samples in the circular axis (54 rpm) of a rotating solubility bath set at 32°C (± 0.5°C) for a period of 48 h. The samples were then centrifuged at 3000 rpm for 15 min, followed by the removal of the supernatant from each sample. The removed supernatant was filtered through a 0.45 µm Millipore® filter and diluted with methanol. A validated high performance liquid chromatographic method was employed to analyse all samples.

Table F1: *Average solubility(mg/mL) at 32ºC of CFZ, INH, PZY and RIF in different solvents (n=3)*

	CFZ	**INH**	**PZY**	**RIF**
	mg/mL			
AVO	12.70	1.76	3.02	10.22
EPO	10.47	0.16	0.68	6.83
OLV	12.10	0.74	0.97	8.56
PALM	27.80	0.78	2.02	12.62
SAFF	21.07	1.84	2.96	7.52
Primary emulsion	3.30	158.17	44.67	10.22

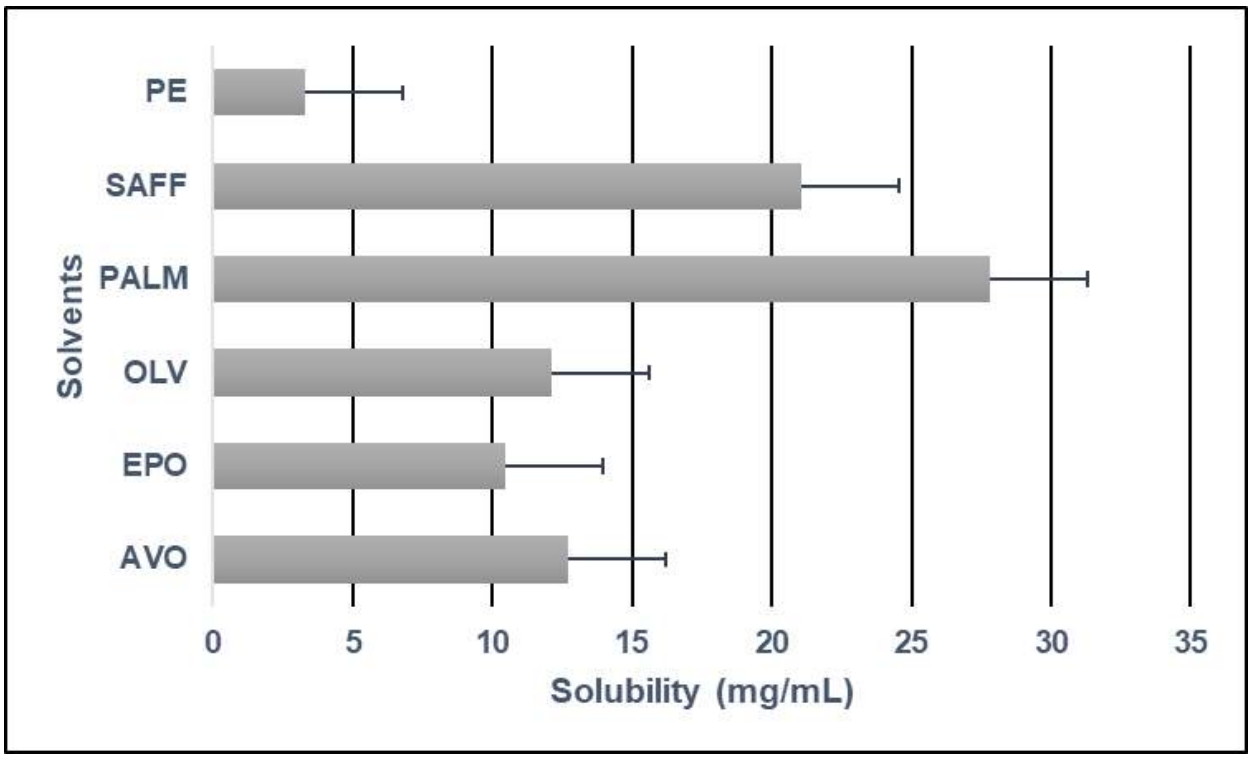


Figure F1: *Solubility of CFZ in different solvents*

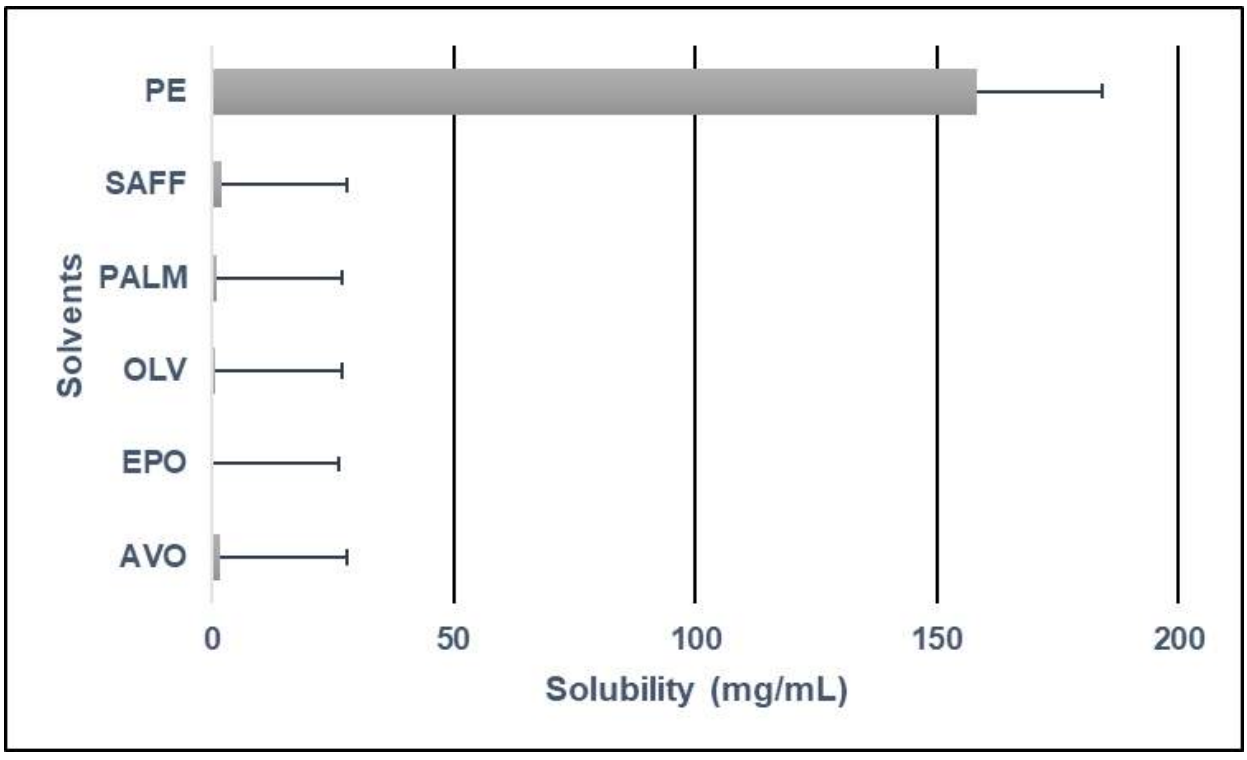


Figure F2: *Solubility of INH in different solvents*

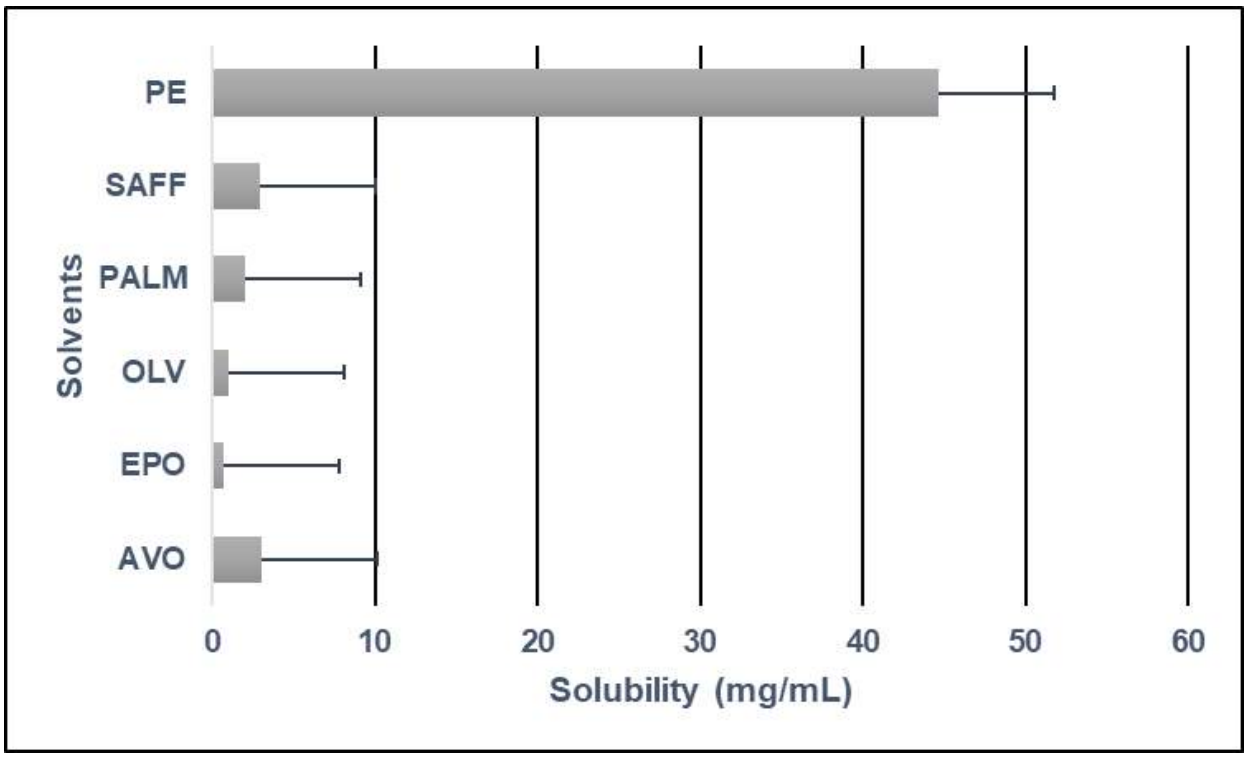


Figure F3: *Solubility of PZY in different solvents*

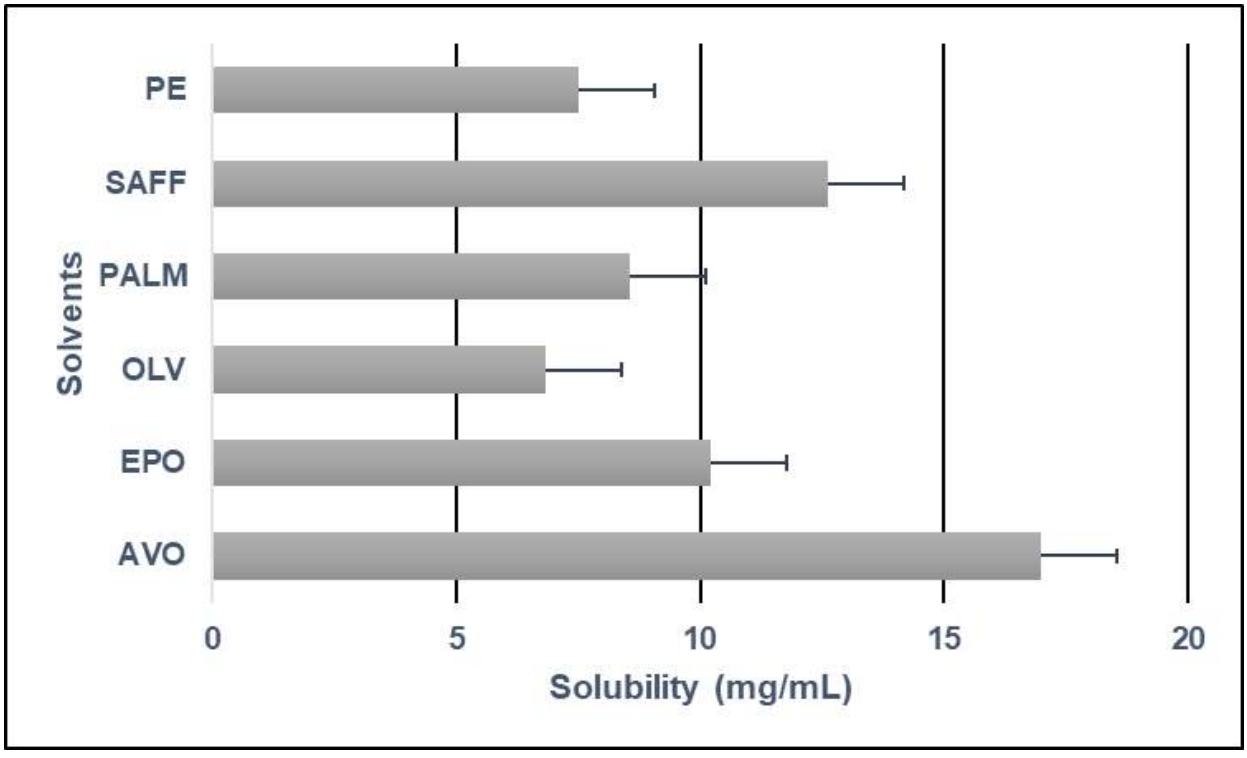


Figure F4: *Solubility of RIF in different solvents*

Annexure G

Summary of Excipient Selection based on the Results of Preformulation Studies

G.1. Introduction

Choosing the correct surface-active agent(s) is essential in order to stabilise the emulsions once formulated. The HLB system allows theoretic quantification of the ability of a surfactant and co-surfactant combination to stabilise an emulsion. Therefore, for the purpose of this study, Span®83 (HLB value of 3.7) and Tween®60 (HLB value of 15) were selected as surfactant and co-surfactant, respectively. A fixed ratio of Span®83:Tween®60 (1:1) was maintained throughout the experiments, as this ratio has demonstrated greater stabilisation of SEDDSs compared to higher ratios that increase the emulsion range but reduce stability, as portrayed by drug precipitation. In addition, as an explorative study this was good starting reference point in terms of surfactant/co-surfactant ratios. Therefore, when sufficient stabilisation was achieved by the fixed ratio of Span®83:Tween®60 (1:1) bentonite clay was excluded as a potential co-stabiliser. Internal- and external oil phases were eliminated from further investigation if clear immiscibility between the two phases were not observed.

Table G1: *Reasons for excipient inclusion or exclusion based on the results of preformulation studies*

Capacity of excipient	Excipient	Reason for inclusion/exclusion
Internal oil phase	Beeswax	***Miscible with external oil phases***
	Linseed oil	***Miscible with external oil phases***
	Transcutol®:PEG (9:1)	**Immiscible with external oil phases**
	Transcutol®	***Without PEG not sufficiently immiscible with external oil phases***
External oil phase	AVO	**Immiscible with internal oil phase**
	EPO	**Immiscible with internal oil phases**
	OLV	**Immiscible with internal oil phases**
	PALM	**Immiscible with internal oil phases**
	SAFF	**Immiscible with internal oil phase**
	Shea butter	***Miscible with internal oil phase***
Surfactant phase	Span®83:Tween®60(1:1)	**Sufficient emulsion stabilisation during construction of pseudo-ternary phase diagrams**
	Bentonite clay	***Sufficient stabilisation achieved without inclusion of bentonite clay***

Annexure H

Microscopic Observation of Primary Emulsion 1 and Checkpoint SDEDDS

H.1. Introduction

All visual examinations of either primary emulsion 1 or the checkpoint SDEDDS were conducted utilising an Olympus microscope (Tokyo, Japan). TEM measurements were performed on a TECNAI G2 (ACI) instrument operated at an accelerating voltage of 200 kV (Hillsboro, OR, United States of America).

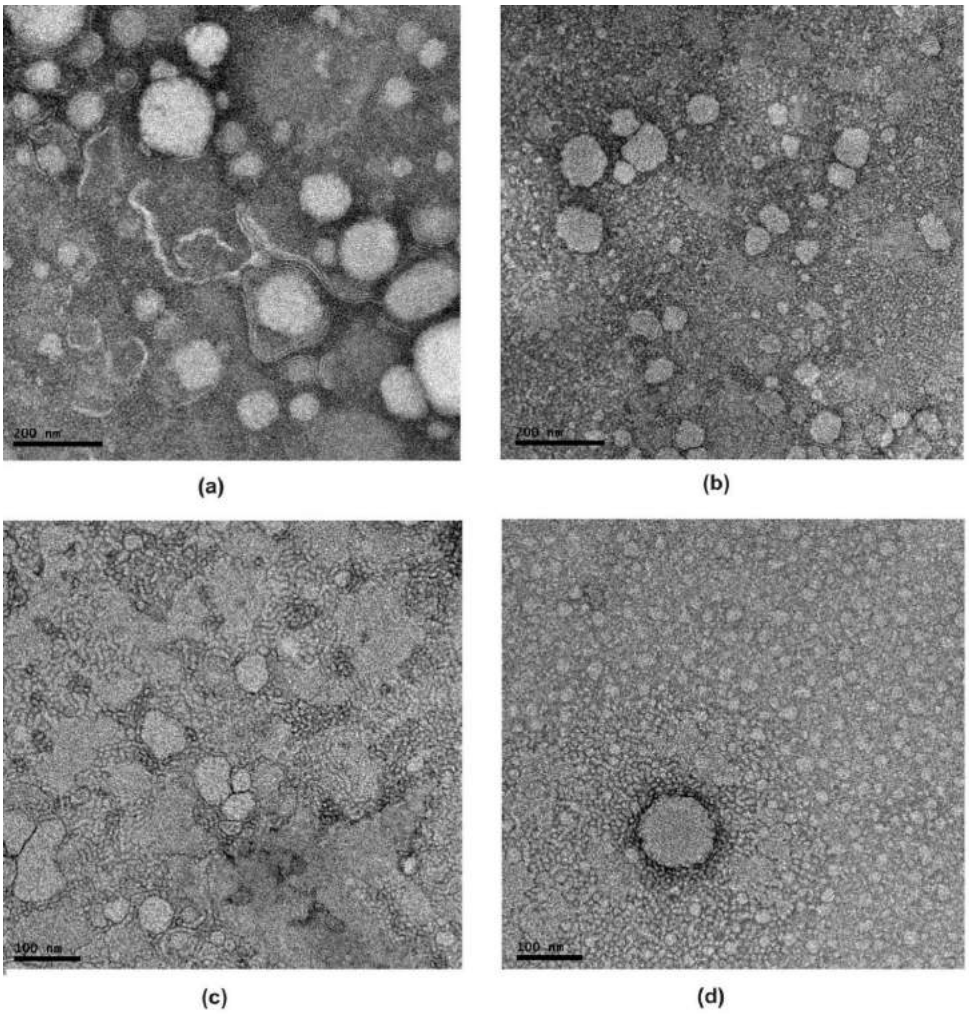


Figure H1: *TEM images of primary emulsion 1, (a) and (b) at a scale of 200 nm, (c) and (d) displayed at 100 nm*

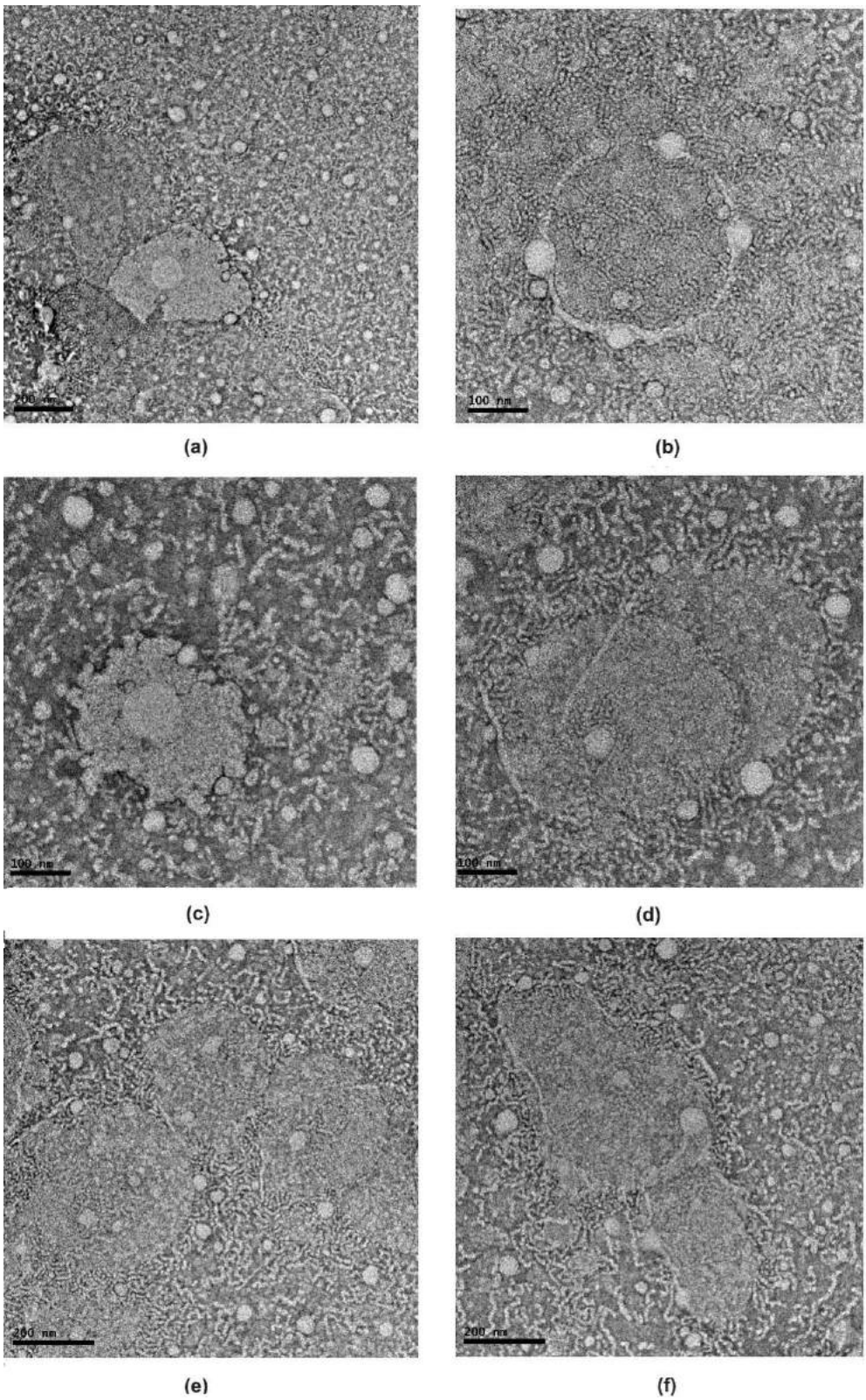


Figure H2: *TEM images of SDEDDS, (a) – (c) demonstrate the mechanism of spontaneous double emulsification where smaller droplets are engulfed to be harboured inside larger droplets at a scale of 200 nm (a) and 100 nm for (b) and (c), (d) – (f) display smaller droplets harboured inside larger droplets of SDEDDS at a scale of 100 nm (d) and (e) and 200 nm (f)*

Figure H3: *Microscopic observation of primary emulsion 1 without incorporation of anti-tubercular drugs*

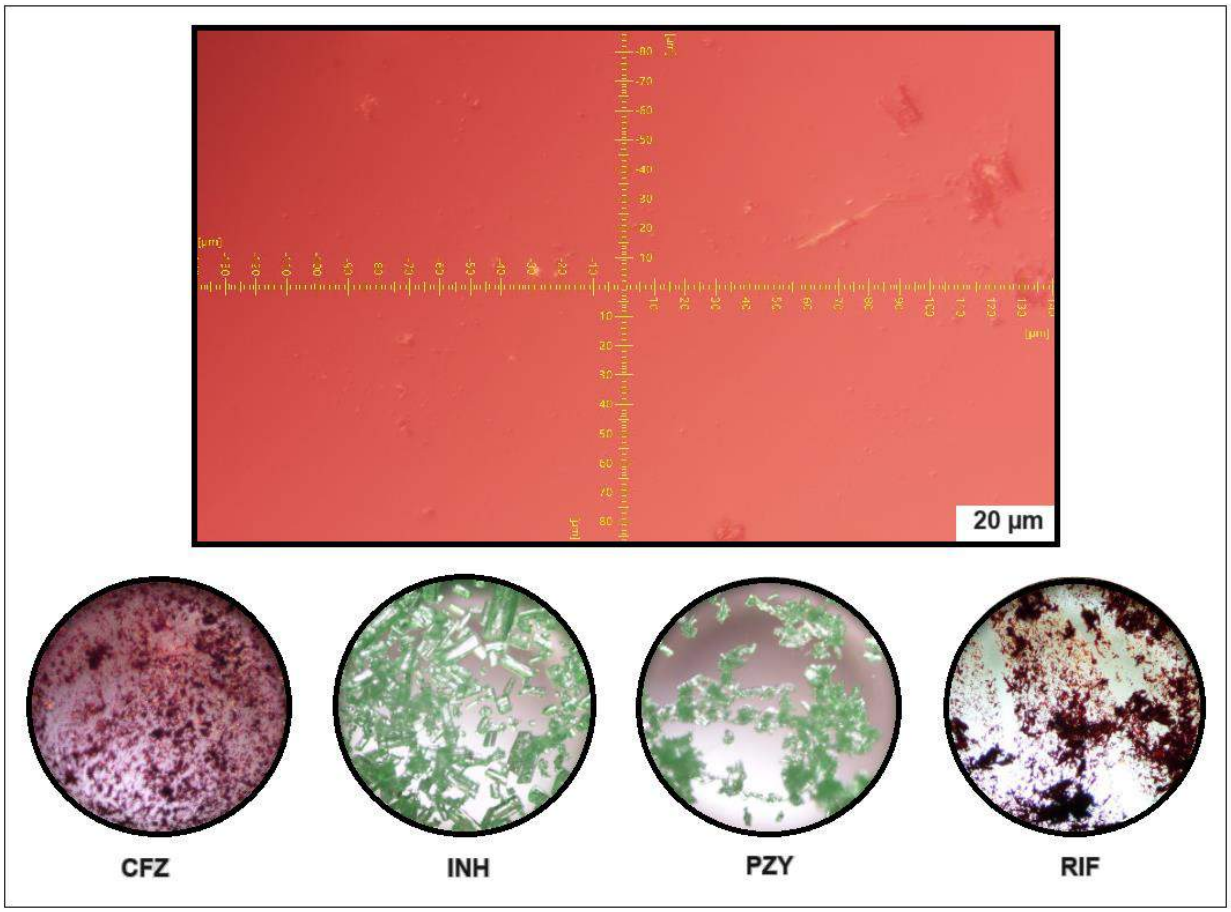


Figure H4: *Microscope image of SDEDDS incorporating CFZ, INH, PZY and RIF accompanied by microscope images of the individual anti-tubercular drugs*

Annexure I

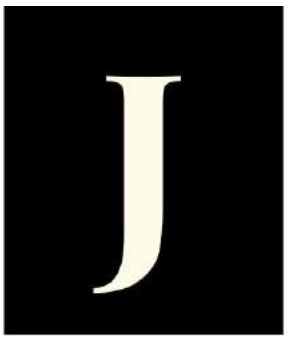
Droplet Size and Droplet Size Distribution of Primary Emulsion 1 and Checkpoint SDEDDS

I.1. Introduction

Droplet size and size distribution were assessed by means of dynamic light scattering performed by a Malvern Zetasizer Nano® ZS (Worcestershire, United Kingdom) at 25°C. All samples were analysed in triplicate.

Table I1: *Average droplet size and droplet size distribution of primary emulsion 1 and check-point SDEDDS (n=3)*

	Primary emulsion 1	**Checkpoint SDEDDS**
Excipient ratios	**(9:9:2)** **Internal oil phase: water: surfactant phase**	**(9:9:5)** **Internal oil phase and surfactant phase (9:2): water: external oil phase**
Average droplet size ± SD	173.90 ± 2.30 nm	92.46 ± 5.52 nm
PDI ± SD	0.236 ± 0.11	0.391 ± 0.05



Annexure J

Instructions for Authors for '*Antibiotics*'

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1. Read the **Aims & Scope** to gain an overview and assess if your manuscript is suitable for this journal;
2. Use the **Microsoft Word template** or **LaTeX template** to prepare your manuscript;
3. Make sure that issues about **publication ethics**, **research ethics**, **copyright**, **authorship**, **figure formats**, **data** and **references format** have been appropriately considered;
4. Ensure that all authors have approved the content of the submitted manuscript.
5. Authors are encouraged to add a **biography** (optional) to the submission and post it to **SciProfiles**.

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Antibiotics has no restrictions on the maximum length of manuscripts, provided that the text is concise and comprehensive. Full experimental details must be provided so that the results can be reproduced. *Antibiotics* requires that authors publish all experimental controls and make full datasets available where possible (see the guidelines on **Supplementary Materials** and references to unpublished data).

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- *Review:* Reviews offer a comprehensive analysis of the existing literature within a field of study, identifying current gaps or problems. They should be critical and constructive and provide recommendations for future research. No new, unpublished data should be presented. The structure can include an Abstract, Keywords, Introduction, Relevant Sections, Discussion, Conclusions, and Future Directions, with a suggested minimum word count of 4000 words.

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- Your references may be in any style, provided that you use the consistent formatting throughout. It is essential to include author(s) name(s), journal or book title, article or chapter title (where required), year of publication, volume and issue (where appropriate) and pagination. DOI numbers (Digital Object Identifier) are not mandatory but highly encouraged. The bibliography software package *EndNote*, ***Zotero***, *Mendeley*, *Reference Manager* are recommended.
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Any prior submissions of the manuscript to MDPI journals must be acknowledged. If this is the case, it is strongly recommended that the previous manuscript ID is provided in the submission system, which will ease your current submission process. The names of proposed and excluded reviewers should be provided in the submission system, not in the cover letter.

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- We confirm that neither the manuscript nor any parts of its content are currently under consideration or published in another journal.
- All authors have approved the manuscript and agree with its submission to (journal name).

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Authors are encouraged to add a biography (maximum 150 words) to the submission and upload it to **SciProfiles**. This should be a single paragraph and should contain the following points:

1. Authors' full names followed by current positions;
2. Education background including institution information and year of graduation (type and level of degree received);
3. Work experience;
4. Current and previous research interests;
5. Memberships of professional societies and awards received.

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General Considerations

- **Research manuscripts** should comprise:
    - **Front matter**: Title, Author list, Affiliations, Abstract, Keywords.
    - **Research manuscript sections**: Introduction, Results, Discussion, Materials and Methods, Conclusions (optional).
    - **Back matter**: Supplementary Materials, Acknowledgments, Author Contributions, Conflicts of Interest, **References**.

Additional point:

Manuscripts describing the use of computational modelling and/or molecular docking programs to predict the structures or activity of new antibiotics will not be considered, unless they present

additional supporting data, such as biological test results (using microorganisms and/or pure protein).

- **Review manuscripts** should comprise the **front matter**, literature review sections and the **back matter**. The template file can also be used to prepare the front and back matter of your review manuscript. It is not necessary to follow the remaining structure. Structured reviews and meta-analyses should use the same structure as research articles and ensure they conform to the **PRISMA** guidelines.
- **Case reports** should include a succinct introduction about the general medical condition or relevant symptoms that will be discussed in the case report; the case presentation including all of the relevant de-identified demographic and descriptive information about the patient(s), and a description of the symptoms, diagnosis, treatment, and outcome; a discussion providing context and any necessary explanation of specific treatment decisions; a conclusion briefly outlining the take-home message and the lessons learned.
- **Graphical Abstract:** A graphical abstract (GA) is an image that appears alongside the text abstract in the Table of Contents. In addition to summarizing the content, it should represent the topic of the article in an attention-grabbing way. Moreover, it should not be exactly the same as the Figure in the paper or just a simple superposition of several subfigures. Note that the GA must be original and unpublished artwork. Any postage stamps, currency from any country, or trademarked items should not be included in it.

The GA should be a high-quality illustration or diagram in any of the following formats: PNG, JPEG, or TIFF. Written text in a GA should be clear and easy to read, using one of the following fonts: Times, Arial, Courier, Helvetica, Ubuntu or Calibri.

The minimum required size for the GA is 560 × 1100 pixels (height × width). The size should be of high quality in order to reproduce well.

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- **Equations:** If you are using Word, please use either the Microsoft Equation Editor or the MathType add-on. Equations should be editable by the editorial office and not appear in a picture format.
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Front Matter

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Back Matter

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Supplementary Materials, Data Deposit and Software Source Code

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New sequence information must be deposited to the appropriate database prior to submission of the manuscript. Accession numbers provided by the database should be included in the submitted manuscript. Manuscripts will not be published until the accession number is provided.

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Research and Publication Ethics

Research Ethics

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1. NSW Department of Primary Industries and Animal Research Review Panel. Three Rs. Available online: **https://www.animalethics.org.au/three-rs**
2. Home Office. Animals (Scientific Procedures) Act 1986. Code of Practice for the Housing and Care of Animals Bred, Supplied or Used for Scientific Purposes. Available online: **https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/388535/CoPanimalsWeb.pdf**
3. American Association for Laboratory Animal Science. The Scientific Basis for Regulation of Animal Care and Use. Available online: **https://www.aalas.org/about-aalas/position-papers/scientific-basis-for-regulation-of-animal-care-and-use**
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The HCT116 cell line was obtained from XXXX. The $MLH1^{+}$ cell line was provided by XXXXX, Ltd. The DLD-1 cell line was obtained from Dr. XXXX. The DR-GFP and SA-GFP reporter plasmids were obtained from Dr. XXX and the Rad51K133A expression vector was obtained from Dr. XXXX.

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Experimental research on plants (either cultivated or wild) including collection of plant material, must comply with institutional, national, or international guidelines. We recommend that authors comply with the **Convention on Biological Diversity** and the **Convention on the Trade in Endangered Species of Wild Fauna and Flora**.

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We encourage our authors to follow the **'Sex and Gender Equity in Research – SAGER – guidelines'** and to include sex and gender considerations where relevant. Authors should use the terms sex (biological attribute) and gender (shaped by social and cultural circumstances) carefully in order to avoid confusing both terms. Article titles and/or abstracts should indicate clearly what sex(es) the study applies to. Authors should also describe in the background, whether sex and/or gender differences may be expected; report how sex and/or gender were accounted for in the design of the study; provide disaggregated data by sex and/or gender, where appropriate; and discuss respective results. If a sex and/or gender analysis was not conducted, the rationale should be given in the Discussion. We suggest that our authors consult the full **guidelines** before submission.

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The editors of this journal enforce a rigorous peer-review process together with strict ethical policies and standards to ensure to add high quality scientific works to the field of scholarly publication. Unfortunately, cases of plagiarism, data falsification, image manipulation, inappropriate authorship credit, and the like, do arise. The editors of *Antibiotics* take such

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Authors wishing to publish their papers in *Antibiotics* must abide to the following:

- Any facts that might be perceived as a possible conflict of interest of the author(s) must be disclosed in the paper prior to submission.
- Authors should accurately present their research findings and include an objective discussion of the significance of their findings.
- Data and methods used in the research need to be presented in sufficient detail in the paper, so that other researchers can replicate the work.
- Raw data should preferably be publicly deposited by the authors before submission of their manuscript. Authors need to at least have the raw data readily available for presentation to the referees and the editors of the journal, if requested. Authors need to ensure appropriate measures are taken so that raw data is retained in full for a reasonable time after publication.
- Simultaneous submission of manuscripts to more than one journal is not tolerated.
- The journal accepts exact translations of previously published work. All submissions of translations must conform with our **policies on translations**.
- If errors and inaccuracies are found by the authors after publication of their paper, they need to be promptly communicated to the editors of this journal so that appropriate actions can be taken. Please refer to our **policy regarding Updating Published Papers**.
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- Plagiarism, data fabrication and image manipulation are not tolerated.
    - **Plagiarism is not acceptable** in *Antibiotics* submissions.

Plagiarism includes copying text, ideas, images, or data from another source, even from your own publications, without giving any credit to the original source.

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- **Image files must not be manipulated or adjusted in any way** that could lead to misinterpretation of the information provided by the original image.

Irregular manipulation includes: 1) introduction, enhancement, moving, or removing features from the original image; 2) grouping of images that should obviously be presented separately (e.g., from different parts of the same gel, or from different gels); or 3) modifying the contrast, brightness or color balance to obscure, eliminate or enhance some information.

If irregular image manipulation is identified and confirmed during the peer review process, we may reject the manuscript. If irregular image manipulation is identified and confirmed after publication, we may correct or retract the paper.

Our in-house editors will investigate any allegations of publication misconduct and may contact the authors' institutions or funders if necessary. If evidence of misconduct is found, appropriate action will be taken to correct or retract the publication. Authors are expected to comply with the best ethical publication practices when publishing with MDPI.

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Authors should ensure that where material is taken from other sources (including their own published writing) the source is clearly cited and that where appropriate permission is obtained.

Authors should not engage in excessive self-citation of their own work.

Authors should not copy references from other publications if they have not read the cited work.

Authors should not preferentially cite their own or their friends', peers', or institution's publications.

Authors should not cite advertisements or advertorial material.

In accordance with COPE guidelines, we expect that "original wording taken directly from publications by other researchers should appear in quotation marks with the appropriate citations." This condition also applies to an author's own work. COPE have produced a discussion document on **citation manipulation** with recommendations for best practice.

Reviewer Suggestions

During the submission process, please suggest three potential reviewers with the appropriate expertise to review the manuscript. The editors will not necessarily approach these referees. Please provide detailed contact information (address, homepage, phone, e-mail address). The

proposed referees should neither be current collaborators of the co-authors nor have published with any of the co-authors of the manuscript within the last three years. Proposed reviewers should be from different institutions to the authors. You may identify appropriate Editorial Board members of the journal as potential reviewers. You may suggest reviewers from among the authors that you frequently cite in your paper.

English Corrections

MDPI provides minor English editing by native English speakers for all accepted papers, included in the APC. The APC does not cover extensive English editing. Submitted papers should be written in good English and require no more than minor English editing before publication. Your paper could be returned to you at the English editing stage of the publication process if extensive editing is required, which could delay the publication of your work. You may choose to use a paid language-editing service, such as MDPI's **Author Services**, before submitting your paper for publication. If you use an alternative service that provides a confirmation certificate, please send a copy to the Editorial Office. Authors from economically developing countries or nations should consider registration with **AuthorAid**, a global research community that provides networking, mentoring, resources and training for researchers.

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Antibiotics accepts submissions that have previously been made available as preprints provided that they have not undergone peer review. A preprint is a draft version of a paper made available online before submission to a journal.

MDPI operates ***Preprints***, a preprint server to which submitted papers can be uploaded directly after completing journal submission. Note that *Preprints* operates independently of the journal and posting a preprint does not affect the peer review process. Check the *Preprints* **instructions for authors** for further information.

Expanded and high-quality conference papers can be considered as articles if they fulfill the following requirements: (1) the paper should be expanded to the size of a research article; (2) the conference paper should be cited and noted on the first page of the paper; (3) if the authors do not hold the copyright of the published conference paper, authors should seek the appropriate permission from the copyright holder; (4) authors are asked to disclose that it is conference paper in their cover letter and include a statement on what has been changed compared to the original conference paper. *Antibiotics* does not publish pilot studies or studies with inadequate statistical power.

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MDPI follows the International Committee of Medical Journal Editors (**ICMJE**) guidelines which state that, in order to qualify for authorship of a manuscript, the following criteria should be observed:

- Substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work; AND
- Drafting the work or reviewing it critically for important intellectual content; AND
- Final approval of the version to be published; AND
- Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Those who contributed to the work but do not qualify for authorship should be listed in the acknowledgments. More detailed guidance on authorship is given by the **International Council of Medical Journal Editors (ICMJE)**.

Any change to the author list should be approved by all authors including any who have been removed from the list. The corresponding author should act as a point of contact between the editor and the other authors and should keep co-authors informed and involve them in major decisions about the publication. We reserve the right to request confirmation that all authors meet the authorship conditions.

For more details about authorship please check **MDPI ethics website**.

Reviewers Recommendation

Authors can recommend potential reviewers. Journal editors will check to make sure there are no conflicts of interest before contacting those reviewers, and will not consider those with competing interests. Reviewers are asked to declare any conflicts of interest. Authors can also enter the names of potential peer reviewers they wish to exclude from consideration in the peer review of their manuscript, during the initial submission progress. The editorial team will respect these requests so long as this does not interfere with the objective and thorough assessment of the submission.

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Lack of Interference with Editorial Decisions

Editorial independence is of utmost importance and MDPI does not interfere with editorial decisions. All articles published by MDPI are peer reviewed and assessed by our independent editorial boards, and MDPI staff are not involved in decisions to accept manuscripts. When making an editorial decision, we expect the academic editor to make their decision based only upon:

- The suitability of selected reviewers;
- Adequacy of reviewer comments and author response;
- Overall scientific quality of the paper.

In all of our journals, in every aspect of operation, MDPI policies are informed by the mission to make science and research findings open and accessible as widely and rapidly as possible.

Editors and Editorial Staff as Authors

Editorial staff or editors shall not be involved in processing their own academic work. Submissions authored by editorial staff/editors will be assigned to at least two independent outside reviewers. Decisions will be made by other Editorial Board Members who do not have a conflict of interest with the author. Journal staff are not involved in the processing of their own work submitted to any MDPI journals.

Conflicts of Interest

According to The International Committee of Medical Journal Editors, “Authors should avoid entering into agreements with study sponsors, both for-profit and non-profit, that interfere with authors’ access to all of the study’s data or that interfere with their ability to analyze and interpret the data and to prepare and publish manuscripts independently when and where they choose.”

All authors must disclose all relationships or interests that could inappropriately influence or bias their work. Examples of potential conflicts of interest include but are not limited to financial interests (such as membership, employment, consultancies, stocks/shares ownership, honoraria, grants or other funding, paid expert testimonies and patent-licensing arrangements) and non-financial interests (such as personal or professional relationships, affiliations, personal beliefs).

Authors can disclose potential conflicts of interest via the online submission system during the submission process. Declarations regarding conflicts of interest can also be collected via the **MDPI disclosure form**. The corresponding author must include a summary statement in the

manuscript in a separate section “Conflicts of Interest” placed just before the reference list. The statement should reflect all the collected potential conflicts of interest disclosures in the form.

See below for examples of disclosures:

Conflicts of Interest: Author A has received research grants from Company A. Author B has received a speaker honorarium from Company X and owns stocks in Company Y. Author C has been involved as a consultant and expert witness in Company Z. Author D is the inventor of patent X.

If no conflicts exist, the authors should state:

Conflicts of Interest: The authors declare no conflicts of interest.

Editorial Procedures and Peer-Review

Pre-check

Immediately after submission, the journal’s Managing Editor will perform the technical pre-check to assess:

- Overall suitability of the manuscript to the journal/section/Special Issue;
- Manuscript adherence to high-quality research and ethical standards;
- Standards of rigor to qualify for further review.

The academic editor (i.e., the Editor-in-Chief in the case of regular submissions, the Guest Editor in the case of Special Issue submissions, or an Editorial Board member in the case of a conflict of interest and of regular submissions if the Editor-in-Chief allows) will be notified of the submission and invited to perform an editorial pre-check. During the editorial pre-check phase, the academic editor will assess the suitability of the submission with respect to the scope of the journal, as well as the overall scientific soundness of the manuscript, including the relevance of the references and the correctness of the applied methodology. Academic editors can decide to reject the manuscript, request revisions before peer-review, or continue with the peer-review process and recommend suitable reviewers.

Peer-Review

Once a manuscript passes the initial checks, it will be assigned to at least two independent experts for peer-review. A single-blind review is applied, where authors' identities are known to reviewers. Peer review comments are confidential and will only be disclosed with the express agreement of the reviewer.

In the case of regular submissions, in-house assistant editors will invite experts, including recommendations by an academic editor. These experts may also include *Editorial Board Members* and Guest Editors of the journal. Potential reviewers suggested by the authors may also be considered. Reviewers should not have published with any of the co-authors during the past three years and should not currently work or collaborate with any of the institutions of the co-authors of the submitted manuscript.

Optional Open Peer-Review

The journal operates optional open peer-review: *Authors are given the option for all review reports and editorial decisions to be published alongside their manuscript. In addition, reviewers can sign their review, i.e., identify themselves in the published review reports.* Authors can alter their choice for open review at any time before publication, but once the paper has been published changes will only be made at the discretion of the *Publisher* and *Editor-in-Chief*. We encourage authors to take advantage of this opportunity as proof of the rigorous process employed in publishing their research. To guarantee impartial refereeing, the names of referees will be revealed only if the referees agree to do so, and after a paper has been accepted for publication.

Editorial Decision and Revision

All the articles, reviews and communications published in MDPI journals go through the peer-review process and receive at least two reviews. The in-house editor will communicate the decision of the academic editor, which will be one of the following:

- **Accept after Minor Revisions:**

  The paper is in principle accepted after revision based on the reviewer's comments. Authors are given five days for minor revisions.
- ***Reconsider after Major Revisions*:**

  The acceptance of the manuscript would depend on the revisions. The author needs to provide a point by point response or provide a rebuttal if some of the reviewer's comments cannot be revised. A maximum of two rounds of major revision per manuscript is normally provided. Authors will be asked to resubmit the revised paper within a suitable time frame, and the revised version will be returned to the reviewer for further comments. If the required revision time is estimated to be longer than 2 months, we will recommend that authors withdraw their manuscript before resubmitting so as to avoid unnecessary time pressure and to ensure that all manuscripts are sufficiently revised.
- ***Reject and Encourage Resubmission*:**

  If additional experiments are needed to support the conclusions, the manuscript will be rejected and the authors will be encouraged to re-submit the paper once further experiments have been conducted.

- ***Reject*:**
  The article has serious flaws, and/or makes no original significant contribution. No offer of resubmission to the journal is provided.

All reviewer comments should be responded to in a point-by-point fashion. Where the authors disagree with a reviewer, they must provide a clear response.

Author Appeals

Authors may appeal a rejection by sending an e-mail to the Editorial Office of the journal. The appeal must provide a detailed justification, including point-by-point responses to the reviewers' and/or Editor's comments using an **appeal form**. Appeals can only be submitted following a "reject and decline resubmission" decision and should be submitted within three months from the decision date. Failure to meet these criteria will result in the appeal not being considered further. The *Managing Editor* will forward the manuscript and related information (including the identities of the referees) to a designated *Editorial Board Member*. The Academic Editor being consulted will be asked to provide an advisory recommendation on the manuscript and may recommend acceptance, further peer-review, or uphold the original rejection decision. This decision will then be validated by the *Editor-in-Chief*. A reject decision at this stage is final and cannot be reversed.

Production and Publication

Once accepted, the manuscript will undergo professional copy-editing, English editing, proofreading by the authors, final corrections, pagination, and, publication on the **www.mdpi.com** website.

Promoting Equity, Diversity and Inclusiveness within MDPI Journals

Our Managing Editors encourage the Editors-in-Chief and Associate Editors to appoint diverse expert Editorial Boards. This is also reflective in our multi-national and inclusive workplace. We are proud to create equal opportunities without regard to gender, ethnicity, sexual orientation, age, religion, or socio-economic status. There is no place for discrimination in our workplace and editors of MDPI journals are to uphold these principles in high regard.

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To improve the reproducibility of scientific research, the **Resource Identification Initiative** aims to provide unique persistent identifiers for key biological resources, including antibodies, cell lines, model organisms and tools.

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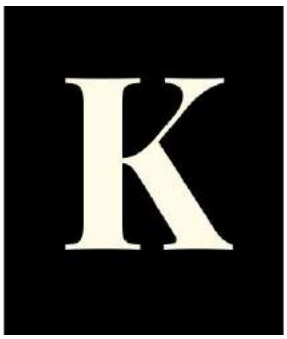

Annexure K

Instructions for Authors for '*Pharmaceutics*'

Submission Checklist

Please:

1. Read the **Aims & Scope** to gain an overview and assess if your manuscript is suitable for this journal;
2. Use the **Microsoft Word template** or **LaTeX template** to prepare your manuscript;
3. Make sure that issues about **publication ethics**, **research ethics**, **copyright**, **authorship**, **figure formats**, **data** and **references format** have been appropriately considered;
4. Ensure that all authors have approved the content of the submitted manuscript.
5. Authors are encouraged to add a **biography** (optional) to the submission and post it to **SciProfiles**.

Manuscript Submission Overview

Types of Publications

Full experimental details must be provided so that the results can be reproduced. *Pharmaceutics* requires that authors publish all experimental controls and make full datasets available where possible (see the guidelines on **Supplementary Materials** and references to unpublished data).

Manuscripts submitted to *Pharmaceutics* should neither be published previously nor be under consideration for publication in another journal. The main article types are listed below and a comprehensive list of article types can be found **here.**

- *Article:* These are original research manuscripts. The work should report scientifically sound experiments and provide a substantial amount of new information. The article should include the most recent and relevant references in the field. The structure should include an Abstract, Keywords, Introduction, Materials and Methods, Results, Discussion, and Conclusions (optional) sections, with a suggested minimum word count of 4000 words.

- *Review:* Reviews offer a comprehensive analysis of the existing literature within a field of study, identifying current gaps or problems. They should be critical and constructive and provide recommendations for future research. No new, unpublished data should be presented. The structure can include an Abstract, Keywords, Introduction, Relevant Sections, Discussion, Conclusions, and Future Directions, with a suggested minimum word count of 4000 words.

Submission Process

Manuscripts for Pharmaceutics should be submitted online at susy.mdpi.com. The submitting author, who is generally the corresponding author, is responsible for the manuscript during the submission and peer-review process. The submitting author must ensure that all eligible co-authors have been included in the author list (read the criteria to qualify for authorship) and that they have all read and approved the submitted version of the manuscript. To submit your manuscript, register and log in to the submission website. Once you have registered, click here to go to the submission form for Pharmaceutics. All co-authors can see the manuscript details in the submission system, if they register and log in using the e-mail address provided during manuscript submission.

Accepted File Formats

Authors are encouraged to use the **Microsoft Word template** or **LaTeX template** to prepare their manuscript. Using the template file will substantially shorten the time to complete copy-editing and publication of accepted manuscripts. The total amount of data for all files must not exceed 120 MB. If this is a problem, please contact the Editorial Office **pharmaceutics@mdpi.com**. Accepted file formats are:

- *Microsoft Word:* Manuscripts prepared in Microsoft Word must be converted into a single file before submission. When preparing manuscripts in Microsoft Word, we encourage you to use the ***Pharmaceutics* Microsoft Word template file**. Please insert your graphics (schemes, figures, *etc.*) in the main text after the paragraph of its first citation.
- *LaTeX:* Manuscripts prepared in LaTeX must be collated into one ZIP folder (including all source files and images, so that the Editorial Office can recompile the submitted PDF). When preparing manuscripts in LaTeX, we encourage you to use the ***Pharmaceutics* LaTeX template files**. You can now also use the online application **writeLaTeX** to submit articles directly to *Pharmaceutics*. The MDPI LaTeX template file should be selected from the **writeLaTeX template gallery**.
- *Supplementary files:* May be any format, but it is recommended that you use common, non-proprietary formats where possible (see **below** for further details).

Disclaimer: Usage of these templates is exclusively intended for submission to the journal for peer-review, and strictly limited to this purpose and it cannot be used for posting online on preprint servers or other websites.

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Pharmaceutics now accepts free format submission:

- We do not have strict formatting requirements, but all manuscripts must contain the required sections: Author Information, Abstract, Keywords, Introduction, Materials & Methods, Results, Conclusions, Figures and Tables with Captions, Funding Information, Author Contributions, Conflict of Interest and other Ethics Statements. Check the Journal ***Instructions for Authors*** for more details.
- Your references may be in any style, provided that you use the consistent formatting throughout. It is essential to include author(s) name(s), journal or book title, article or chapter title (where required), year of publication, volume and issue (where appropriate) and pagination. DOI numbers (Digital Object Identifier) are not mandatory but highly encouraged. The bibliography software package *EndNote*, ***Zotero***, *Mendeley*, *Reference Manager* are recommended.
- When your manuscript reaches the revision stage, you will be requested to format the manuscript according to the journal guidelines.

Cover Letter

A cover letter must be included with each manuscript submission. It should be concise and explain why the content of the paper is significant, placing the findings in the context of existing work. It should explain why the manuscript fits the scope of the journal.

Any prior submissions of the manuscript to MDPI journals must be acknowledged. If this is the case, it is strongly recommended that the previous manuscript ID is provided in the submission system, which will ease your current submission process. The names of proposed and excluded reviewers should be provided in the submission system, not in the cover letter.

All cover letters are required to include the statements:

- We confirm that neither the manuscript nor any parts of its content are currently under consideration or published in another journal.
- All authors have approved the manuscript and agree with its submission to (journal name).

Author Identification

Authors are encouraged to add a biography (maximum 150 words) to the submission and upload it to **SciProfiles**. This should be a single paragraph and should contain the following points:

1. Authors' full names followed by current positions;
2. Education background including institution information and year of graduation (type and level of degree received);
3. Work experience;
4. Current and previous research interests;
5. Memberships of professional societies and awards received.

If a manuscript is accepted for publication, we will add an icon linking to your online **ORCID** profile in the final version of the published paper.

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All authors should list their current affiliation and the affiliation where most research was carried out for the preparation of their manuscript. We recommend adding as primary the affiliation where most of the research was conducted or supported, but please check with your institution for any contractual agreement requirements.

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If one or all the authors are not currently affiliated with a university, institution or company, or have not been during the development of the manuscript, they should list themselves as an "Independent Researcher".

Manuscript Preparation

General Considerations

- **Research manuscripts** should comprise:
    - **Front matter**: Title, Author list, Affiliations, Abstract, Keywords.
    - **Research manuscript sections**: Introduction, Materials and Methods, Results, Discussion, Conclusions (optional).
    - **Back matter**: Supplementary Materials, Acknowledgments, Author Contributions, Conflicts of Interest, **References**.
- **Review manuscripts** should comprise the **front matter**, literature review sections and the **back matter**. The template file can also be used to prepare the front and back matter of your review manuscript. It is not necessary to follow the remaining structure. Structured

reviews and meta-analyses should use the same structure as research articles and ensure they conform to the **PRISMA** guidelines.

Graphical Abstract:

A graphical abstract (GA) is an image that appears alongside the text abstract in the Table of Contents. In addition to summarizing the content, it should represent the topic of the article in an attention-grabbing way. Moreover, it should not be exactly the same as the Figure in the paper or just a simple superposition of several subfigures. Note that the GA must be original and unpublished artwork. Any postage stamps, currency from any country, or trademarked items should not be included in it.

The GA should be a high-quality illustration or diagram in any of the following formats: PNG, JPEG, or TIFF. Written text in a GA should be clear and easy to read, using one of the following fonts: Times, Arial, Courier, Helvetica, Ubuntu or Calibri.

The minimum required size for the GA is 560 × 1100 pixels (height × width). The size should be of high quality in order to reproduce well.

- **Acronyms/Abbreviations/Initialisms** should be defined the first time they appear in each of three sections: the abstract; the main text; the first figure or table. When defined for the first time, the acronym/abbreviation/initialism should be added in parentheses after the written-out form.
- **SI Units** (International System of Units) should be used. Imperial, US customary and other units should be converted to SI units whenever possible.
- **Accession numbers** of RNA, DNA and protein sequences used in the manuscript should be provided in the Materials and Methods section. Also see the section on **Deposition of Sequences and Expression Data**.
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- **Preregistration:** Where authors have preregistered studies or analysis plans, links to the preregistration must be provided in the manuscript.

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Front Matter

These sections should appear in all manuscript types:

- **Title:** The title of your manuscript should be concise, specific and relevant. It should identify if the study reports (human or animal) trial data, or is a systematic review, meta-analysis or replication study. When gene or protein names are included, the abbreviated name rather than full name should be used. Please do not include abbreviated or short forms of the title, such as a running title or head. These will be removed by our Editorial Office.
- **Author List and Affiliations:** Authors' full first and last names must be provided. The initials of any middle names can be added. The PubMed/MEDLINE standard format is used for affiliations: complete address information including city, zip code, state/province, and country. At least one author should be designated as the corresponding author. The email addresses of all authors will be displayed on published papers, and hidden by Captcha on the website as standard. It is the responsibility of the corresponding author to ensure that consent for the display of email addresses is obtained from all authors. If an author (other than the corresponding author) does not wish to have their email addresses displayed in this way, the corresponding author must indicate as such during proofreading. After acceptance, updates to author names or affiliations may not be permitted. Equal Contributions: authors who have contributed equally should be marked with a superscript symbol (†). The symbol must be included below the affiliations, and the following statement added: “These authors contributed equally to this work”. The equal roles of authors should also be adequately disclosed in the author contributions statement. Please read the criteria to qualify for authorship.
- **Abstract:** The abstract should be a total of about 200 words maximum. The abstract should be a single paragraph and should follow the style of structured abstracts, but without headings: 1) Background: Place the question addressed in a broad context and highlight the purpose of the study; 2) Methods: Describe briefly the main methods or treatments applied. Include any relevant preregistration numbers, and species and strains of any animals used; 3) Results: Summarize the article's main findings; and 4) Conclusion: Indicate the main conclusions or interpretations. The abstract should be an objective representation of the article: it must not contain results which are not presented and substantiated in the main text and should not exaggerate the main conclusions.

- **Keywords:** Three to ten pertinent keywords need to be added after the abstract. We recommend that the keywords are specific to the article, yet reasonably common within the subject discipline.

Research Manuscript Sections

- **Introduction:** The introduction should briefly place the study in a broad context and highlight why it is important. It should define the purpose of the work and its significance, including specific hypotheses being tested. The current state of the research field should be reviewed carefully and key publications cited. Please highlight controversial and diverging hypotheses when necessary. Finally, briefly mention the main aim of the work and highlight the main conclusions. Keep the introduction comprehensible to scientists working outside the topic of the paper.
- **Materials and Methods:** They should be described with sufficient detail to allow others to replicate and build on published results. New methods and protocols should be described in detail while well-established methods can be briefly described and appropriately cited. Give the name and version of any software used and make clear whether computer code used is available. Include any pre-registration codes.
- **Results:** Provide a concise and precise description of the experimental results, their interpretation as well as the experimental conclusions that can be drawn.
- **Discussion:** Authors should discuss the results and how they can be interpreted in perspective of previous studies and of the working hypotheses. The findings and their implications should be discussed in the broadest context possible and limitations of the work highlighted. Future research directions may also be mentioned. This section may be combined with Results.
- **Conclusions:** This section is not mandatory but can be added to the manuscript if the discussion is unusually long or complex.
- **Patents:** This section is not mandatory but may be added if there are patents resulting from the work reported in this manuscript.

Back Matter

- **Supplementary Materials:** Describe any supplementary material published online alongside the manuscript (figure, tables, video, spreadsheets, etc.). Please indicate the name and title of each element as follows Figure S1: title, Table S1: title, etc.
- **Author Contributions:** Each author is expected to have made substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data; or the creation of new software used in the work; or have drafted the work or substantively revised it; AND has approved the submitted version (and version substantially edited by

journal staff that involves the author's contribution to the study); AND agrees to be personally accountable for the author's own contributions and for ensuring that questions related to the accuracy or integrity of any part of the work, even ones in which the author was not personally involved, are appropriately investigated, resolved, and documented in the literature. For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used "Conceptualization, X.X. and Y.Y.; Methodology, X.X.; Software, X.X.; Validation, X.X., Y.Y. and Z.Z.; Formal Analysis, X.X.; Investigation, X.X.; Resources, X.X.; Data Curation, X.X.; Writing – Original Draft Preparation, X.X.; Writing – Review & Editing, X.X.; Visualization, X.X.; Supervision, X.X.; Project Administration, X.X.; Funding Acquisition, Y.Y.", please turn to the **CRediT taxonomy** for the term explanation. For more background on CRediT, see **here**. "**Authorship must include and be limited to those who have contributed substantially to the work. Please read the section concerning the criteria to qualify for authorship carefully**".

- **Funding:** All sources of funding of the study should be disclosed. Clearly indicate grants that you have received in support of your research work and if you received funds to cover publication costs. Note that some funders will not refund article processing charges (APC) if the funder and grant number are not clearly and correctly identified in the paper. Funding information can be entered separately into the submission system by the authors during submission of their manuscript. Such funding information, if available, will be deposited to FundRef if the manuscript is finally published.

  Please add: "This research received no external funding" or "This research was funded by [name of funder] grant number [xxx]" and "The APC was funded by [XXX]" in this section. Check carefully that the details given are accurate and use the standard spelling of funding agency names at https://search.crossref.org/funding, any errors may affect your future funding.
- **Institutional Review Board Statement:** In this section, please add the Institutional Review Board Statement and approval number for studies involving humans or animals. Please note that the Editorial Office might ask you for further information. Please add "The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Review Board (or Ethics Committee) of NAME OF INSTITUTE (protocol code XXX and date of approval)." OR "Ethical review and approval were waived for this study, due to REASON (please provide a detailed justification)." OR "Not applicable" for studies not involving humans or animals. You might also choose to exclude this statement if the study did not involve humans or animals.
- **Informed Consent Statement:** Any research article describing a study involving humans should contain this statement. Please add "Informed consent was obtained from all subjects involved in the study." OR "Patient consent was waived due to REASON (please provide a

detailed justification).” OR “Not applicable” for studies not involving humans. You might also choose to exclude this statement if the study did not involve humans. Written informed consent for publication must be obtained from participating patients who can be identified (including by the patients themselves). Please state “Written informed consent has been obtained from the patient(s) to publish this paper” if applicable.

- **Data Availability Statement:** In this section, please provide details regarding where data supporting reported results can be found, including links to publicly archived datasets analyzed or generated during the study. Please refer to suggested Data Availability Statements in section “**MDPI Research Data Policies**”. You might choose to exclude this statement if the study did not report any data.
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- **References:** References must be numbered in order of appearance in the text (including table captions and figure legends) and listed individually at the end of the manuscript. We recommend preparing the references with a bibliography software package, such as **EndNote**, **ReferenceManager** or **Zotero** to avoid typing mistakes and duplicated references. We encourage citations to data, computer code and other citable research material. If available online, you may use reference style 9. below.
- Citations and References in Supplementary files are permitted provided that they also appear in the main text and in the reference list.

In the text, reference numbers should be placed in square brackets [ ], and placed before the punctuation; for example [1], [1–3] or [1,3]. For embedded citations in the text with pagination, use both parentheses and brackets to indicate the reference number and page numbers; for example [5] (p. 10). or [6] (pp. 101–105).

The reference list should include the full title, as recommended by the ACS style guide. Style files for **Endnote** and **Zotero** are available.

References should be described as follows, depending on the type of work:

Journal Articles:

1. Author 1, A.B.; Author 2, C.D. Title of the article. *Abbreviated Journal Name* **Year**, *Volume*, page range.

Books and Book Chapters:

2. Author 1, A.; Author 2, B. *Book Title*, 3rd ed.; Publisher: Publisher Location, Country, Year; pp. 154–196.

3. Author 1, A.; Author 2, B. Title of the chapter. In *Book Title*, 2nd ed.; Editor 1, A., Editor 2, B., Eds.; Publisher: Publisher Location, Country, Year; Volume 3, pp. 154–196.

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5. Author 1, A.B.; Author 2, C. Title of Unpublished Work. *Abbreviated Journal Name* year, *phrase indicating stage of publication* (*submitted*; *accepted*; *in press*).

Unpublished materials not intended for publication:

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Conference Proceedings:

7. Author 1, A.B.; Author 2, C.D.; Author 3, E.F. Title of Presentation. In *Title of the Collected Work* (if available), Proceedings of the Name of the Conference, Location of Conference, Country, Date of Conference; Editor 1, Editor 2, Eds. (if available); Publisher: City, Country, Year (if available); Abstract Number (optional), Pagination (optional).

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8. Author 1, A.B. Title of Thesis. Level of Thesis, Degree-Granting University, Location of University, Date of Completion.

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9. Title of Site. Available online: URL (accessed on Day Month Year). Unlike published works, websites may change over time or disappear, so we encourage you create an archive of the cited

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See the **Reference List and Citations Guide** for more detailed information.

Preparing Figures, Schemes and Tables

- File for Figures and Schemes must be provided during submission in a single zip archive and at a sufficiently high resolution (minimum 1000 pixels width/height, or a resolution of 300 dpi or higher). Common formats are accepted, however, TIFF, JPEG, EPS and PDF are preferred.
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- All Figures, Schemes and Tables should be inserted into the main text close to their first citation and must be numbered following their number of appearance (Figure 1, Scheme I, Figure 2, Scheme II, Table 1, *etc.*).
- All Figures, Schemes and Tables should have a short explanatory title and caption.
- All table columns should have an explanatory heading. To facilitate the copy-editing of larger tables, smaller fonts may be used, but no less than 8 pt. in size. Authors should use the Table option of Microsoft Word to create tables.
- Authors are encouraged to prepare figures and schemes in color (RGB at 8-bit per channel). There is no additional cost for publishing full color graphics.

Original Images for Blots and Gels Requirements

For the main text, please ensure that:

- All experimental samples and controls used for one comparative analysis are run on the same blot/gel.
- Image processing methods, such as adjusting the brightness or contrast, do not alter or distort the information in the figure and are applied to every pixel. High-contrast blots/gels are discouraged.
- Cropped blots/gels present in the main text retain all important information and bands.
- You have checked figures for duplications and ensured the figure legends are clear and accurate. Please include all relevant information in the figure legends and clearly indicate any re-arrangement of lanes.

In order to ensure the integrity and scientific validity of blots (including, but not limited to, Western blots) and the reporting of gel data, original, uncropped and unadjusted images should be uploaded as Supporting Information files at the time of initial submission.

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Supplementary Materials, Data Deposit and Software Source Code

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Where ethical, legal or privacy issues are present, data should not be shared. The authors should make any limitations clear in the Data Availability Statement upon submission. Authors should ensure that data shared are in accordance with consent provided by participants on the use of confidential data.

Data Availability Statements provide details regarding where data supporting reported results can be found, including links to publicly archived datasets analyzed or generated during the study.

Below are suggested Data Availability Statements:

- Data available in a publicly accessible repository
  The data presented in this study are openly available in [repository name e.g., FigShare] at [**doi**], reference number [reference number].
- Data available in a publicly accessible repository that does not issue DOIs
  Publicly available datasets were analyzed in this study. This data can be found here: [link/accession number]

- Data available on request due to restrictions eg privacy or ethical
  The data presented in this study are available on request from the corresponding author. The data are not publicly available due to [insert reason here]
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  Restrictions apply to the availability of these data. Data was obtained from [third party] and are available [from the authors/at URL] with the permission of [third party].
- Data sharing not applicable
  No new data were created or analyzed in this study. Data sharing is not applicable to this article.
- Data is contained within the article or supplementary material
  The data presented in this study are available in [insert article or supplementary material here]

Data citation:

- [dataset] Authors. Year. Dataset title; Data repository or archive; Version (if any); Persistent identifier (e.g., DOI).

Computer Code and Software

For work where novel computer code was developed, authors should release the code either by depositing in a recognized, public repository such as **GitHub** or uploading as supplementary information to the publication. The name, version, corporation and location information for all software used should be clearly indicated. Please include all the parameters used to run software/programs analyses.

Supplementary Material

Additional data and files can be uploaded as "Supplementary Files" during the manuscript submission process. The supplementary files will also be available to the referees as part of the peer-review process. Any file format is acceptable; however, we recommend that common, non-proprietary formats are used where possible. For more information on supplementary materials, please refer to https://www.mdpi.com/authors/layout#_bookmark83.

References in Supplementary Files

Citations and References in Supplementary files are permitted provided that they also appear in the reference list of the main text.

Unpublished Data

Restrictions on data availability should be noted during submission and in the manuscript. "Data not shown" should be avoided: authors are encouraged to publish all observations related to the submitted manuscript as Supplementary Material. "Unpublished data" intended for publication in a manuscript that is either planned, "in preparation" or "submitted" but not yet accepted, should

be cited in the text and a reference should be added in the References section. "Personal Communication" should also be cited in the text and reference added in the References section. (see also the MDPI reference list and citations style guide).

Remote Hosting and Large Data Sets

Data may be deposited with specialized service providers or institutional/subject repositories, preferably those that use the DataCite mechanism. Large data sets and files greater than 60 MB must be deposited in this way. For a list of other repositories specialized in scientific and experimental data, please consult **databib.org** or **re3data.org**. The data repository name, link to the data set (URL) and accession number, doi or handle number of the data set must be provided in the paper. The journal **Data** also accepts submissions of data set papers.

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New sequence information must be deposited to the appropriate database prior to submission of the manuscript. Accession numbers provided by the database should be included in the submitted manuscript. Manuscripts will not be published until the accession number is provided.

- *New nucleic acid sequences* must be deposited into an acceptable repository such as **GenBank**, **EMBL**, or **DDBJ**. Sequences should be submitted to only one database.
- *New high throughput sequencing (HTS) datasets* (RNA-seq, ChIP-Seq, degradome analysis, …) must be deposited either in the **GEO database** or in the NCBI's **Sequence Read Archive (SRA)**.
- *New microarray data* must be deposited either in the **GEO** or the **ArrayExpress** databases. The "Minimal Information About a Microarray Experiment" (MIAME) guidelines published by the Microarray Gene Expression Data Society must be followed.
- *New protein sequences* obtained by protein sequencing must be submitted to UniProt (submission tool **SPIN**). Annotated protein structure and its reference sequence must be submitted to **RCSB of Protein Data Bank**.

All sequence names and the accession numbers provided by the databases must be provided in the Materials and Methods section of the article.

Deposition of Proteomics Data

Methods used to generate the proteomics data should be described in detail and we encourage authors to adhere to the "**Minimum Information About a Proteomics Experiment**". All generated mass spectrometry raw data must be deposited in the appropriate public database such as **ProteomeXchange**, **PRIDE** or **jPOST**. At the time of submission, please include all relevant information in the materials and methods section, such as repository where the data was submitted and link, data set identifier, username and password needed to access the data.

Research and Publication Ethics

Research Ethics

Research Involving Human Subjects

When reporting on research that involves human subjects, human material, human tissues, or human data, authors must declare that the investigations were carried out following the rules of the Declaration of Helsinki of 1975 (**https://www.wma.net/what-we-do/medical-ethics/declaration-of-helsinki/**), revised in 2013. According to point 23 of this declaration, an approval from the local institutional review board (IRB) or other appropriate ethics committee must be obtained before undertaking the research to confirm the study meets national and international guidelines. As a minimum, a statement including the project identification code, date of approval, and name of the ethics committee or institutional review board must be stated in Section 'Institutional Review Board Statement' of the article.

Example of an ethical statement: "All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of XXX (Project identification code)."

For non-interventional studies (e.g. surveys, questionnaires, social media research), all participants must be fully informed if the anonymity is assured, why the research is being conducted, how their data will be used and if there are any risks associated. As with all research involving humans, ethical approval from an appropriate ethics committee must be obtained prior to conducting the study. If ethical approval is not required, authors must either provide an exemption from the ethics committee or are encouraged to cite the local or national legislation that indicates ethics approval is not required for this type of study. Where a study has been granted exemption, the name of the ethics committee which provided this should be stated in Section 'Institutional Review Board Statement' with a full explanation regarding why ethical approval was not required.

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specific age, ethnicity, or occupation where they are not relevant to the conclusions. A **template permission form** is available to download. A blank version of the form used to obtain permission (without the patient names or signature) must be uploaded with your submission. Editors reserve the right to reject any submission that does not meet these requirements.

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If the study reports research involving vulnerable groups, an additional check may be performed. The submitted manuscript will be scrutinized by the editorial office and upon request, documentary evidence (blank consent forms and any related discussion documents from the ethics board) must be supplied. Additionally, when studies describe groups by race, ethnicity, gender, disability, disease, etc., explanation regarding why such categorization was needed must be clearly stated in the article.

Ethical Guidelines for the Use of Animals in Research

The editors will require that the benefits potentially derived from any research causing harm to animals are significant in relation to any cost endured by animals, and that procedures followed are unlikely to cause offense to the majority of readers. Authors should particularly ensure that their research complies with the commonly-accepted '3Rs [1]':

- Replacement of animals by alternatives wherever possible,
- Reduction in number of animals used, and
- Refinement of experimental conditions and procedures to minimize the harm to animals.

Authors must include details on housing, husbandry and pain management in their manuscript.

For further guidance authors should refer to the Code of Practice for the Housing and Care of Animals Used in Scientific Procedures [2], American Association for Laboratory Animal Science [3] or European Animal Research Association [4].

If national legislation requires it, studies involving vertebrates or higher invertebrates must only be carried out after obtaining approval from the appropriate ethics committee. As a minimum, the project identification code, date of approval and name of the ethics committee or institutional review board should be stated in Section 'Institutional Review Board Statement'. Research

procedures must be carried out in accordance with national and institutional regulations. Statements on animal welfare should confirm that the study complied with all relevant legislation. Clinical studies involving animals and interventions outside of routine care require ethics committee oversight as per the American Veterinary Medical Association. If the study involved client-owned animals, informed client consent must be obtained and certified in the manuscript report of the research. Owners must be fully informed if there are any risks associated with the procedures and that the research will be published. If available, a high standard of veterinary care must be provided. Authors are responsible for correctness of the statements provided in the manuscript.

If ethical approval is not required by national laws, authors must provide an exemption from the ethics committee, if one is available. Where a study has been granted exemption, the name of the ethics committee that provided this should be stated in Section 'Institutional Review Board Statement' with a full explanation on why the ethical approval was not required.

If no animal ethics committee is available to review applications, authors should be aware that the ethics of their research will be evaluated by reviewers and editors. Authors should provide a statement justifying the work from an ethical perspective, using the same utilitarian framework that is used by ethics committees. Authors may be asked to provide this even if they have received ethical approval.

MDPI endorses the ARRIVE guidelines (**arriveguidelines.org/**) for reporting experiments using live animals. Authors and reviewers must use the ARRIVE guidelines as a checklist, which can be found at: **https://arriveguidelines.org/sites/arrive/files/documents/ARRIVE%20Compliance%20Questionnaire.pdf**. Editors reserve the right to ask for the checklist and to reject submissions that do not adhere to these guidelines, to reject submissions based on ethical or animal welfare concerns or if the procedure described does not appear to be justified by the value of the work presented.

1. NSW Department of Primary Industries and Animal Research Review Panel. Three Rs. Available online: **https://www.animalethics.org.au/three-rs**
2. Home Office. Animals (Scientific Procedures) Act 1986. Code of Practice for the Housing and Care of Animals Bred, Supplied or Used for Scientific Purposes. Available online: **https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/388535/CoPanimalsWeb.pdf**

3. American Association for Laboratory Animal Science. The Scientific Basis for Regulation of Animal Care and Use. Available online: **https://www.aalas.org/about-aalas/position-papers/scientific-basis-for-regulation-of-animal-care-and-use**
4. European Animal Research Association. EU regulations on animal research. Available online: **https://www.eara.eu/animal-research-law**

Research Involving Cell Lines

Methods sections for submissions reporting on research with cell lines should state the origin of any cell lines. For established cell lines the provenance should be stated and references must also be given to either a published paper or to a commercial source. If previously unpublished *de novo* cell lines were used, including those gifted from another laboratory, details of institutional review board or ethics committee approval must be given, and confirmation of written informed consent must be provided if the line is of human origin.

An example of Ethical Statements:

The HCT116 cell line was obtained from XXXX. The $MLH1^{+}$ cell line was provided by XXXXX, Ltd. The DLD-1 cell line was obtained from Dr. XXXX. The DR-GFP and SA-GFP reporter plasmids were obtained from Dr. XXX and the Rad51K133A expression vector was obtained from Dr. XXXX.

Research Involving Plants

Experimental research on plants (either cultivated or wild) including collection of plant material, must comply with institutional, national, or international guidelines. We recommend that authors comply with the **Convention on Biological Diversity** and the **Convention on the Trade in Endangered Species of Wild Fauna and Flora**.

For each submitted manuscript supporting genetic information and origin must be provided. For research manuscripts involving rare and non-model plants (other than, e.g., *Arabidopsis thaliana, Nicotiana benthamiana, Oryza sativa*, or many other typical model plants), voucher specimens must be deposited in an accessible herbarium or museum. Vouchers may be requested for review by future investigators to verify the identity of the material used in the study (especially if taxonomic rearrangements occur in the future). They should include details of the populations sampled on the site of collection (GPS coordinates), date of collection, and document the part(s) used in the study where appropriate. For rare, threatened or endangered species this can be waived but it is necessary for the author to describe this in the cover letter.

Editors reserve the rights to reject any submission that does not meet these requirements.

An example of Ethical Statements:

Torenia fournieri plants were used in this study. White-flowered Crown White (CrW) and violet-flowered Crown Violet (CrV) cultivars selected from 'Crown Mix' (XXX Company, City, Country) were kindly provided by Dr. XXX (XXX Institute, City, Country).

Arabidopis mutant lines (SALKxxxx, SAILxxxx,…) were kindly provided by Dr. XXX, institute, city, country).

Clinical Trials Registration

Registration

MDPI follows the International Committee of Medical Journal Editors (ICMJE) **guidelines** which require and recommend registration of clinical trials in a public trials registry at or before the time of first patient enrolment as a condition of consideration for publication.

Purely observational studies do not require registration. A clinical trial not only refers to studies that take place in a hospital or involve pharmaceuticals, but also refer to all studies which involve participant randomization and group classification in the context of the intervention under assessment.

Authors are strongly encouraged to pre-register clinical trials with an international clinical trials register and cite a reference to the registration in the Methods section. Suitable databases include **clinicaltrials.gov**, **the EU Clinical Trials Register** and those listed by the World Health Organisation **International Clinical Trials Registry Platform**.

Approval to conduct a study from an independent local, regional, or national review body is not equivalent to prospective clinical trial registration. MDPI reserves the right to decline any paper without trial registration for further peer-review. However, if the study protocol has been published before the enrolment, the registration can be waived with correct citation of the published protocol.

CONSORT Statement

MDPI requires a completed CONSORT 2010 **checklist** and **flow diagram** as a condition of submission when reporting the results of a randomized trial. Templates for these can be found here or on the CONSORT website (**http://www.consort-statement.org**) which also describes several CONSORT checklist extensions for different designs and types of data beyond two group parallel trials. At minimum, your article should report the content addressed by each item of the checklist.

Sex and Gender in Research

We encourage our authors to follow the **'Sex and Gender Equity in Research – SAGER – guidelines'** and to include sex and gender considerations where relevant. Authors should use the terms sex (biological attribute) and gender (shaped by social and cultural circumstances) carefully in order to avoid confusing both terms. Article titles and/or abstracts should indicate clearly what sex(es) the study applies to. Authors should also describe in the background, whether sex and/or gender differences may be expected; report how sex and/or gender were accounted for in the design of the study; provide disaggregated data by sex and/or gender, where appropriate; and discuss respective results. If a sex and/or gender analysis was not conducted, the rationale should be given in the Discussion. We suggest that our authors consult the full **guidelines** before submission.

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- Any facts that might be perceived as a possible conflict of interest of the author(s) must be disclosed in the paper prior to submission.
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- Data and methods used in the research need to be presented in sufficient detail in the paper, so that other researchers can replicate the work.
- Raw data should preferably be publicly deposited by the authors before submission of their manuscript. Authors need to at least have the raw data readily available for presentation to the referees and the editors of the journal, if requested. Authors need to ensure appropriate measures are taken so that raw data is retained in full for a reasonable time after publication.
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During the submission process, please suggest three potential reviewers with the appropriate expertise to review the manuscript. The editors will not necessarily approach these referees. Please provide detailed contact information (address, homepage, phone, e-mail address). The proposed referees should neither be current collaborators of the co-authors nor have published with any of the co-authors of the manuscript within the last three years. Proposed reviewers should be from different institutions to the authors. You may identify appropriate Editorial Board members of the journal as potential reviewers. You may suggest reviewers from among the authors that you frequently cite in your paper.

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Pharmaceutics accepts submissions that have previously been made available as preprints provided that they have not undergone peer review. A preprint is a draft version of a paper made available online before submission to a journal.

MDPI operates ***Preprints***, a preprint server to which submitted papers can be uploaded directly after completing journal submission. Note that *Preprints* operates independently of the journal and posting a preprint does not affect the peer review process. Check the *Preprints* **instructions for authors** for further information.

Expanded and high-quality conference papers can be considered as articles if they fulfill the following requirements: (1) the paper should be expanded to the size of a research article; (2) the conference paper should be cited and noted on the first page of the paper; (3) if the authors do not hold the copyright of the published conference paper, authors should seek the appropriate permission from the copyright holder; (4) authors are asked to disclose that it is conference paper in their cover letter and include a statement on what has been changed compared to the original conference paper. *Pharmaceutics* does not publish pilot studies or studies with inadequate statistical power.

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MDPI follows the International Committee of Medical Journal Editors (**ICMJE**) guidelines which state that, in order to qualify for authorship of a manuscript, the following criteria should be observed:

- Substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work; AND
- Drafting the work or reviewing it critically for important intellectual content; AND
- Final approval of the version to be published; AND

- Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Those who contributed to the work but do not qualify for authorship should be listed in the acknowledgments. More detailed guidance on authorship is given by the **International Council of Medical Journal Editors (ICMJE)**.

Any change to the author list should be approved by all authors including any who have been removed from the list. The corresponding author should act as a point of contact between the editor and the other authors and should keep co-authors informed and involve them in major decisions about the publication. We reserve the right to request confirmation that all authors meet the authorship conditions.

For more details about authorship please check **MDPI ethics website**.

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Authors can recommend potential reviewers. Journal editors will check to make sure there are no conflicts of interest before contacting those reviewers, and will not consider those with competing interests. Reviewers are asked to declare any conflicts of interest. Authors can also enter the names of potential peer reviewers they wish to exclude from consideration in the peer review of their manuscript, during the initial submission progress. The editorial team will respect these requests so long as this does not interfere with the objective and thorough assessment of the submission.

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Lack of Interference with Editorial Decisions

Editorial independence is of utmost importance and MDPI does not interfere with editorial decisions. All articles published by MDPI are peer reviewed and assessed by our independent editorial boards, and MDPI staff are not involved in decisions to accept manuscripts. When making an editorial decision, we expect the academic editor to make their decision based only upon:

- The suitability of selected reviewers;
- Adequacy of reviewer comments and author response;
- Overall scientific quality of the paper.

In all of our journals, in every aspect of operation, MDPI policies are informed by the mission to make science and research findings open and accessible as widely and rapidly as possible.

Editors and Editorial Staff as Authors

Editorial staff or editors shall not be involved in processing their own academic work. Submissions authored by editorial staff/editors will be assigned to at least two independent outside reviewers. Decisions will be made by other Editorial Board Members who do not have a conflict of interest with the author. Journal staff are not involved in the processing of their own work submitted to any MDPI journals.

Conflicts of Interest

According to The International Committee of Medical Journal Editors, “Authors should avoid entering into agreements with study sponsors, both for-profit and non-profit, that interfere with authors’ access to all of the study’s data or that interfere with their ability to analyze and interpret the data and to prepare and publish manuscripts independently when and where they choose.”

All authors must disclose all relationships or interests that could inappropriately influence or bias their work. Examples of potential conflicts of interest include but are not limited to financial interests (such as membership, employment, consultancies, stocks/shares ownership, honoraria, grants or other funding, paid expert testimonies and patent-licensing arrangements) and non-financial interests (such as personal or professional relationships, affiliations, personal beliefs).

Authors can disclose potential conflicts of interest via the online submission system during the submission process. Declarations regarding conflicts of interest can also be collected via the **MDPI disclosure form**. The corresponding author must include a summary statement in the manuscript in a separate section “Conflicts of Interest” placed just before the reference list. The statement should reflect all the collected potential conflicts of interest disclosures in the form.

See below for examples of disclosures:

Conflicts of Interest: Author A has received research grants from Company A. Author B has received a speaker honorarium from Company X and owns stocks in Company Y. Author C has been involved as a consultant and expert witness in Company Z. Author D is the inventor of patent X.

If no conflicts exist, the authors should state:

Conflicts of Interest: The authors declare no conflicts of interest.

Editorial Procedures and Peer-Review

Pre-check

Immediately after submission, the journal's Managing Editor will perform the technical pre-check to assess:

- Overall suitability of the manuscript to the journal/section/Special Issue;
- Manuscript adherence to high-quality research and ethical standards;
- Standards of rigor to qualify for further review.

The academic editor (i.e., the Editor-in-Chief in the case of regular submissions, the Guest Editor in the case of Special Issue submissions, or an Editorial Board member in the case of a conflict of interest and of regular submissions if the Editor-in-Chief allows) will be notified of the submission and invited to perform an editorial pre-check. During the editorial pre-check phase, the academic editor will assess the suitability of the submission with respect to the scope of the journal, as well as the overall scientific soundness of the manuscript, including the relevance of the references and the correctness of the applied methodology. Academic editors can decide to reject the manuscript, request revisions before peer-review, or continue with the peer-review process and recommend suitable reviewers.

Peer-Review

Once a manuscript passes the initial checks, it will be assigned to at least two independent experts for peer-review. A single-blind review is applied, where authors' identities are known to reviewers. Peer review comments are confidential and will only be disclosed with the express agreement of the reviewer.

In the case of regular submissions, in-house assistant editors will invite experts, including recommendations by an academic editor. These experts may also include *Editorial Board Members* and Guest Editors of the journal. Potential reviewers suggested by the authors may also be considered. Reviewers should not have published with any of the co-authors during the past three years and should not currently work or collaborate with any of the institutions of the co-authors of the submitted manuscript.

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The journal operates optional open peer-review: Authors are given the option for all review reports and editorial decisions to be published alongside their manuscript. In addition, reviewers can sign their review, i.e., identify themselves in the published review reports. Authors can alter their choice for open review at any time before publication, but once the paper has been published changes will only be made at the discretion of the Publisher and Editor-in-Chief. We encourage authors to take advantage of this opportunity as proof of the rigorous process employed in publishing their

research. To guarantee impartial refereeing, the names of referees will be revealed only if the referees agree to do so, and after a paper has been accepted for publication.

Editorial Decision and Revision

All the articles, reviews and communications published in MDPI journals go through the peer-review process and receive at least two reviews. The in-house editor will communicate the decision of the academic editor, which will be one of the following:

- ***Accept after Minor Revisions*:**
  The paper is in principle accepted after revision based on the reviewer's comments. Authors are given five days for minor revisions.
- ***Reconsider after Major Revisions*:**
  The acceptance of the manuscript would depend on the revisions. The author needs to provide a point by point response or provide a rebuttal if some of the reviewer's comments cannot be revised. A maximum of two rounds of major revision per manuscript is normally provided. Authors will be asked to resubmit the revised paper within a suitable time frame, and the revised version will be returned to the reviewer for further comments. If the required revision time is estimated to be longer than 2 months, we will recommend that authors withdraw their manuscript before resubmitting so as to avoid unnecessary time pressure and to ensure that all manuscripts are sufficiently revised.
- ***Reject and Encourage Resubmission*:**
  If additional experiments are needed to support the conclusions, the manuscript will be rejected and the authors will be encouraged to re-submit the paper once further experiments have been conducted.
- ***Reject*:**
  The article has serious flaws, and/or makes no original significant contribution. No offer of resubmission to the journal is provided.

All reviewer comments should be responded to in a point-by-point fashion. Where the authors disagree with a reviewer, they must provide a clear response.

Author Appeals

Authors may appeal a rejection by sending an e-mail to the Editorial Office of the journal. The appeal must provide a detailed justification, including point-by-point responses to the reviewers' and/or Editor's comments using an **appeal form**. Appeals can only be submitted following a "reject and decline resubmission" decision and should be submitted within three months from the decision date. Failure to meet these criteria will result in the appeal not being considered further. The *Managing Editor* will forward the manuscript and related information (including the identities of the referees) to a designated *Editorial Board Member*. The Academic Editor being consulted

will be asked to provide an advisory recommendation on the manuscript and may recommend acceptance, further peer-review, or uphold the original rejection decision. This decision will then be validated by the *Editor-in-Chief*. A reject decision at this stage is final and cannot be reversed.

Production and Publication

Once accepted, the manuscript will undergo professional copy-editing, English editing, proofreading by the authors, final corrections, pagination, and, publication on the **www.mdpi.com** website.

Promoting Equity, Diversity, and Inclusiveness within MDPI Journals

Our Managing Editors encourage the Editors-in-Chief and Associate Editors to appoint diverse expert Editorial Boards. This is also reflective in our multi-national and inclusive workplace. We are proud to create equal opportunities without regard to gender, ethnicity, sexual orientation, age, religion, or socio-economic status. There is no place for discrimination in our workplace and editors of MDPI journals are to uphold these principles in high regard.

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To improve the reproducibility of scientific research, the **Resource Identification Initiative** aims to provide unique persistent identifiers for key biological resources, including antibodies, cell lines, model organisms and tools.

We encourage authors to include unique identifiers - RRIDs- provided by the **Resource Identification Portal** in the dedicated section of the manuscript.

To help authors quickly find the correct identifiers for their materials, there is a single **website** where all resource types can be found and a ‘cite this’ button next to each resource, that contains a proper citation text that should be included in the methods section of the manuscript.

Annexure L

Instructions for Authors for '*Pharmaceuticals*'

Submission Checklist

Please:

1. Read the **Aims & Scope** to gain an overview and assess if your manuscript is suitable for this journal;
2. Use the **Microsoft Word template** or **LaTeX template** to prepare your manuscript;
3. Make sure that issues about **publication ethics**, **research ethics**, **copyright**, **authorship**, **figure formats**, **data** and **references format** have been appropriately considered;
4. Ensure that all authors have approved the content of the submitted manuscript.
5. Authors are encouraged to add a **biography** (optional) to the submission and post it to **SciProfiles**.

Manuscript Submission Overview

Types of Publications

Full experimental details must be provided so that the results can be reproduced. *Pharmaceuticals* requires that authors publish all experimental controls and make full datasets available where possible (see the guidelines on **Supplementary Materials** and references to unpublished data).

Manuscripts submitted to *Pharmaceuticals* should neither be published previously nor be under consideration for publication in another journal. The main article types are listed below and a comprehensive list of article types can be found **here.**

- *Article:* These are original research manuscripts. The work should report scientifically sound experiments and provide a substantial amount of new information. The article should include the most recent and relevant references in the field. The structure should include an Abstract, Keywords, Introduction, Materials and Methods, Results, Discussion, and Conclusions (optional) sections, with a suggested minimum word count of 4000 words.

- *Review:* Reviews offer a comprehensive analysis of the existing literature within a field of study, identifying current gaps or problems. They should be critical and constructive and provide recommendations for future research. No new, unpublished data should be presented. The structure can include an Abstract, Keywords, Introduction, Relevant Sections, Discussion, Conclusions, and Future Directions, with a suggested minimum word count of 4000 words.

Submission Process

Manuscripts for *Pharmaceuticals* should be submitted online at **susy.mdpi.com**. The submitting author, who is generally the corresponding author, is responsible for the manuscript during the submission and peer-review process. The submitting author must ensure that all eligible co-authors have been included in the author list (read the **criteria to qualify for authorship**) and that they have all read and approved the submitted version of the manuscript. To submit your manuscript, register and log in to the **submission website**. Once you have registered, **click here to go to the submission form for *Pharmaceuticals***. All co-authors can see the manuscript details in the submission system, if they register and log in using the e-mail address provided during manuscript submission.

Accepted File Formats

Authors are encouraged to use the **Microsoft Word template** or **LaTeX template** to prepare their manuscript. Using the template file will substantially shorten the time to complete copy-editing and publication of accepted manuscripts. The total amount of data for all files must not exceed 120 MB. If this is a problem, please contact the Editorial Office **pharmaceuticals@mdpi.com**. Accepted file formats are:

- *Microsoft Word:* Manuscripts prepared in Microsoft Word must be converted into a single file before submission. When preparing manuscripts in Microsoft Word, we encourage you to use the ***Pharmaceuticals* Microsoft Word template file**. Please insert your graphics (schemes, figures, *etc.*) in the main text after the paragraph of its first citation.
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- *Supplementary files:* May be any format, but it is recommended that you use common, non-proprietary formats where possible (see **below** for further details).

Disclaimer: Usage of these templates is exclusively intended for submission to the journal for peer-review, and strictly limited to this purpose and it cannot be used for posting online on preprint servers or other websites.

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- We do not have strict formatting requirements, but all manuscripts must contain the required sections: Author Information, Abstract, Keywords, Introduction, Materials & Methods, Results, Conclusions, Figures and Tables with Captions, Funding Information, Author Contributions, Conflict of Interest and other Ethics Statements. Check the Journal ***Instructions for Authors*** for more details.
- Your references may be in any style, provided that you use the consistent formatting throughout. It is essential to include author(s) name(s), journal or book title, article or chapter title (where required), year of publication, volume and issue (where appropriate) and pagination. DOI numbers (Digital Object Identifier) are not mandatory but highly encouraged. The bibliography software package *EndNote*, ***Zotero***, *Mendeley*, *Reference Manager* are recommended.
- When your manuscript reaches the revision stage, you will be requested to format the manuscript according to the journal guidelines.

Cover Letter

A cover letter must be included with each manuscript submission. It should be concise and explain why the content of the paper is significant, placing the findings in the context of existing work. It should explain why the manuscript fits the scope of the journal.

Any prior submissions of the manuscript to MDPI journals must be acknowledged. If this is the case, it is strongly recommended that the previous manuscript ID is provided in the submission system, which will ease your current submission process. The names of proposed and excluded reviewers should be provided in the submission system, not in the cover letter.

All cover letters are required to include the statements:

- We confirm that neither the manuscript nor any parts of its content are currently under consideration or published in another journal.
- All authors have approved the manuscript and agree with its submission to (journal name).

Author Identification

Authors are encouraged to add a biography (maximum 150 words) to the submission and upload it to **SciProfiles**. This should be a single paragraph and should contain the following points:

1. Authors' full names followed by current positions;

2. Education background including institution information and year of graduation (type and level of degree received);
3. Work experience;
4. Current and previous research interests;
5. Memberships of professional societies and awards received.

If a manuscript is accepted for publication, we will add an icon linking to your online **ORCID** profile in the final version of the published paper.

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All authors should list their current affiliation and the affiliation where most research was carried out for the preparation of their manuscript. We recommend adding as primary the affiliation where most of the research was conducted or supported, but please check with your institution for any contractual agreement requirements.

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Independent Researcher

If one or all the authors are not currently affiliated with a university, institution or company, or have not been during the development of the manuscript, they should list themselves as an "Independent Researcher".

Manuscript Preparation

General Considerations

- **Research manuscripts** should comprise:
    - **Front matter**: Title, Author list, Affiliations, Abstract, Keywords.
    - **Research manuscript sections**: Introduction, Results, Discussion, Materials and Methods, Conclusions (optional).
    - **Back matter**: Supplementary Materials, Acknowledgments, Author Contributions, Conflicts of Interest, **References**.
- **Review manuscripts** should comprise the **front matter**, literature review sections and the **back matter**. The template file can also be used to prepare the front and back matter of your review manuscript. It is not necessary to follow the remaining structure. Structured reviews and meta-analyses should use the same structure as research articles and ensure they conform to the **PRISMA** guidelines.

- **Case reports** should include a succinct introduction about the general medical condition or relevant symptoms that will be discussed in the case report; the case presentation including all of the relevant de-identified demographic and descriptive information about the patient(s), and a description of the symptoms, diagnosis, treatment, and outcome; a discussion providing context and any necessary explanation of specific treatment decisions; a conclusion briefly outlining the take-home message and the lessons learned.
- **Graphical Abstract:** A graphical abstract (GA) is an image that appears alongside the text abstract in the Table of Contents. In addition to summarizing the content, it should represent the topic of the article in an attention-grabbing way. Moreover, it should not be exactly the same as the Figure in the paper or just a simple superposition of several subfigures. Note that the GA must be original and unpublished artwork. Any postage stamps, currency from any country, or trademarked items should not be included in it.

The GA should be a high-quality illustration or diagram in any of the following formats: PNG, JPEG, or TIFF. Written text in a GA should be clear and easy to read, using one of the following fonts: Times, Arial, Courier, Helvetica, Ubuntu or Calibri.

The minimum required size for the GA is 560 × 1100 pixels (height × width). The size should be of high quality in order to reproduce well.

- **Acronyms/Abbreviations/Initialisms** should be defined the first time they appear in each of three sections: the abstract; the main text; the first figure or table. When defined for the first time, the acronym/abbreviation/initialism should be added in parentheses after the written-out form.
- **SI Units** (International System of Units) should be used. Imperial, US customary and other units should be converted to SI units whenever possible.
- **Accession numbers** of RNA, DNA and protein sequences used in the manuscript should be provided in the Materials and Methods section. Also see the section on **Deposition of Sequences and Expression Data**.
- **Equations:** If you are using Word, please use either the Microsoft Equation Editor or the MathType add-on. Equations should be editable by the editorial office and not appear in a picture format.
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- **Preregistration:** Where authors have preregistered studies or analysis plans, links to the preregistration must be provided in the manuscript.
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Front Matter

These sections should appear in all manuscript types

- **Title:** The title of your manuscript should be concise, specific and relevant. It should identify if the study reports (human or animal) trial data, or is a systematic review, meta-analysis or replication study. When gene or protein names are included, the abbreviated name rather than full name should be used. Please do not include abbreviated or short forms of the title, such as a running title or head. These will be removed by our Editorial Office.
- **Author List and Affiliations:** Authors' full first and last names must be provided. The initials of any middle names can be added. The PubMed/MEDLINE standard format is used for affiliations: complete address information including city, zip code, state/province, and country. At least one author should be designated as the corresponding author. The email addresses of all authors will be displayed on published papers, and hidden by Captcha on the website as standard. It is the responsibility of the corresponding author to ensure that consent for the display of email addresses is obtained from all authors. If an author (other than the corresponding author) does not wish to have their email addresses displayed in this way, the corresponding author must indicate as such during proofreading. After acceptance, updates to author names or affiliations may not be permitted. Equal Contributions: authors who have contributed equally should be marked with a superscript symbol (†). The symbol must be included below the affiliations, and the following statement added: “These authors contributed equally to this work”. The equal roles of authors should also be adequately disclosed in the author contributions statement. Please read the criteria to qualify for authorship.
- **Abstract:** The abstract should be a total of about 200 words maximum. The abstract should be a single paragraph and should follow the style of structured abstracts, but without headings: 1) Background: Place the question addressed in a broad context and highlight the purpose of the study; 2) Methods: Describe briefly the main methods or treatments applied. Include any relevant preregistration numbers, and species and strains of any animals used; 3) Results: Summarize the article's main findings; and 4) Conclusion: Indicate the main conclusions or interpretations. The abstract should be an objective representation of the article: it must not contain results which are not presented and substantiated in the main text and should not exaggerate the main conclusions.

- **Keywords:** Three to ten pertinent keywords need to be added after the abstract. We recommend that the keywords are specific to the article, yet reasonably common within the subject discipline.

Research Manuscript Sections

- **Introduction:** The introduction should briefly place the study in a broad context and highlight why it is important. It should define the purpose of the work and its significance, including specific hypotheses being tested. The current state of the research field should be reviewed carefully and key publications cited. Please highlight controversial and diverging hypotheses when necessary. Finally, briefly mention the main aim of the work and highlight the main conclusions. Keep the introduction comprehensible to scientists working outside the topic of the paper.
- **Results:** Provide a concise and precise description of the experimental results, their interpretation as well as the experimental conclusions that can be drawn.
- **Discussion:** Authors should discuss the results and how they can be interpreted in perspective of previous studies and of the working hypotheses. The findings and their implications should be discussed in the broadest context possible and limitations of the work highlighted. Future research directions may also be mentioned. This section may be combined with Results.
- **Materials and Methods:** They should be described with sufficient detail to allow others to replicate and build on published results. New methods and protocols should be described in detail while well-established methods can be briefly described and appropriately cited. Give the name and version of any software used and make clear whether computer code used is available. Include any pre-registration codes.
- **Conclusions:** This section is not mandatory but can be added to the manuscript if the discussion is unusually long or complex.
- **Patents:** This section is not mandatory but may be added if there are patents resulting from the work reported in this manuscript.

Back Matter

- **Supplementary Materials:** Describe any supplementary material published online alongside the manuscript (figure, tables, video, spreadsheets, etc.). Please indicate the name and title of each element as follows Figure S1: title, Table S1: title, etc.
- **Author Contributions:** Each author is expected to have made substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data; or the creation of new software used in the work; or have drafted the work or substantively revised it; AND has approved the submitted version (and version

substantially edited by journal staff that involves the author's contribution to the study); AND agrees to be personally accountable for the author's own contributions and for ensuring that questions related to the accuracy or integrity of any part of the work, even ones in which the author was not personally involved, are appropriately investigated, resolved, and documented in the literature.
For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used "Conceptualization, X.X. and Y.Y.; Methodology, X.X.; Software, X.X.; Validation, X.X., Y.Y. and Z.Z.; Formal Analysis, X.X.; Investigation, X.X.; Resources, X.X.; Data Curation, X.X.; Writing – Original Draft Preparation, X.X.; Writing – Review & Editing, X.X.; Visualization, X.X.; Supervision, X.X.; Project Administration, X.X.; Funding Acquisition, Y.Y.", please turn to the **CRediT taxonomy** for the term explanation. For more background on CRediT, see **here**. "**Authorship must include and be limited to those who have contributed substantially to the work. Please read the section concerning the criteria to qualify for authorship carefully**".

- **Funding:** All sources of funding of the study should be disclosed. Clearly indicate grants that you have received in support of your research work and if you received funds to cover publication costs. Note that some funders will not refund article processing charges (APC) if the funder and grant number are not clearly and correctly identified in the paper. Funding information can be entered separately into the submission system by the authors during submission of their manuscript. Such funding information, if available, will be deposited to FundRef if the manuscript is finally published. Please add: "This research received no external funding" or "This research was funded by [name of funder] grant number [xxx]" and "The APC was funded by [XXX]" in this section. Check carefully that the details given are accurate and use the standard spelling of funding agency names at https://search.crossref.org/funding, any errors may affect your future funding.
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- **Informed Consent Statement:** Any research article describing a study involving humans should contain this statement. Please add "Informed consent was obtained from all

subjects involved in the study." OR "Patient consent was waived due to REASON (please provide a detailed justification)." OR "Not applicable" for studies not involving humans. You might also choose to exclude this statement if the study did not involve humans. Written informed consent for publication must be obtained from participating patients who can be identified (including by the patients themselves). Please state "Written informed consent has been obtained from the patient(s) to publish this paper" if applicable.

- **Data Availability Statement:** In this section, please provide details regarding where data supporting reported results can be found, including links to publicly archived datasets analyzed or generated during the study. Please refer to suggested Data Availability Statements in section "**MDPI Research Data Policies**". You might choose to exclude this statement if the study did not report any data.
- **Acknowledgments:** In this section you can acknowledge any support given which is not covered by the author contribution or funding sections. This may include administrative and technical support, or donations in kind (e.g., materials used for experiments).
- **Conflicts of Interest:** Authors must identify and declare any personal circumstances or interest that may be perceived as influencing the representation or interpretation of reported research results. If there is no conflict of interest, please state "The authors declare no conflict of interest." Any role of the funding sponsors in the choice of research project; design of the study; in the collection, analyses or interpretation of data; in the writing of the manuscript; or in the decision to publish the results must be declared in this section. *Pharmaceuticals* does not publish studies funded partially or fully by the tobacco industry. Any projects funded by industry must pay special attention to the full declaration of funder involvement. If there is no role, please state "The sponsors had no role in the design, execution, interpretation, or writing of the study". For more details please see **Conflict of Interest**.
- **References:** References must be numbered in order of appearance in the text (including table captions and figure legends) and listed individually at the end of the manuscript. We recommend preparing the references with a bibliography software package, such as **EndNote**, **ReferenceManager** or **Zotero** to avoid typing mistakes and duplicated references. We encourage citations to data, computer code and other citable research material. If available online, you may use reference style 9. below.
- Citations and References in Supplementary files are permitted provided that they also appear in the main text and in the reference list.

In the text, reference numbers should be placed in square brackets [ ], and placed before the punctuation; for example [1], [1–3] or [1,3]. For embedded citations in the text with pagination,

use both parentheses and brackets to indicate the reference number and page numbers; for example [5] (p. 10). or [6] (pp. 101–105).

The reference list should include the full title, as recommended by the ACS style guide. Style files for **Endnote** and **Zotero** are available.

References should be described as follows, depending on the type of work:

Journal Articles:

1. Author 1, A.B.; Author 2, C.D. Title of the article. *Abbreviated Journal Name* **Year**, *Volume*, page range.

Books and Book Chapters:

2. Author 1, A.; Author 2, B. *Book Title*, 3rd ed.; Publisher: Publisher Location, Country, Year; pp. 154–196.

3. Author 1, A.; Author 2, B. Title of the chapter. In *Book Title*, 2nd ed.; Editor 1, A., Editor 2, B., Eds.; Publisher: Publisher Location, Country, Year; Volume 3, pp. 154–196.

Unpublished materials intended for publication:

4. Author 1, A.B.; Author 2, C. Title of Unpublished Work (optional). Correspondence Affiliation, City, State, Country. year, *status* (*manuscript in preparation*; *to be submitted*).

5. Author 1, A.B.; Author 2, C. Title of Unpublished Work. *Abbreviated Journal Name* year, *phrase indicating stage of publication* (*submitted*; *accepted*; *in press*).

Unpublished materials not intended for publication:

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7. Author 1, A.B.; Author 2, C.D.; Author 3, E.F. Title of Presentation. In *Title of the Collected Work* (if available), Proceedings of the Name of the Conference, Location of Conference, Country, Date of Conference; Editor 1, Editor 2, Eds. (if available); Publisher: City, Country, Year (if available); Abstract Number (optional), Pagination (optional).

Thesis:

8. Author 1, A.B. Title of Thesis. Level of Thesis, Degree-Granting University, Location of University, Date of Completion.

Websites:

9. Title of Site. Available online: URL (accessed on Day Month Year).

Unlike published works, websites may change over time or disappear, so we encourage you create an archive of the cited website using a service such as **WebCite**. Archived websites should be cited using the link provided as follows:

10. Title of Site. URL (archived on Day Month Year).

See the **Reference List and Citations Guide** for more detailed information.

Preparing Figures, Schemes and Tables

- File for Figures and Schemes must be provided during submission in a single zip archive and at a sufficiently high resolution (minimum 1000 pixels width/height, or a resolution of 300 dpi or higher). Common formats are accepted, however, TIFF, JPEG, EPS and PDF are preferred.
- *Pharmaceuticals* can publish multimedia files in articles or as supplementary materials. Please contact the editorial office for further information.
- All Figures, Schemes and Tables should be inserted into the main text close to their first citation and must be numbered following their number of appearance (Figure 1, Scheme I, Figure 2, Scheme II, Table 1, *etc.*).
- All Figures, Schemes and Tables should have a short explanatory title and caption.
- All table columns should have an explanatory heading. To facilitate the copy-editing of larger tables, smaller fonts may be used, but no less than 8 pt. in size. Authors should use the Table option of Microsoft Word to create tables.
- Authors are encouraged to prepare figures and schemes in color (RGB at 8-bit per channel). There is no additional cost for publishing full color graphics.

Original Images for Blots and Gels Requirements

For the main text, please ensure that:

- All experimental samples and controls used for one comparative analysis are run on the same blot/gel.
- Image processing methods, such as adjusting the brightness or contrast, do not alter or distort the information in the figure and are applied to every pixel. High-contrast blots/gels are discouraged.
- Cropped blots/gels present in the main text retain all important information and bands.
- You have checked figures for duplications and ensured the figure legends are clear and accurate. Please include all relevant information in the figure legends and clearly indicate any re-arrangement of lanes.

In order to ensure the integrity and scientific validity of blots (including, but not limited to, Western blots) and the reporting of gel data, original, uncropped and unadjusted images should be uploaded as Supporting Information files at the time of initial submission.

A single PDF file or a zip folder including all the original images reported in the main figure and supplemental figures should be prepared. Authors should annotate each original image, corresponding to the figure in the main article or supplementary materials, and label each lane or loading order. All experimental samples and controls used for one comparative analysis should be run on the same blot/gel image. For quantitative analyses, please provide the blots/gels for each independent biological replicate used in the analysis.

Supplementary Materials, Data Deposit and Software Source Code

MDPI Research Data Policies

MDPI is committed to supporting open scientific exchange and enabling our authors to achieve best practices in sharing and archiving research data. We encourage all authors of articles published in MDPI journals to share their research data. Individual journal guidelines can be found at the journal 'Instructions for Authors' page. Data sharing policies concern the minimal dataset that supports the central findings of a published study. Generated data should be publicly available and cited in accordance with journal guidelines.

MDPI data policies are informed by **TOP Guidelines** and **FAIR Principles**.

Where ethical, legal or privacy issues are present, data should not be shared. The authors should make any limitations clear in the Data Availability Statement upon submission. Authors should ensure that data shared are in accordance with consent provided by participants on the use of confidential data.

Data Availability Statements provide details regarding where data supporting reported results can be found, including links to publicly archived datasets analyzed or generated during the study.

Below are suggested Data Availability Statements:

- Data available in a publicly accessible repository. The data presented in this study are openly available in [repository name e.g., FigShare] at [**doi**], reference number [reference number].
- Data available in a publicly accessible repository that does not issue DOIs Publicly available datasets were analyzed in this study. This data can be found here: [link/accession number]

- Data available on request due to restrictions eg privacy or ethical. The data presented in this study are available on request from the corresponding author. The data are not publicly available due to [insert reason here]
- 3rd Party Data
  Restrictions apply to the availability of these data. Data was obtained from [third party] and are available [from the authors/at URL] with the permission of [third party].
- Data sharing not applicable
  No new data were created or analyzed in this study. Data sharing is not applicable to this article.
- Data is contained within the article or supplementary material
  The data presented in this study are available in [insert article or supplementary material here]

Data citation:

- [dataset] Authors. Year. Dataset title; Data repository or archive; Version (if any); Persistent identifier (e.g., DOI).

Computer Code and Software

For work where novel computer code was developed, authors should release the code either by depositing in a recognized, public repository such as **GitHub** or uploading as supplementary information to the publication. The name, version, corporation and location information for all software used should be clearly indicated. Please include all the parameters used to run software/programs analyses.

Supplementary Material

Additional data and files can be uploaded as "Supplementary Files" during the manuscript submission process. The supplementary files will also be available to the referees as part of the peer-review process. Any file format is acceptable; however, we recommend that common, non-proprietary formats are used where possible. For more information on supplementary materials, please refer to **https://www.mdpi.com/authors/layout#_bookmark83**.

References in Supplementary Files

Citations and References in Supplementary files are permitted provided that they also appear in the reference list of the main text.

Unpublished Data

Restrictions on data availability should be noted during submission and in the manuscript. "Data not shown" should be avoided: authors are encouraged to publish all observations related to the submitted manuscript as Supplementary Material. "Unpublished data" intended for publication in

a manuscript that is either planned, "in preparation" or "submitted" but not yet accepted, should be cited in the text and a reference should be added in the References section. "Personal Communication" should also be cited in the text and reference added in the References section. (see also the MDPI reference list and citations style guide).

Remote Hosting and Large Data Sets

Data may be deposited with specialized service providers or institutional/subject repositories, preferably those that use the DataCite mechanism. Large data sets and files greater than 60 MB must be deposited in this way. For a list of other repositories specialized in scientific and experimental data, please consult **databib.org** or **re3data.org**. The data repository name, link to the data set (URL) and accession number, doi or handle number of the data set must be provided in the paper. The journal **Data** also accepts submissions of data set papers.

Deposition of Sequences and Expression Data

New sequence information must be deposited to the appropriate database prior to submission of the manuscript. Accession numbers provided by the database should be included in the submitted manuscript. Manuscripts will not be published until the accession number is provided.

- *New nucleic acid sequences* must be deposited into an acceptable repository such as **GenBank**, **EMBL**, or **DDBJ**. Sequences should be submitted to only one database.
- *New high throughput sequencing (HTS) datasets* (RNA-seq, ChIP-Seq, degradome analysis, …) must be deposited either in the **GEO database** or in the NCBI's **Sequence Read Archive (SRA)**.
- *New microarray data* must be deposited either in the **GEO** or the **ArrayExpress** databases.The "Minimal Information About a Microarray Experiment" (MIAME) guidelines published by the Microarray Gene Expression Data Society must be followed.
- *New protein sequences* obtained by protein sequencing must be submitted to UniProt (submission tool **SPIN**). Annotated protein structure and its reference sequence must be submitted to **RCSB of Protein Data Bank**.

All sequence names and the accession numbers provided by the databases must be provided in the Materials and Methods section of the article.

Deposition of Proteomics Data

Methods used to generate the proteomics data should be described in detail and we encourage authors to adhere to the "**Minimum Information About a Proteomics Experiment**". All generated mass spectrometry raw data must be deposited in the appropriate public database such as **ProteomeXchange**, **PRIDE** or **jPOST**. At the time of submission, please include all

relevant information in the materials and methods section, such as repository where the data was submitted and link, data set identifier, username and password needed to access the data.

Research and Publication Ethics

Research Ethics

Research Involving Human Subjects

When reporting on research that involves human subjects, human material, human tissues, or human data, authors must declare that the investigations were carried out following the rules of the Declaration of Helsinki of 1975 (**https://www.wma.net/what-we-do/medical-ethics/declaration-of-helsinki/**), revised in 2013. According to point 23 of this declaration, an approval from the local institutional review board (IRB) or other appropriate ethics committee must be obtained before undertaking the research to confirm the study meets national and international guidelines. As a minimum, a statement including the project identification code, date of approval, and name of the ethics committee or institutional review board must be stated in Section 'Institutional Review Board Statement' of the article.

Example of an ethical statement: "All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of XXX (Project identification code)."

For non-interventional studies (e.g. surveys, questionnaires, social media research), all participants must be fully informed if the anonymity is assured, why the research is being conducted, how their data will be used and if there are any risks associated. As with all research involving humans, ethical approval from an appropriate ethics committee must be obtained prior to conducting the study. If ethical approval is not required, authors must either provide an exemption from the ethics committee or are encouraged to cite the local or national legislation that indicates ethics approval is not required for this type of study. Where a study has been granted exemption, the name of the ethics committee which provided this should be stated in Section 'Institutional Review Board Statement' with a full explanation regarding why ethical approval was not required.

A written informed consent for publication must be obtained from participating patients. Data relating to individual participants must be described in detail, but private information identifying participants need not be included unless the identifiable materials are of relevance to the research (for example, photographs of participants' faces that show a particular symptom). Patients' initials or other personal identifiers must not appear in any images. For manuscripts that include any case details, personal information, and/or images of patients, authors must obtain signed

informed consent for publication from patients (or their relatives/guardians) before submitting to an MDPI journal. Patient details must be anonymized as far as possible, e.g., do not mention specific age, ethnicity, or occupation where they are not relevant to the conclusions. A **template permission form** is available to download. A blank version of the form used to obtain permission (without the patient names or signature) must be uploaded with your submission. Editors reserve the right to reject any submission that does not meet these requirements.

You may refer to our sample form and provide an appropriate form after consulting with your affiliated institution. For the purposes of publishing in MDPI journals, a consent, permission, or release form should include unlimited permission for publication in all formats (including print, electronic, and online), in sublicensed and reprinted versions (including translations and derived works), and in other works and products under open access license. To respect patients' and any other individual's privacy, please do not send signed forms. The journal reserves the right to ask authors to provide signed forms if necessary.

If the study reports research involving vulnerable groups, an additional check may be performed. The submitted manuscript will be scrutinized by the editorial office and upon request, documentary evidence (blank consent forms and any related discussion documents from the ethics board) must be supplied. Additionally, when studies describe groups by race, ethnicity, gender, disability, disease, etc., explanation regarding why such categorization was needed must be clearly stated in the article.

Ethical Guidelines for the Use of Animals in Research

The editors will require that the benefits potentially derived from any research causing harm to animals are significant in relation to any cost endured by animals, and that procedures followed are unlikely to cause offense to the majority of readers. Authors should particularly ensure that their research complies with the commonly-accepted '3Rs [1]':

- Replacement of animals by alternatives wherever possible,
- Reduction in number of animals used, and
- Refinement of experimental conditions and procedures to minimize the harm to animals.

Authors must include details on housing, husbandry and pain management in their manuscript.

For further guidance authors should refer to the Code of Practice for the Housing and Care of Animals Used in Scientific Procedures [2], American Association for Laboratory Animal Science [3] or European Animal Research Association [4].

If national legislation requires it, studies involving vertebrates or higher invertebrates must only be carried out after obtaining approval from the appropriate ethics committee. As a minimum, the

project identification code, date of approval and name of the ethics committee or institutional review board should be stated in Section 'Institutional Review Board Statement'. Research procedures must be carried out in accordance with national and institutional regulations. Statements on animal welfare should confirm that the study complied with all relevant legislation. Clinical studies involving animals and interventions outside of routine care require ethics committee oversight as per the American Veterinary Medical Association. If the study involved client-owned animals, informed client consent must be obtained and certified in the manuscript report of the research. Owners must be fully informed if there are any risks associated with the procedures and that the research will be published. If available, a high standard of veterinary care must be provided. Authors are responsible for correctness of the statements provided in the manuscript.

If ethical approval is not required by national laws, authors must provide an exemption from the ethics committee, if one is available. Where a study has been granted exemption, the name of the ethics committee that provided this should be stated in Section 'Institutional Review Board Statement' with a full explanation on why the ethical approval was not required.

If no animal ethics committee is available to review applications, authors should be aware that the ethics of their research will be evaluated by reviewers and editors. Authors should provide a statement justifying the work from an ethical perspective, using the same utilitarian framework that is used by ethics committees. Authors may be asked to provide this even if they have received ethical approval.

MDPI endorses the ARRIVE guidelines (**arriveguidelines.org/**) for reporting experiments using live animals. Authors and reviewers must use the ARRIVE guidelines as a checklist, which can be found at **https://arriveguidelines.org/sites/arrive/files/documents/ARRIVE%20Compliance%20Questionnaire.pdf**. Editors reserve the right to ask for the checklist and to reject submissions that do not adhere to these guidelines, to reject submissions based on ethical or animal welfare concerns or if the procedure described does not appear to be justified by the value of the work presented.

1. NSW Department of Primary Industries and Animal Research Review Panel. Three Rs. Available online: **https://www.animalethics.org.au/three-rs**
2. Home Office. Animals (Scientific Procedures) Act 1986. Code of Practice for the Housing and Care of Animals Bred, Supplied or Used for Scientific Purposes. Available online: **https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/388535/CoPanimalsWeb.pdf**

3. American Association for Laboratory Animal Science. The Scientific Basis for Regulation of Animal Care and Use. Available online: **https://www.aalas.org/about-aalas/position-papers/scientific-basis-for-regulation-of-animal-care-and-use**
4. European Animal Research Association. EU regulations on animal research. Available online: **https://www.eara.eu/animal-research-law**

Research Involving Cell Lines

Methods sections for submissions reporting on research with cell lines should state the origin of any cell lines. For established cell lines the provenance should be stated and references must also be given to either a published paper or to a commercial source. If previously unpublished *de novo* cell lines were used, including those gifted from another laboratory, details of institutional review board or ethics committee approval must be given, and confirmation of written informed consent must be provided if the line is of human origin.

An example of Ethical Statements:

The HCT116 cell line was obtained from XXXX. The $MLH1^{+}$ cell line was provided by XXXXX, Ltd. The DLD-1 cell line was obtained from Dr. XXXX. The DR-GFP and SA-GFP reporter plasmids were obtained from Dr. XXX and the Rad51K133A expression vector was obtained from Dr. XXXX.

Research Involving Plants

Experimental research on plants (either cultivated or wild) including collection of plant material, must comply with institutional, national, or international guidelines. We recommend that authors comply with the **Convention on Biological Diversity** and the **Convention on the Trade in Endangered Species of Wild Fauna and Flora**.

For each submitted manuscript supporting genetic information and origin must be provided. For research manuscripts involving rare and non-model plants (other than, e.g., *Arabidopsis thaliana, Nicotiana benthamiana, Oryza sativa*, or many other typical model plants), voucher specimens must be deposited in an accessible herbarium or museum. Vouchers may be requested for review by future investigators to verify the identity of the material used in the study (especially if taxonomic rearrangements occur in the future). They should include details of the populations sampled on the site of collection (GPS coordinates), date of collection, and document the part(s) used in the study where appropriate. For rare, threatened or endangered species this can be waived but it is necessary for the author to describe this in the cover letter.

Editors reserve the rights to reject any submission that does not meet these requirements.

An example of Ethical Statements:

Torenia fournieri plants were used in this study. White-flowered Crown White (CrW) and violet-flowered Crown Violet (CrV) cultivars selected from 'Crown Mix' (XXX Company, City, Country) were kindly provided by Dr. XXX (XXX Institute, City, Country).

Arabidopis mutant lines (SALKxxxx, SAILxxxx,…) were kindly provided by Dr. XXX, institute, city, country).

Clinical Trials Registration

Registration

MDPI follows the International Committee of Medical Journal Editors (ICMJE) **guidelines** which require and recommend registration of clinical trials in a public trials registry at or before the time of first patient enrollment as a condition of consideration for publication.

Purely observational studies do not require registration. A clinical trial not only refers to studies that take place in a hospital or involve pharmaceuticals, but also refer to all studies which involve participant randomization and group classification in the context of the intervention under assessment.

Authors are strongly encouraged to pre-register clinical trials with an international clinical trials register and cite a reference to the registration in the Methods section. Suitable databases include **clinicaltrials.gov**, **the EU Clinical Trials Register** and those listed by the World Health Organisation **International Clinical Trials Registry Platform**.

Approval to conduct a study from an independent local, regional, or national review body is not equivalent to prospective clinical trial registration. MDPI reserves the right to decline any paper without trial registration for further peer-review. However, if the study protocol has been published before the enrolment, the registration can be waived with correct citation of the published protocol.

CONSORT Statement

MDPI requires a completed CONSORT 2010 **checklist** and **flow diagram** as a condition of submission when reporting the results of a randomized trial. Templates for these can be found here or on the CONSORT website (**http://www.consort-statement.org**) which also describes several CONSORT checklist extensions for different designs and types of data beyond two group parallel trials. At minimum, your article should report the content addressed by each item of the checklist.

Sex and Gender in Research

We encourage our authors to follow the **'Sex and Gender Equity in Research – SAGER – guidelines'** and to include sex and gender considerations where relevant. Authors should use

the terms sex (biological attribute) and gender (shaped by social and cultural circumstances) carefully in order to avoid confusing both terms. Article titles and/or abstracts should indicate clearly what sex(es) the study applies to. Authors should also describe in the background, whether sex and/or gender differences may be expected; report how sex and/or gender were accounted for in the design of the study; provide disaggregated data by sex and/or gender, where appropriate; and discuss respective results. If a sex and/or gender analysis was not conducted, the rationale should be given in the Discussion. We suggest that our authors consult the full **guidelines** before submission.

Borders and Territories

Potential disputes over borders and territories may have particular relevance for authors in describing their research or in an author or editor correspondence address, and should be respected. Content decisions are an editorial matter and where there is a potential or perceived dispute or complaint, the editorial team will attempt to find a resolution that satisfies parties involved.

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Publication Ethics Statement

Pharmaceuticals is a member of the Committee on Publication Ethics (**COPE**). We fully adhere to its **Code of Conduct** and to its **Best Practice Guidelines**.

The editors of this journal enforce a rigorous peer-review process together with strict ethical policies and standards to ensure to add high quality scientific works to the field of scholarly publication. Unfortunately, cases of plagiarism, data falsification, image manipulation, inappropriate authorship credit, and the like, do arise. The editors of *Pharmaceuticals* take such publishing ethics issues very seriously and are trained to proceed in such cases with a zero tolerance policy.

Authors wishing to publish their papers in *Pharmaceuticals* must abide to the following:

- Any facts that might be perceived as a possible conflict of interest of the author(s) must be disclosed in the paper prior to submission.
- Authors should accurately present their research findings and include an objective discussion of the significance of their findings.
- Data and methods used in the research need to be presented in sufficient detail in the paper, so that other researchers can replicate the work.
- Raw data should preferably be publicly deposited by the authors before submission of their manuscript. Authors need to at least have the raw data readily available for

presentation to the referees and the editors of the journal, if requested. Authors need to ensure appropriate measures are taken so that raw data is retained in full for a reasonable time after publication.

- Simultaneous submission of manuscripts to more than one journal is not tolerated.
- The journal accepts exact translations of previously published work. All submissions of translations must conform with our **policies on translations**.
- If errors and inaccuracies are found by the authors after publication of their paper, they need to be promptly communicated to the editors of this journal so that appropriate actions can be taken. Please refer to our **policy regarding Updating Published Papers**.
- Your manuscript should not contain any information that has already been published. If you include already published figures or images, please obtain the necessary permission from the copyright holder to publish under the CC-BY license. For further information, see the **Rights and Permissions** page.
- Plagiarism, data fabrication and image manipulation are not tolerated.
    - **Plagiarism is not acceptable** in *Pharmaceuticals* submissions.

Plagiarism includes copying text, ideas, images, or data from another source, even from your own publications, without giving any credit to the original source.

Reuse of text that is copied from another source must be between quotes and the original source must be cited. If a study's design or the manuscript's structure or language has been inspired by previous works, these works must be explicitly cited.

All MDPI submissions are checked for plagiarism using the industry standard software iThenticate. If plagiarism is detected during the peer review process, the manuscript may be rejected. If plagiarism is detected after publication, an investigation will take place and action taken in accordance with our policies.

- **Image files must not be manipulated or adjusted in any way** that could lead to misinterpretation of the information provided by the original image.

Irregular manipulation includes: 1) introduction, enhancement, moving, or removing features from the original image; 2) grouping of images that should obviously be presented separately (e.g., from different parts of the same gel, or from different gels); or 3) modifying the contrast, brightness or color balance to obscure, eliminate or enhance some information.

If irregular image manipulation is identified and confirmed during the peer review process, we may reject the manuscript. If irregular image manipulation is identified and confirmed after publication, we may correct or retract the paper.

Our in-house editors will investigate any allegations of publication misconduct and may contact the authors' institutions or funders if necessary. If evidence of misconduct is found, appropriate action will be taken to correct or retract the publication. Authors are expected to comply with the best ethical publication practices when publishing with MDPI.

Citation Policy

Authors should ensure that where material is taken from other sources (including their own published writing) the source is clearly cited and that where appropriate permission is obtained.

Authors should not engage in excessive self-citation of their own work.

Authors should not copy references from other publications if they have not read the cited work.

Authors should not preferentially cite their own or their friends', peers', or institution's publications.

Authors should not cite advertisements or advertorial material.

In accordance with COPE guidelines, we expect that "original wording taken directly from publications by other researchers should appear in quotation marks with the appropriate citations." This condition also applies to an author's own work. COPE have produced a discussion document on **citation manipulation** with recommendations for best practice.

Instructions for Articles Focusing on Plant Extracts or Herbal Mixtures

All research articles submitted to the Natural Products section should fulfil the following requirements:

1. Provide complete details of any methodology used for isolation and purification.
2. Latin binomial nomenclature and authority should be provided after each common name the first time it is referred to in the text.
3. For manuscripts based on plant extracts or pure compounds loaded into nanoparticles, details for the full characterization of nanoparticles must be provided (stability, encapsulation efficiency, release of loaded material, etc.).
4. The NMR spectra used for structure elucidation must be attached as supplementary material.
5. For isolated compounds, the purity should be calculated (by HPLC, qNMR, etc.).
6. Complete characterization of mixture components must be included. Articles **must conform to at least 2** of the following points:

- Structure elucidation of the mixture components (by MS, LC–MS, NMR).
- Composition ratio of each component (HPLC, LC–MS, or other chromatography techniques. A minimum of 2 methods is required.).

- Focus on robust method development and validation (in the case of a methodology article, mechanistic studies are not necessary).

7. In vitro/in vivo testing. Articles **must contain examples of at least 3** of the following points:
   - High-throughput or in silico screening.
   - Target identification (identification of a receptor, enzyme, metabolic pathway, etc.).
   - IC_{50} curves or MIC when antimicrobial activity is investigated by disk diffusion.
   - Two independent methods are used to determine activity.
   - Metabolism or toxicology studies.

8. Correct Identification of Natural Products

The correct identification of the active components of extracts obtained from natural sources is a key part of the scientific information; thus, the journal will not accept papers reporting on only the activity of extracts while lacking any chemical characterization of the responsible components.

Reports on previously undescribed compounds must include, as supplementary data, ^{1}H and either ^{13}C or key 2D NMR spectra described in the discussion. The identification of known compounds in extracts should be adequately supported by chromatographic and/or spectroscopic data (e.g., LC/GC retention times and/or UV, mass spectra) as well as comparison with data of authentic samples or previously published values. When previously reported compounds are isolated and used in biological activity assays, the ^{1}H NMR spectrum should be given in the supplementary data as proof of purity. Authors should consider very carefully potential sources of artifacts and contaminants resulting from extraction procedures or sample handling.

Where possible, compounds should be purified to at least 95% purity. Whatever the claimed purity, it must be fully supported by appropriate analytical techniques, and this purity must be taken into account when reporting and comparing biological activities.

9. Correct Identification and Characterization of Chemical Compounds

The correct identification of chemical compounds is a key part of the scientific information; thus, the journal will not accept papers lacking chemical characterization. For this reason, if the manuscript reports on the synthesis and/or extraction and/or characterization of chemical compounds, the authors are strongly encouraged to fill out and submit the **Chemical Characterization Checklist**, marking the performed analyses. The Checklist will be available to Editors and Reviewers to support them in assessing the completeness and robustness of compound characterization. The inclusion of original spectra in the supplementary material is highly recommended. Links to deposited data in publicly accessible repositories are also acceptable.

Organic Compounds

Reports on previously undescribed organic compounds should include, as supplementary data, ^{1}H, ^{13}C and/or other key heteronuclear or 2D NMR spectra, together with high-resolution mass spectrometry (HRMS) or elemental analysis. The identification of known compounds in extracts must be adequately supported with chromatographic and/or spectroscopic data (e.g., LC/GC retention times and/or UV, mass spectra) as well as comparison with data of authentic samples or previously published values. When reporting and comparing biological or catalyst activity, the claimed purity must be fully supported by appropriate analytical techniques, e.g., NMR spectra, HPLC data, etc. Authors should consider very carefully potential sources of artifacts and contaminants resulting from extraction procedures or sample handling.

Inorganic/Organometallic Compounds

Reports on previously undescribed inorganic/organometallic compounds must provide adequate data to establish their identity and degree of purity. Whenever possible, the supplementary material should include data from elemental analysis, X-ray single crystallography, or powder diffraction. However, please note that an X-ray single crystal structure may not be sufficient for the characterization of new compounds and that the X-ray powder diffractogram (simulated is acceptable), should be provided. Additional characterization of particular samples, such as via NMR spectroscopy, mass spectrometry, infrared spectroscopy, electronic spectroscopy, etc., should also be included, if available.

Reviewer Suggestions

During the submission process, please suggest three potential reviewers with the appropriate expertise to review the manuscript. The editors will not necessarily approach these referees. Please provide detailed contact information (address, homepage, phone, e-mail address). The proposed referees should neither be current collaborators of the co-authors nor have published with any of the co-authors of the manuscript within the last three years. Proposed reviewers should be from different institutions to the authors. You may identify appropriate Editorial Board members of the journal as potential reviewers. You may suggest reviewers from among the authors that you frequently cite in your paper.

English Corrections

MDPI provides minor English editing by native English speakers for all accepted papers, included in the APC. The APC does not cover extensive English editing. Submitted papers should be written in good English and require no more than minor English editing before publication. Your paper could be returned to you at the English editing stage of the publication process if extensive editing is required, which could delay the publication of your work. You may choose to use a paid language-editing service, such as MDPI's **Author Services**, before submitting your paper for

publication. If you use an alternative service that provides a confirmation certificate, please send a copy to the Editorial Office. Authors from economically developing countries or nations should consider registration with **AuthorAid**, a global research community that provides networking, mentoring, resources and training for researchers.

Preprints and Conference Papers

Pharmaceuticals accepts submissions that have previously been made available as preprints provided that they have not undergone peer review. A preprint is a draft version of a paper made available online before submission to a journal.

MDPI operates ***Preprints***, a preprint server to which submitted papers can be uploaded directly after completing journal submission. Note that *Preprints* operates independently of the journal and posting a preprint does not affect the peer review process. Check the *Preprints* **instructions for authors** for further information.

Expanded and high-quality conference papers can be considered as articles if they fulfill the following requirements: (1) the paper should be expanded to the size of a research article; (2) the conference paper should be cited and noted on the first page of the paper; (3) if the authors do not hold the copyright of the published conference paper, authors should seek the appropriate permission from the copyright holder; (4) authors are asked to disclose that it is conference paper in their cover letter and include a statement on what has been changed compared to the original conference paper. *Pharmaceuticals* does not publish pilot studies or studies with inadequate statistical power.

Unpublished conference papers that do not meet the above conditions are recommended to be submitted to the **Proceedings Series journals**.

Authorship

MDPI follows the International Committee of Medical Journal Editors (**ICMJE**) guidelines which state that, in order to qualify for authorship of a manuscript, the following criteria should be observed:

- Substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work; AND
- Drafting the work or reviewing it critically for important intellectual content; AND
- Final approval of the version to be published; AND
- Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Those who contributed to the work but do not qualify for authorship should be listed in the acknowledgments. More detailed guidance on authorship is given by the **International Council of Medical Journal Editors (ICMJE)**.

Any change to the author list should be approved by all authors including any who have been removed from the list. The corresponding author should act as a point of contact between the editor and the other authors and should keep co-authors informed and involve them in major decisions about the publication. We reserve the right to request confirmation that all authors meet the authorship conditions.

For more details about authorship please check **MDPI ethics website**.

Reviewers Recommendation

Authors can recommend potential reviewers. Journal editors will check to make sure there are no conflicts of interest before contacting those reviewers, and will not consider those with competing interests. Reviewers are asked to declare any conflicts of interest. Authors can also enter the names of potential peer reviewers they wish to exclude from consideration in the peer review of their manuscript, during the initial submission progress. The editorial team will respect these requests so long as this does not interfere with the objective and thorough assessment of the submission.

Editorial Independence

Lack of Interference with Editorial Decisions

Editorial independence is of utmost importance and MDPI does not interfere with editorial decisions. All articles published by MDPI are peer reviewed and assessed by our independent editorial boards, and MDPI staff are not involved in decisions to accept manuscripts. When making an editorial decision, we expect the academic editor to make their decision based only upon:

- The suitability of selected reviewers;
- Adequacy of reviewer comments and author response;
- Overall scientific quality of the paper.

In all of our journals, in every aspect of operation, MDPI policies are informed by the mission to make science and research findings open and accessible as widely and rapidly as possible.

Editors and Editorial Staff as Authors

Editorial staff or editors shall not be involved in processing their own academic work. Submissions authored by editorial staff/editors will be assigned to at least two independent outside reviewers. Decisions will be made by other Editorial Board Members who do not have a conflict of interest

with the author. Journal staff are not involved in the processing of their own work submitted to any MDPI journals.

Conflicts of Interest

According to The International Committee of Medical Journal Editors, “Authors should avoid entering into agreements with study sponsors, both for-profit and non-profit, that interfere with authors’ access to all of the study’s data or that interfere with their ability to analyze and interpret the data and to prepare and publish manuscripts independently when and where they choose.”

All authors must disclose all relationships or interests that could inappropriately influence or bias their work. Examples of potential conflicts of interest include but are not limited to financial interests (such as membership, employment, consultancies, stocks/shares ownership, honoraria, grants or other funding, paid expert testimonies and patent-licensing arrangements) and non-financial interests (such as personal or professional relationships, affiliations, personal beliefs).

Authors can disclose potential conflicts of interest via the online submission system during the submission process. Declarations regarding conflicts of interest can also be collected via the **MDPI disclosure form**. The corresponding author must include a summary statement in the manuscript in a separate section “Conflicts of Interest” placed just before the reference list. The statement should reflect all the collected potential conflicts of interest disclosures in the form.

See below for examples of disclosures:

Conflicts of Interest: Author A has received research grants from Company A. Author B has received a speaker honorarium from Company X and owns stocks in Company Y. Author C has been involved as a consultant and expert witness in Company Z. Author D is the inventor of patent X.

If no conflicts exist, the authors should state:

Conflicts of Interest: The authors declare no conflicts of interest.

Editorial Procedures and Peer-Review

Pre-check

Immediately after submission, the journal’s Managing Editor will perform the technical pre-check to assess:

- Overall suitability of the manuscript to the journal/section/Special Issue;
- Manuscript adherence to high-quality research and ethical standards;
- Standards of rigor to qualify for further review.

The academic editor (i.e., the Editor-in-Chief in the case of regular submissions, the Guest Editor in the case of Special Issue submissions, or an Editorial Board member in the case of a conflict of interest and of regular submissions if the Editor-in-Chief allows) will be notified of the submission and invited to perform an editorial pre-check. During the editorial pre-check phase, the academic editor will assess the suitability of the submission with respect to the scope of the journal, as well as the overall scientific soundness of the manuscript, including the relevance of the references and the correctness of the applied methodology. Academic editors can decide to reject the manuscript, request revisions before peer-review, or continue with the peer-review process and recommend suitable reviewers.

Peer-Review

Once a manuscript passes the initial checks, it will be assigned to at least two independent experts for peer-review. A single-blind review is applied, where authors' identities are known to reviewers. Peer review comments are confidential and will only be disclosed with the express agreement of the reviewer.

In the case of regular submissions, in-house assistant editors will invite experts, including recommendations by an academic editor. These experts may also include *Editorial Board Members* and Guest Editors of the journal. Potential reviewers suggested by the authors may also be considered. Reviewers should not have published with any of the co-authors during the past three years and should not currently work or collaborate with any of the institutions of the co-authors of the submitted manuscript.

Optional Open Peer-Review

The journal operates optional open peer-review: *Authors are given the option for all review reports and editorial decisions to be published alongside their manuscript. In addition, reviewers can sign their review, i.e., identify themselves in the published review reports.* Authors can alter their choice for open review at any time before publication, but once the paper has been published changes will only be made at the discretion of the *Publisher* and *Editor-in-Chief*. We encourage authors to take advantage of this opportunity as proof of the rigorous process employed in publishing their research. To guarantee impartial refereeing, the names of referees will be revealed only if the referees agree to do so, and after a paper has been accepted for publication.

Editorial Decision and Revision

All the articles, reviews and communications published in MDPI journals go through the peer-review process and receive at least two reviews. The in-house editor will communicate the decision of the academic editor, which will be one of the following:

- *Accept after Minor Revisions*:
  The paper is in principle accepted after revision based on the reviewer's comments. Authors are given five days for minor revisions.
- *Reconsider after Major Revisions*:
  The acceptance of the manuscript would depend on the revisions. The author needs to provide a point by point response or provide a rebuttal if some of the reviewer's comments cannot be revised. A maximum of two rounds of major revision per manuscript is normally provided. Authors will be asked to resubmit the revised paper within a suitable time frame, and the revised version will be returned to the reviewer for further comments. If the required revision time is estimated to be longer than 2 months, we will recommend that authors withdraw their manuscript before resubmitting so as to avoid unnecessary time pressure and to ensure that all manuscripts are sufficiently revised.
- *Reject and Encourage Resubmission*:
  If additional experiments are needed to support the conclusions, the manuscript will be rejected and the authors will be encouraged to re-submit the paper once further experiments have been conducted.
- *Reject*:
  The article has serious flaws, and/or makes no original significant contribution. No offer of resubmission to the journal is provided.

All reviewer comments should be responded to in a point-by-point fashion. Where the authors disagree with a reviewer, they must provide a clear response.

Author Appeals

Authors may appeal a rejection by sending an e-mail to the Editorial Office of the journal. The appeal must provide a detailed justification, including point-by-point responses to the reviewers' and/or Editor's comments using an **appeal form**. Appeals can only be submitted following a "reject and decline resubmission" decision and should be submitted within three months from the decision date. Failure to meet these criteria will result in the appeal not being considered further. The *Managing Editor* will forward the manuscript and related information (including the identities of the referees) to a designated *Editorial Board Member*. The Academic Editor being consulted will be asked to provide an advisory recommendation on the manuscript and may recommend acceptance, further peer-review, or uphold the original rejection decision. This decision will then be validated by the *Editor-in-Chief*. A reject decision at this stage is final and cannot be reversed.

Production and Publication

Once accepted, the manuscript will undergo professional copy-editing, English editing, proofreading by the authors, final corrections, pagination, and, publication on the **www.mdpi.com** website.

Promoting Equity, Diversity and Inclusiveness within MDPI Journals

Our Managing Editors encourage the Editors-in-Chief and Associate Editors to appoint diverse expert Editorial Boards. This is also reflective in our multi-national and inclusive workplace. We are proud to create equal opportunities without regard to gender, ethnicity, sexual orientation, age, religion, or socio-economic status. There is no place for discrimination in our workplace and editors of MDPI journals are to uphold these principles in high regard.

Resource Identification Initiative

To improve the reproducibility of scientific research, the **Resource Identification Initiative** aims to provide unique persistent identifiers for key biological resources, including antibodies, cell lines, model organisms and tools.

We encourage authors to include unique identifiers - RRIDs- provided by the **Resource Identification Portal** in the dedicated section of the manuscript.

To help authors quickly find the correct identifiers for their materials, there is a single **website** where all resource types can be found and a ‘cite this’ button next to each resource, that contains a proper citation text that should be included in the methods section of the manuscript.

Annexure M

Instructions for Authors for *'Methods and Protocols'*

Submission Checklist

Please:

1. Read the **Aims & Scope** to gain an overview and assess if your manuscript is suitable for this journal;
2. Use the **Microsoft Word template (Protocol)** or **Microsoft Word template (types of papers other than Protocols)** or **LaTeX template** to prepare your manuscript;
3. Make sure that issues about **publication ethics**, **research ethics**, **copyright**, **authorship**, **figure formats**, **data** and **references format** have been appropriately considered;
4. Ensure that all authors have approved the content of the submitted manuscript.
5. Authors are encouraged to add a **biography** (optional) to the submission and post it to **SciProfiles**.
6. Consider submitting a **videoarticle** along with your manuscript.

Manuscript Submission Overview

Types of Publications

Manuscripts submitted to *MPs* should neither be published previously nor be under consideration for publication in another journal. The main article types are listed below and a comprehensive list of article types can be found **here.**

- *Article:* These are original research manuscripts. The work should report scientifically sound experiments and provide a substantial amount of new information. The article should include the most recent and relevant references in the field. The structure should include an Abstract, Keywords, Introduction, Materials and Methods, Results, Discussion, and Conclusions (optional) sections, with a suggested minimum word count of 4000 words.
- *Protocol:* Protocols provide a detailed step-by-step description of a method. They should be proven to be robust and reproducible and should accompany a previously published

article that uses this method. Any materials and equipment used should be explicitly listed. Conditions, quantities, concentrations, etc., should be given. Critical timepoints and steps, as well as warnings, should be emphasized in the text. The structure should include an Abstract, Keywords, Introduction, Experimental Design, Materials and Equipment, Detailed Procedure, and Expected Results, with a suggested minimum word count of 4000 words.

- *Technical Note:* Technical notes are brief articles focused on a new technique, method, or procedure. These should describe important modifications or unique applications for the described method. Technical notes can also be used for describing a new software tool or computational method. The structure should include an Abstract, Keywords, Introduction, Materials and Methods, Results, Discussion, and Conclusions, with a suggested minimum word count of 3500 words.
- *Review:* Reviews offer a comprehensive analysis of the existing literature within a field of study, identifying current gaps or problems. They should be critical and constructive and provide recommendations for future research. No new, unpublished data should be presented. The structure can include an Abstract, Keywords, Introduction, Relevant Sections, Discussion, Conclusions, and Future Directions, with a suggested minimum word count of 4000 words.
- *Comments:* This journal encourages the submission of Comments on currently active areas of research. It's meant to be an open forum for discussion on already published protocols or methods.

Videoarticles

Videoarticles are detailed, step-by-step experimental demonstrations that illustrate new methods or protocols by presenting the material, equipment and actions required to conduct the experiment described in a Protocol or Benchmark paper. They increase the reproducibility and visibility of newly developed protocols/methods and can also be used as teaching aids. We welcome the submission of Videoarticles demonstrating new methods/protocols in any scientific discipline covered by Methods and Protocols.

Videoarticles will be published alongside textual methods/protocols under a Creative Commons **CC BY** license, meaning that they are freely accessible and downloadable, and can be reused if correctly cited. We encourage sharing of Videoarticles, including via social media. In order for your research to reach the broadest audience possible, only Videoarticles in English will be considered for publication. All Videoarticles will be checked for editorial suitability and quality, as well as for correspondence to the Protocol or Benchmark paper.

Please note that your video should not contain any music, sound effects or background talking, nor overt product promotion. Before preparing your Videoarticle, please read the **Guidelines** and **Tips** for Videoarticle production; the slides to introduce the different sections in Videoarticles can be downloaded **here**.

Submission Process

Manuscripts for *MPs* should be submitted online at **susy.mdpi.com**. The submitting author, who is generally the corresponding author, is responsible for the manuscript during the submission and peer-review process. The submitting author must ensure that all eligible co-authors have been included in the author list (read the **criteria to qualify for authorship**) and that they have all read and approved the submitted version of the manuscript. To submit your manuscript, register and log in to the **submission website**. Once you have registered, **click here to go to the submission form for *MPs***. All co-authors can see the manuscript details in the submission system, if they register and log in using the e-mail address provided during manuscript submission.

Accepted File Formats

Authors are encouraged to use the **Microsoft Word template** or **LaTeX template** to prepare their manuscript. Using the template file will substantially shorten the time to complete copy-editing and publication of accepted manuscripts. The total amount of data for all files must not exceed 120 MB. If this is a problem, please contact the Editorial Office **mps@mdpi.com**. Accepted file formats are:

- *Microsoft Word:* Manuscripts prepared in Microsoft Word must be converted into a single file before submission. When preparing manuscripts in Microsoft Word, we encourage you to use the ***MPs* Microsoft Word template file**. Please insert your graphics (schemes, figures, *etc.*) in the main text after the paragraph of its first citation.
- *LaTeX:* Manuscripts prepared in LaTeX must be collated into one ZIP folder (including all source files and images, so that the Editorial Office can recompile the submitted PDF). When preparing manuscripts in LaTeX, we encourage you to use the ***MPs* LaTeX template files**. You can now also use the online application **writeLaTeX** to submit articles directly to *MPs*. The MDPI LaTeX template file should be selected from the **writeLaTeX template gallery**.
- *Supplementary files:* May be any format, but it is recommended that you use common, non-proprietary formats where possible (see **below** for further details).

Disclaimer: Usage of these templates is exclusively intended for submission to the journal for peer-review, and strictly limited to this purpose and it cannot be used for posting online on preprint servers or other websites.

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MPs now accepts free format submission:

- We do not have strict formatting requirements, but all manuscripts must contain the required sections: Author Information, Abstract, Keywords, Introduction, Materials & Methods, Results, Conclusions, Figures and Tables with Captions, Funding Information, Author Contributions, Conflict of Interest and other Ethics Statements. Check the Journal ***Instructions for Authors*** for more details.
- Your references may be in any style, provided that you use the consistent formatting throughout. It is essential to include author(s) name(s), journal or book title, article or chapter title (where required), year of publication, volume and issue (where appropriate) and pagination. DOI numbers (Digital Object Identifier) are not mandatory but highly encouraged. The bibliography software package *EndNote*, ***Zotero***, *Mendeley*, *Reference Manager* are recommended.
- When your manuscript reaches the revision stage, you will be requested to format the manuscript according to the journal guidelines.

Cover Letter

A cover letter must be included with each manuscript submission. It should be concise and explain why the content of the paper is significant, placing the findings in the context of existing work. It should explain why the manuscript fits the scope of the journal.

Any prior submissions of the manuscript to MDPI journals must be acknowledged. If this is the case, it is strongly recommended that the previous manuscript ID is provided in the submission system, which will ease your current submission process. The names of proposed and excluded reviewers should be provided in the submission system, not in the cover letter.

All cover letters are required to include the statements:

- We confirm that neither the manuscript nor any parts of its content are currently under consideration or published in another journal.
- All authors have approved the manuscript and agree with its submission to (journal name).

Author Identification

Authors are encouraged to add a biography (maximum 150 words) to the submission and upload it to **SciProfiles**. This should be a single paragraph and should contain the following points:

1. Authors' full names followed by current positions;
2. Education background including institution information and year of graduation (type and level of degree received);
3. Work experience;

4. Current and previous research interests;
5. Memberships of professional societies and awards received.

If a manuscript is accepted for publication, we will add an icon linking to your online **ORCID** profile in the final version of the published paper.

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All authors should list their current affiliation and the affiliation where most research was carried out for the preparation of their manuscript. We recommend adding as primary the affiliation where most of the research was conducted or supported, but please check with your institution for any contractual agreement requirements.

It is very important that author names and affiliations are correct. Incorrect information can mean a lack of proper attribution or incorrect citation and can even lead to problems with promotion or funding. After the publication of an article, updates or corrections to the author's address or affiliation may not be permitted.

Independent Researcher

If one or all the authors are not currently affiliated with a university, institution or company, or have not been during the development of the manuscript, they should list themselves as an "Independent Researcher".

Manuscript Preparation

General Considerations

- **Research manuscripts** should comprise:
    - **Front matter**: Title, Author list, Affiliations, Abstract, Keywords.
    - **Research manuscript sections**: Introduction, Materials and Methods, Results, Discussion, Conclusions (optional).
    - **Back matter**: Supplementary Materials, Acknowledgments, Author Contributions, Conflicts of Interest, **References**.
- **Review manuscripts** should comprise the **front matter**, literature review sections and the **back matter**. The template file can also be used to prepare the front and back matter of your review manuscript. It is not necessary to follow the remaining structure. Structured reviews and meta-analyses should use the same structure as research articles and ensure they conform to the **PRISMA** guidelines.

Graphical Abstract:

A graphical abstract (GA) is an image that appears alongside the text abstract in the Table of Contents. In addition to summarizing the content, it should represent the topic of the article in an attention-grabbing way. Moreover, it should not be exactly the same as the Figure in the paper or just a simple superposition of several subfigures. Note that the GA must be original and unpublished artwork. Any postage stamps, currency from any country, or trademarked items should not be included in it.

The GA should be a high-quality illustration or diagram in any of the following formats: PNG, JPEG, or TIFF. Written text in a GA should be clear and easy to read, using one of the following fonts: Times, Arial, Courier, Helvetica, Ubuntu or Calibri.

The minimum required size for the GA is 560 × 1100 pixels (height × width). The size should be of high quality in order to reproduce well.

- **Acronyms/Abbreviations/Initialisms** should be defined the first time they appear in each of three sections: the abstract; the main text; the first figure or table. When defined for the first time, the acronym/abbreviation/initialism should be added in parentheses after the written-out form.
- **SI Units** (International System of Units) should be used. Imperial, US customary and other units should be converted to SI units whenever possible.
- **Accession numbers** of RNA, DNA and protein sequences used in the manuscript should be provided in the Materials and Methods section. Also see the section on **Deposition of Sequences and Expression Data**.
- **Equations:** If you are using Word, please use either the Microsoft Equation Editor or the MathType add-on. Equations should be editable by the editorial office and not appear in a picture format.
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Front Matter

These sections should appear in all manuscript types

- **Title:** The title of your manuscript should be concise, specific and relevant. It should identify if the study reports (human or animal) trial data, or is a systematic review, meta-analysis or replication study. When gene or protein names are included, the abbreviated name rather than full name should be used. Please do not include abbreviated or short forms of the title, such as a running title or head. These will be removed by our Editorial Office.
- **Author List and Affiliations:** Authors' full first and last names must be provided. The initials of any middle names can be added. The PubMed/MEDLINE standard format is used for affiliations: complete address information including city, zip code, state/province, and country. At least one author should be designated as the corresponding author. The email addresses of all authors will be displayed on published papers, and hidden by Captcha on the website as standard. It is the responsibility of the corresponding author to ensure that consent for the display of email addresses is obtained from all authors. If an author (other than the corresponding author) does not wish to have their email addresses displayed in this way, the corresponding author must indicate as such during proofreading. After acceptance, updates to author names or affiliations may not be permitted. Equal Contributions: authors who have contributed equally should be marked with a superscript symbol (†). The symbol must be included below the affiliations, and the following statement added: “These authors contributed equally to this work”. The equal roles of authors should also be adequately disclosed in the author contributions statement. Please read the criteria to qualify for authorship.
- **Abstract:** The abstract should be a total of about 200 words maximum. The abstract should be a single paragraph and should follow the style of structured abstracts, but without headings: 1) Background: Place the question addressed in a broad context and highlight the purpose of the study; 2) Methods: Describe briefly the main methods or treatments applied. Include any relevant preregistration numbers, and species and strains of any animals used; 3) Results: Summarize the article's main findings; and 4) Conclusion: Indicate the main conclusions or interpretations. The abstract should be an objective representation of the article: it must not contain results which are not presented and substantiated in the main text and should not exaggerate the main conclusions.
- **Keywords:** Three to ten pertinent keywords need to be added after the abstract. We recommend that the keywords are specific to the article, yet reasonably common within the subject discipline.

Research Manuscript Sections

- **Introduction:** The introduction should briefly place the study in a broad context and highlight why it is important. It should define the purpose of the work and its significance, including specific hypotheses being tested. The current state of the research field should be reviewed carefully and key publications cited. Please highlight controversial and diverging hypotheses when necessary. Finally, briefly mention the main aim of the work and highlight the main conclusions. Keep the introduction comprehensible to scientists working outside the topic of the paper.
- **Materials and Methods:** They should be described with sufficient detail to allow others to replicate and build on published results. New methods and protocols should be described in detail while well-established methods can be briefly described and appropriately cited. Give the name and version of any software used and make clear whether computer code used is available. Include any pre-registration codes.
- **Results:** Provide a concise and precise description of the experimental results, their interpretation as well as the experimental conclusions that can be drawn.
- **Discussion:** Authors should discuss the results and how they can be interpreted in perspective of previous studies and of the working hypotheses. The findings and their implications should be discussed in the broadest context possible and limitations of the work highlighted. Future research directions may also be mentioned. This section may be combined with Results.
- **Conclusions:** This section is not mandatory but can be added to the manuscript if the discussion is unusually long or complex.
- **Patents:** This section is not mandatory but may be added if there are patents resulting from the work reported in this manuscript.

Back Matter

- **Supplementary Materials:** Describe any supplementary material published online alongside the manuscript (figure, tables, video, spreadsheets, etc.). Please indicate the name and title of each element as follows Figure S1: title, Table S1: title, etc.
- **Author Contributions:** Each author is expected to have made substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data; or the creation of new software used in the work; or have drafted the work or substantively revised it; AND has approved the submitted version (and version substantially edited by journal staff that involves the author's contribution to the study); AND agrees to be personally accountable for the author's own contributions and for ensuring that questions related to the accuracy or integrity of any part of the work, even ones in which the author was not personally involved, are appropriately investigated,

resolved, and documented in the literature.
For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used "Conceptualization, X.X. and Y.Y.; Methodology, X.X.; Software, X.X.; Validation, X.X., Y.Y. and Z.Z.; Formal Analysis, X.X.; Investigation, X.X.; Resources, X.X.; Data Curation, X.X.; Writing – Original Draft Preparation, X.X.; Writing – Review & Editing, X.X.; Visualization, X.X.; Supervision, X.X.; Project Administration, X.X.; Funding Acquisition, Y.Y.", please turn to the **CRediT taxonomy** for the term explanation. For more background on CRediT, see **here**. "**Authorship must include and be limited to those who have contributed substantially to the work. Please read the section concerning the criteria to qualify for authorship carefully**".

- **Funding:** All sources of funding of the study should be disclosed. Clearly indicate grants that you have received in support of your research work and if you received funds to cover publication costs. Note that some funders will not refund article processing charges (APC) if the funder and grant number are not clearly and correctly identified in the paper. Funding information can be entered separately into the submission system by the authors during submission of their manuscript. Such funding information, if available, will be deposited to FundRef if the manuscript is finally published. Please add: “This research received no external funding” or “This research was funded by [name of funder] grant number [xxx]” and “The APC was funded by [XXX]” in this section. Check carefully that the details given are accurate and use the standard spelling of funding agency names at https://search.crossref.org/funding, any errors may affect your future funding.
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can be identified (including by the patients themselves). Please state “Written informed consent has been obtained from the patient(s) to publish this paper” if applicable.

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- **Conflicts of Interest:** Authors must identify and declare any personal circumstances or interest that may be perceived as influencing the representation or interpretation of reported research results. If there is no conflict of interest, please state "The authors declare no conflict of interest." Any role of the funding sponsors in the choice of research project; design of the study; in the collection, analyses or interpretation of data; in the writing of the manuscript; or in the decision to publish the results must be declared in this section. *MPs* does not publish studies funded partially or fully by the tobacco industry. Any projects funded by industry must pay special attention to the full declaration of funder involvement. If there is no role, please state “The sponsors had no role in the design, execution, interpretation, or writing of the study”. For more details please see **Conflict of Interest**.
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In the text, reference numbers should be placed in square brackets [ ], and placed before the punctuation; for example [1], [1–3] or [1,3]. For embedded citations in the text with pagination, use both parentheses and brackets to indicate the reference number and page numbers; for example [5] (p. 10). or [6] (pp. 101–105).

The reference list should include the full title, as recommended by the ACS style guide. Style files for **Endnote** and **Zotero** are available.

References should be described as follows, depending on the type of work:

Journal Articles:

1. Author 1, A.B.; Author 2, C.D. Title of the article. *Abbreviated Journal Name* **Year**, *Volume*, page range.

Books and Book Chapters:

2. Author 1, A.; Author 2, B. *Book Title*, 3rd ed.; Publisher: Publisher Location, Country, Year; pp. 154–196.

3. Author 1, A.; Author 2, B. Title of the chapter. In *Book Title*, 2nd ed.; Editor 1, A., Editor 2, B., Eds.; Publisher: Publisher Location, Country, Year; Volume 3, pp. 154–196.

Unpublished materials intended for publication:

4. Author 1, A.B.; Author 2, C. Title of Unpublished Work (optional). Correspondence Affiliation, City, State, Country. year, *status* (*manuscript in preparation*; *to be submitted*).

5. Author 1, A.B.; Author 2, C. Title of Unpublished Work. *Abbreviated Journal Name* year, *phrase indicating stage of publication* (*submitted*; *accepted*; *in press*).

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Conference Proceedings:

7. Author 1, A.B.; Author 2, C.D.; Author 3, E.F. Title of Presentation. In *Title of the Collected Work* (if available), Proceedings of the Name of the Conference, Location of Conference, Country, Date of Conference; Editor 1, Editor 2, Eds. (if available); Publisher: City, Country, Year (if available); Abstract Number (optional), Pagination (optional).

Thesis:

8. Author 1, A.B. Title of Thesis. Level of Thesis, Degree-Granting University, Location of University, Date of Completion.

Websites:

9. Title of Site. Available online: URL (accessed on Day Month Year). Unlike published works, websites may change over time or disappear, so we encourage you create an archive of the cited website using a service such as **WebCite**. Archived websites should be cited using the link provided as follows: 10. Title of Site. URL (archived on Day Month Year).

See the **Reference List and Citations Guide** for more detailed information.

Preparing Figures, Schemes and Tables

- File for Figures and Schemes must be provided during submission in a single zip archive and at a sufficiently high resolution (minimum 1000 pixels width/height, or a resolution of 300 dpi or higher). Common formats are accepted, however, TIFF, JPEG, EPS and PDF are preferred.
- *MPs* can publish multimedia files in articles or as supplementary materials. Please contact the editorial office for further information.
- All Figures, Schemes and Tables should be inserted into the main text close to their first citation and must be numbered following their number of appearance (Figure 1, Scheme I, Figure 2, Scheme II, Table 1, *etc.*).
- All Figures, Schemes and Tables should have a short explanatory title and caption.
- All table columns should have an explanatory heading. To facilitate the copy-editing of larger tables, smaller fonts may be used, but no less than 8 pt. in size. Authors should use the Table option of Microsoft Word to create tables.
- Authors are encouraged to prepare figures and schemes in color (RGB at 8-bit per channel). There is no additional cost for publishing full color graphics.

Original Images for Blots and Gels Requirements

For the main text, please ensure that:

- All experimental samples and controls used for one comparative analysis are run on the same blot/gel.
- Image processing methods, such as adjusting the brightness or contrast, do not alter or distort the information in the figure and are applied to every pixel. High-contrast blots/gels are discouraged.
- Cropped blots/gels present in the main text retain all important information and bands.
- You have checked figures for duplications and ensured the figure legends are clear and accurate. Please include all relevant information in the figure legends and clearly indicate any re-arrangement of lanes.

In order to ensure the integrity and scientific validity of blots (including, but not limited to, Western blots) and the reporting of gel data, original, uncropped and unadjusted images should be uploaded as Supporting Information files at the time of initial submission.

A single PDF file or a zip folder including all the original images reported in the main figure and supplemental figures should be prepared. Authors should annotate each original image, corresponding to the figure in the main article or supplementary materials, and label each lane or loading order. All experimental samples and controls used for one comparative analysis should

be run on the same blot/gel image. For quantitative analyses, please provide the blots/gels for each independent biological replicate used in the analysis.

Supplementary Materials, Data Deposit and Software Source Code

MDPI Research Data Policies

MDPI is committed to supporting open scientific exchange and enabling our authors to achieve best practices in sharing and archiving research data. We encourage all authors of articles published in MDPI journals to share their research data. Individual journal guidelines can be found at the journal 'Instructions for Authors' page. Data sharing policies concern the minimal dataset that supports the central findings of a published study. Generated data should be publicly available and cited in accordance with journal guidelines.

MDPI data policies are informed by **TOP Guidelines** and **FAIR Principles**.

Where ethical, legal or privacy issues are present, data should not be shared. The authors should make any limitations clear in the Data Availability Statement upon submission. Authors should ensure that data shared are in accordance with consent provided by participants on the use of confidential data.

Data Availability Statements provide details regarding where data supporting reported results can be found, including links to publicly archived datasets analyzed or generated during the study.

Below are suggested Data Availability Statements:

- Data available in a publicly accessible repository
  The data presented in this study are openly available in [repository name e.g., FigShare] at [**doi**], reference number [reference number].
- Data available in a publicly accessible repository that does not issue DOIs
  Publicly available datasets were analyzed in this study. This data can be found here: [link/accession number]
- Data available on request due to restrictions eg privacy or ethical
  The data presented in this study are available on request from the corresponding author. The data are not publicly available due to [insert reason here]
- 3rd Party Data
  Restrictions apply to the availability of these data. Data was obtained from [third party] and are available [from the authors/at URL] with the permission of [third party].
- Data sharing not applicable
  No new data were created or analyzed in this study. Data sharing is not applicable to this article.

- Data is contained within the article or supplementary material
  The data presented in this study are available in [insert article or supplementary material here]

Data citation:

- [dataset] Authors. Year. Dataset title; Data repository or archive; Version (if any); Persistent identifier (e.g., DOI).

Computer Code and Software

For work where novel computer code was developed, authors should release the code either by depositing in a recognized, public repository such as **GitHub** or uploading as supplementary information to the publication. The name, version, corporation and location information for all software used should be clearly indicated. Please include all the parameters used to run software/programs analyses.

Supplementary Material

Additional data and files can be uploaded as "Supplementary Files" during the manuscript submission process. The supplementary files will also be available to the referees as part of the peer-review process. Any file format is acceptable; however, we recommend that common, non-proprietary formats are used where possible. For more information on supplementary materials, please refer to **https://www.mdpi.com/authors/layout#_bookmark83**.

References in Supplementary Files

Citations and References in Supplementary files are permitted provided that they also appear in the reference list of the main text.

Unpublished Data

Restrictions on data availability should be noted during submission and in the manuscript. "Data not shown" should be avoided: authors are encouraged to publish all observations related to the submitted manuscript as Supplementary Material. "Unpublished data" intended for publication in a manuscript that is either planned, "in preparation" or "submitted" but not yet accepted, should be cited in the text and a reference should be added in the References section. "Personal Communication" should also be cited in the text and reference added in the References section. (see also the MDPI reference list and citations style guide).

Remote Hosting and Large Data Sets

Data may be deposited with specialized service providers or institutional/subject repositories, preferably those that use the DataCite mechanism. Large data sets and files greater than 60 MB must be deposited in this way. For a list of other repositories specialized in scientific and

experimental data, please consult **databib.org** or **re3data.org**. The data repository name, link to the data set (URL) and accession number, doi or handle number of the data set must be provided in the paper. The journal **Data** also accepts submissions of data set papers.

Deposition of Sequences and Expression Data

New sequence information must be deposited to the appropriate database prior to submission of the manuscript. Accession numbers provided by the database should be included in the submitted manuscript. Manuscripts will not be published until the accession number is provided.

- *New nucleic acid sequences* must be deposited into an acceptable repository such as **GenBank**, **EMBL**, or **DDBJ**. Sequences should be submitted to only one database.
- *New high throughput sequencing (HTS) datasets* (RNA-seq, ChIP-Seq, degradome analysis, …) must be deposited either in the **GEO database** or in the NCBI's **Sequence Read Archive (SRA)**.
- *New microarray data* must be deposited either in the **GEO** or the **ArrayExpress** databases.The "Minimal Information About a Microarray Experiment" (MIAME) guidelines published by the Microarray Gene Expression Data Society must be followed.
- *New protein sequences* obtained by protein sequencing must be submitted to UniProt (submission tool **SPIN**). Annotated protein structure and its reference sequence must be submitted to **RCSB of Protein Data Bank**.

All sequence names and the accession numbers provided by the databases must be provided in the Materials and Methods section of the article.

Deposition of Proteomics Data

Methods used to generate the proteomics data should be described in detail and we encourage authors to adhere to the "**Minimum Information About a Proteomics Experiment**". All generated mass spectrometry raw data must be deposited in the appropriate public database such as **ProteomeXchange**, **PRIDE** or **jPOST**. At the time of submission, please include all relevant information in the materials and methods section, such as repository where the data was submitted and link, data set identifier, username and password needed to access the data.

Research and Publication Ethics

Research Ethics

Research Involving Human Subjects

When reporting on research that involves human subjects, human material, human tissues, or human data, authors must declare that the investigations were carried out following the rules of the Declaration of Helsinki of 1975 (**https://www.wma.net/what-we-do/medical-ethics/declaration-of-helsinki/**), revised in 2013. According to point 23 of this declaration, an approval from the local institutional review board (IRB) or other appropriate ethics committee must be obtained before undertaking the research to confirm the study meets national and international guidelines. As a minimum, a statement including the project identification code, date of approval, and name of the ethics committee or institutional review board must be stated in Section 'Institutional Review Board Statement' of the article.

Example of an ethical statement: "All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of XXX (Project identification code)."

For non-interventional studies (e.g. surveys, questionnaires, social media research), all participants must be fully informed if the anonymity is assured, why the research is being conducted, how their data will be used and if there are any risks associated. As with all research involving humans, ethical approval from an appropriate ethics committee must be obtained prior to conducting the study. If ethical approval is not required, authors must either provide an exemption from the ethics committee or are encouraged to cite the local or national legislation that indicates ethics approval is not required for this type of study. Where a study has been granted exemption, the name of the ethics committee which provided this should be stated in Section 'Institutional Review Board Statement' with a full explanation regarding why ethical approval was not required.

A written informed consent for publication must be obtained from participating patients. Data relating to individual participants must be described in detail, but private information identifying participants need not be included unless the identifiable materials are of relevance to the research (for example, photographs of participants' faces that show a particular symptom). Patients' initials or other personal identifiers must not appear in any images. For manuscripts that include any case details, personal information, and/or images of patients, authors must obtain signed informed consent for publication from patients (or their relatives/guardians) before submitting to an MDPI journal. Patient details must be anonymized as far as possible, e.g., do not mention

specific age, ethnicity, or occupation where they are not relevant to the conclusions. A **template permission form** is available to download. A blank version of the form used to obtain permission (without the patient names or signature) must be uploaded with your submission. Editors reserve the right to reject any submission that does not meet these requirements.

You may refer to our sample form and provide an appropriate form after consulting with your affiliated institution. For the purposes of publishing in MDPI journals, a consent, permission, or release form should include unlimited permission for publication in all formats (including print, electronic, and online), in sublicensed and reprinted versions (including translations and derived works), and in other works and products under open access license. To respect patients' and any other individual's privacy, please do not send signed forms. The journal reserves the right to ask authors to provide signed forms if necessary.

If the study reports research involving vulnerable groups, an additional check may be performed. The submitted manuscript will be scrutinized by the editorial office and upon request, documentary evidence (blank consent forms and any related discussion documents from the ethics board) must be supplied. Additionally, when studies describe groups by race, ethnicity, gender, disability, disease, etc., explanation regarding why such categorization was needed must be clearly stated in the article.

Ethical Guidelines for the Use of Animals in Research

The editors will require that the benefits potentially derived from any research causing harm to animals are significant in relation to any cost endured by animals, and that procedures followed are unlikely to cause offense to the majority of readers. Authors should particularly ensure that their research complies with the commonly-accepted '3Rs [1]':

- Replacement of animals by alternatives wherever possible,
- Reduction in number of animals used, and
- Refinement of experimental conditions and procedures to minimize the harm to animals.

Authors must include details on housing, husbandry and pain management in their manuscript.

For further guidance authors should refer to the Code of Practice for the Housing and Care of Animals Used in Scientific Procedures [2], American Association for Laboratory Animal Science [3] or European Animal Research Association [4].

If national legislation requires it, studies involving vertebrates or higher invertebrates must only be carried out after obtaining approval from the appropriate ethics committee. As a minimum, the project identification code, date of approval and name of the ethics committee or institutional review board should be stated in Section 'Institutional Review Board Statement'. Research

procedures must be carried out in accordance with national and institutional regulations. Statements on animal welfare should confirm that the study complied with all relevant legislation. Clinical studies involving animals and interventions outside of routine care require ethics committee oversight as per the American Veterinary Medical Association. If the study involved client-owned animals, informed client consent must be obtained and certified in the manuscript report of the research. Owners must be fully informed if there are any risks associated with the procedures and that the research will be published. If available, a high standard of veterinary care must be provided. Authors are responsible for correctness of the statements provided in the manuscript.

If ethical approval is not required by national laws, authors must provide an exemption from the ethics committee, if one is available. Where a study has been granted exemption, the name of the ethics committee that provided this should be stated in Section 'Institutional Review Board Statement' with a full explanation on why the ethical approval was not required.

If no animal ethics committee is available to review applications, authors should be aware that the ethics of their research will be evaluated by reviewers and editors. Authors should provide a statement justifying the work from an ethical perspective, using the same utilitarian framework that is used by ethics committees. Authors may be asked to provide this even if they have received ethical approval.

MDPI endorses the ARRIVE guidelines (**arriveguidelines.org/**) for reporting experiments using live animals. Authors and reviewers must use the ARRIVE guidelines as a checklist, which can be found at **https://arriveguidelines.org/sites/arrive/files/documents/ARRIVE%20Compliance%20Questionnaire.pdf**. Editors reserve the right to ask for the checklist and to reject submissions that do not adhere to these guidelines, to reject submissions based on ethical or animal welfare concerns or if the procedure described does not appear to be justified by the value of the work presented.

1. NSW Department of Primary Industries and Animal Research Review Panel. Three Rs. Available online: **https://www.animalethics.org.au/three-rs**
2. Home Office. Animals (Scientific Procedures) Act 1986. Code of Practice for the Housing and Care of Animals Bred, Supplied or Used for Scientific Purposes. Available online: **https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/388535/CoPanimalsWeb.pdf**

3. American Association for Laboratory Animal Science. The Scientific Basis for Regulation of Animal Care and Use. Available online: **https://www.aalas.org/about-aalas/position-papers/scientific-basis-for-regulation-of-animal-care-and-use**
4. European Animal Research Association. EU regulations on animal research. Available online: **https://www.eara.eu/animal-research-law**

Research Involving Cell Lines

Methods sections for submissions reporting on research with cell lines should state the origin of any cell lines. For established cell lines the provenance should be stated and references must also be given to either a published paper or to a commercial source. If previously unpublished *de novo* cell lines were used, including those gifted from another laboratory, details of institutional review board or ethics committee approval must be given, and confirmation of written informed consent must be provided if the line is of human origin.

An example of Ethical Statements:

The HCT116 cell line was obtained from XXXX. The $MLH1^{+}$ cell line was provided by XXXXX, Ltd. The DLD-1 cell line was obtained from Dr. XXXX. The DR-GFP and SA-GFP reporter plasmids were obtained from Dr. XXX and the Rad51K133A expression vector was obtained from Dr. XXXX.

Research Involving Plants

Experimental research on plants (either cultivated or wild) including collection of plant material, must comply with institutional, national, or international guidelines. We recommend that authors comply with the **Convention on Biological Diversity** and the **Convention on the Trade in Endangered Species of Wild Fauna and Flora**.

For each submitted manuscript supporting genetic information and origin must be provided. For research manuscripts involving rare and non-model plants (other than, e.g., *Arabidopsis thaliana, Nicotiana benthamiana, Oryza sativa*, or many other typical model plants), voucher specimens must be deposited in an accessible herbarium or museum. Vouchers may be requested for review by future investigators to verify the identity of the material used in the study (especially if taxonomic rearrangements occur in the future). They should include details of the populations sampled on the site of collection (GPS coordinates), date of collection, and document the part(s) used in the study where appropriate. For rare, threatened or endangered species this can be waived but it is necessary for the author to describe this in the cover letter.

Editors reserve the rights to reject any submission that does not meet these requirements.

An example of Ethical Statements:

Torenia fournieri plants were used in this study. White-flowered Crown White (CrW) and violet-flowered Crown Violet (CrV) cultivars selected from 'Crown Mix' (XXX Company, City, Country) were kindly provided by Dr. XXX (XXX Institute, City, Country).

Arabidopis mutant lines (SALKxxxx, SAILxxxx,…) were kindly provided by Dr. XXX, institute, city, country).

Clinical Trials Registration

Registration

MDPI follows the International Committee of Medical Journal Editors (ICMJE) **guidelines** which require and recommend registration of clinical trials in a public trials registry at or before the time of first patient enrollment as a condition of consideration for publication.

Purely observational studies do not require registration. A clinical trial not only refers to studies that take place in a hospital or involve pharmaceuticals, but also refer to all studies which involve participant randomization and group classification in the context of the intervention under assessment.

Authors are strongly encouraged to pre-register clinical trials with an international clinical trials register and cite a reference to the registration in the Methods section. Suitable databases include **clinicaltrials.gov**, **the EU Clinical Trials Register** and those listed by the World Health Organisation **International Clinical Trials Registry Platform**.

Approval to conduct a study from an independent local, regional, or national review body is not equivalent to prospective clinical trial registration. MDPI reserves the right to decline any paper without trial registration for further peer-review. However, if the study protocol has been published before the enrolment, the registration can be waived with correct citation of the published protocol.

CONSORT Statement

MDPI requires a completed CONSORT 2010 **checklist** and **flow diagram** as a condition of submission when reporting the results of a randomized trial. Templates for these can be found here or on the CONSORT website (**http://www.consort-statement.org**) which also describes several CONSORT checklist extensions for different designs and types of data beyond two group parallel trials. At minimum, your article should report the content addressed by each item of the checklist.

Sex and Gender in Research

We encourage our authors to follow the **'Sex and Gender Equity in Research – SAGER – guidelines'** and to include sex and gender considerations where relevant. Authors should use

the terms sex (biological attribute) and gender (shaped by social and cultural circumstances) carefully in order to avoid confusing both terms. Article titles and/or abstracts should indicate clearly what sex(es) the study applies to. Authors should also describe in the background, whether sex and/or gender differences may be expected; report how sex and/or gender were accounted for in the design of the study; provide disaggregated data by sex and/or gender, where appropriate; and discuss respective results. If a sex and/or gender analysis was not conducted, the rationale should be given in the Discussion. We suggest that our authors consult the full **guidelines** before submission.

Borders and Territories

Potential disputes over borders and territories may have particular relevance for authors in describing their research or in an author or editor correspondence address, and should be respected. Content decisions are an editorial matter and where there is a potential or perceived dispute or complaint, the editorial team will attempt to find a resolution that satisfies parties involved.

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Publication Ethics Statement

MPs is a member of the Committee on Publication Ethics (**COPE**). We fully adhere to its **Code of Conduct** and to its **Best Practice Guidelines**.

The editors of this journal enforce a rigorous peer-review process together with strict ethical policies and standards to ensure to add high quality scientific works to the field of scholarly publication. Unfortunately, cases of plagiarism, data falsification, image manipulation, inappropriate authorship credit, and the like, do arise. The editors of *MPs* take such publishing ethics issues very seriously and are trained to proceed in such cases with a zero tolerance policy.

Authors wishing to publish their papers in *MPs* must abide to the following:

- Any facts that might be perceived as a possible conflict of interest of the author(s) must be disclosed in the paper prior to submission.
- Authors should accurately present their research findings and include an objective discussion of the significance of their findings.
- Data and methods used in the research need to be presented in sufficient detail in the paper, so that other researchers can replicate the work.
- Raw data should preferably be publicly deposited by the authors before submission of their manuscript. Authors need to at least have the raw data readily available for presentation to the referees and the editors of the journal, if requested. Authors need to

ensure appropriate measures are taken so that raw data is retained in full for a reasonable time after publication.

- Simultaneous submission of manuscripts to more than one journal is not tolerated.
- The journal accepts exact translations of previously published work. All submissions of translations must conform with our **policies on translations**.
- If errors and inaccuracies are found by the authors after publication of their paper, they need to be promptly communicated to the editors of this journal so that appropriate actions can be taken. Please refer to our **policy regarding Updating Published Papers**.
- Your manuscript should not contain any information that has already been published. If you include already published figures or images, please obtain the necessary permission from the copyright holder to publish under the CC-BY license. For further information, see the **Rights and Permissions** page.
- Plagiarism, data fabrication and image manipulation are not tolerated.
    - **Plagiarism is not acceptable** in *MPs* submissions.

Plagiarism includes copying text, ideas, images, or data from another source, even from your own publications, without giving any credit to the original source.

Reuse of text that is copied from another source must be between quotes and the original source must be cited. If a study's design or the manuscript's structure or language has been inspired by previous works, these works must be explicitly cited.

All MDPI submissions are checked for plagiarism using the industry standard software iThenticate. If plagiarism is detected during the peer review process, the manuscript may be rejected. If plagiarism is detected after publication, an investigation will take place and action taken in accordance with our policies.

- **Image files must not be manipulated or adjusted in any way** that could lead to misinterpretation of the information provided by the original image.

Irregular manipulation includes: 1) introduction, enhancement, moving, or removing features from the original image; 2) grouping of images that should obviously be presented separately (e.g., from different parts of the same gel, or from different gels); or 3) modifying the contrast, brightness or color balance to obscure, eliminate or enhance some information.

If irregular image manipulation is identified and confirmed during the peer review process, we may reject the manuscript. If irregular image manipulation is identified and confirmed after publication, we may correct or retract the paper.

Our in-house editors will investigate any allegations of publication misconduct and may contact the authors' institutions or funders if necessary. If evidence of misconduct is found, appropriate action will be taken to correct or retract the publication. Authors are expected to comply with the best ethical publication practices when publishing with MDPI.

Citation Policy

Authors should ensure that where material is taken from other sources (including their own published writing) the source is clearly cited and that where appropriate permission is obtained.

Authors should not engage in excessive self-citation of their own work.

Authors should not copy references from other publications if they have not read the cited work.

Authors should not preferentially cite their own or their friends', peers', or institution's publications.

Authors should not cite advertisements or advertorial material.

In accordance with COPE guidelines, we expect that "original wording taken directly from publications by other researchers should appear in quotation marks with the appropriate citations." This condition also applies to an author's own work. COPE have produced a discussion document on **citation manipulation** with recommendations for best practice.

Reviewer Suggestions

During the submission process, please suggest three potential reviewers with the appropriate expertise to review the manuscript. The editors will not necessarily approach these referees. Please provide detailed contact information (address, homepage, phone, e-mail address). The proposed referees should neither be current collaborators of the co-authors nor have published with any of the co-authors of the manuscript within the last three years. Proposed reviewers should be from different institutions to the authors. You may identify appropriate Editorial Board members of the journal as potential reviewers. You may suggest reviewers from among the authors that you frequently cite in your paper.

English Corrections

MDPI provides minor English editing by native English speakers for all accepted papers, included in the APC. The APC does not cover extensive English editing. Submitted papers should be written in good English and require no more than minor English editing before publication. Your paper could be returned to you at the English editing stage of the publication process if extensive editing is required, which could delay the publication of your work. You may choose to use a paid language-editing service, such as MDPI's **Author Services**, before submitting your paper for publication. If you use an alternative service that provides a confirmation certificate, please send

a copy to the Editorial Office. Authors from economically developing countries or nations should consider registration with **AuthorAid**, a global research community that provides networking, mentoring, resources and training for researchers.

Preprints and Conference Papers

MPs accepts submissions that have previously been made available as preprints provided that they have not undergone peer review. A preprint is a draft version of a paper made available online before submission to a journal.

MDPI operates ***Preprints***, a preprint server to which submitted papers can be uploaded directly after completing journal submission. Note that *Preprints* operates independently of the journal and posting a preprint does not affect the peer review process. Check the *Preprints* **instructions for authors** for further information.

Expanded and high-quality conference papers can be considered as articles if they fulfill the following requirements: (1) the paper should be expanded to the size of a research article; (2) the conference paper should be cited and noted on the first page of the paper; (3) if the authors do not hold the copyright of the published conference paper, authors should seek the appropriate permission from the copyright holder; (4) authors are asked to disclose that it is conference paper in their cover letter and include a statement on what has been changed compared to the original conference paper. *MPs* does not publish pilot studies or studies with inadequate statistical power.

Unpublished conference papers that do not meet the above conditions are recommended to be submitted to the **Proceedings Series journals**.

Authorship

MDPI follows the International Committee of Medical Journal Editors (**ICMJE**) guidelines which state that, in order to qualify for authorship of a manuscript, the following criteria should be observed:

- Substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work; AND
- Drafting the work or reviewing it critically for important intellectual content; AND
- Final approval of the version to be published; AND
- Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Those who contributed to the work but do not qualify for authorship should be listed in the acknowledgments. More detailed guidance on authorship is given by the **International Council of Medical Journal Editors (ICMJE)**.

Any change to the author list should be approved by all authors including any who have been removed from the list. The corresponding author should act as a point of contact between the editor and the other authors and should keep co-authors informed and involve them in major decisions about the publication. We reserve the right to request confirmation that all authors meet the authorship conditions.

For more details about authorship please check **MDPI ethics website**.

Reviewers Recommendation

Authors can recommend potential reviewers. Journal editors will check to make sure there are no conflicts of interest before contacting those reviewers, and will not consider those with competing interests. Reviewers are asked to declare any conflicts of interest. Authors can also enter the names of potential peer reviewers they wish to exclude from consideration in the peer review of their manuscript, during the initial submission progress. The editorial team will respect these requests so long as this does not interfere with the objective and thorough assessment of the submission.

Editorial Independence

Lack of Interference with Editorial Decisions

Editorial independence is of utmost importance and MDPI does not interfere with editorial decisions. All articles published by MDPI are peer reviewed and assessed by our independent editorial boards, and MDPI staff are not involved in decisions to accept manuscripts. When making an editorial decision, we expect the academic editor to make their decision based only upon:

- The suitability of selected reviewers;
- Adequacy of reviewer comments and author response;
- Overall scientific quality of the paper.

In all of our journals, in every aspect of operation, MDPI policies are informed by the mission to make science and research findings open and accessible as widely and rapidly as possible.

Editors and Editorial Staff as Authors

Editorial staff or editors shall not be involved in processing their own academic work. Submissions authored by editorial staff/editors will be assigned to at least two independent outside reviewers. Decisions will be made by other Editorial Board Members who do not have a conflict of interest

with the author. Journal staff are not involved in the processing of their own work submitted to any MDPI journals.

Conflicts of Interest

According to The International Committee of Medical Journal Editors, “Authors should avoid entering into agreements with study sponsors, both for-profit and non-profit, that interfere with authors’ access to all of the study’s data or that interfere with their ability to analyze and interpret the data and to prepare and publish manuscripts independently when and where they choose.”

All authors must disclose all relationships or interests that could inappropriately influence or bias their work. Examples of potential conflicts of interest include but are not limited to financial interests (such as membership, employment, consultancies, stocks/shares ownership, honoraria, grants or other funding, paid expert testimonies and patent-licensing arrangements) and non-financial interests (such as personal or professional relationships, affiliations, personal beliefs).

Authors can disclose potential conflicts of interest via the online submission system during the submission process. Declarations regarding conflicts of interest can also be collected via the **MDPI disclosure form**. The corresponding author must include a summary statement in the manuscript in a separate section “Conflicts of Interest” placed just before the reference list. The statement should reflect all the collected potential conflicts of interest disclosures in the form.

See below for examples of disclosures:

Conflicts of Interest: Author A has received research grants from Company A. Author B has received a speaker honorarium from Company X and owns stocks in Company Y. Author C has been involved as a consultant and expert witness in Company Z. Author D is the inventor of patent X.

If no conflicts exist, the authors should state:

Conflicts of Interest: The authors declare no conflicts of interest.

Editorial Procedures and Peer-Review

Pre-check

Immediately after submission, the journal’s Managing Editor will perform the technical pre-check to assess:

- Overall suitability of the manuscript to the journal/section/Special Issue;
- Manuscript adherence to high-quality research and ethical standards;
- Standards of rigor to qualify for further review.

The academic editor (i.e., the Editor-in-Chief in the case of regular submissions, the Guest Editor in the case of Special Issue submissions, or an Editorial Board member in the case of a conflict of interest and of regular submissions if the Editor-in-Chief allows) will be notified of the submission and invited to perform an editorial pre-check. During the editorial pre-check phase, the academic editor will assess the suitability of the submission with respect to the scope of the journal, as well as the overall scientific soundness of the manuscript, including the relevance of the references and the correctness of the applied methodology. Academic editors can decide to reject the manuscript, request revisions before peer-review, or continue with the peer-review process and recommend suitable reviewers.

Peer-Review

Once a manuscript passes the initial checks, it will be assigned to at least two independent experts for peer-review. A single-blind review is applied, where authors' identities are known to reviewers. Peer review comments are confidential and will only be disclosed with the express agreement of the reviewer.

In the case of regular submissions, in-house assistant editors will invite experts, including recommendations by an academic editor. These experts may also include *Editorial Board Members* and Guest Editors of the journal. Potential reviewers suggested by the authors may also be considered. Reviewers should not have published with any of the co-authors during the past three years and should not currently work or collaborate with any of the institutions of the co-authors of the submitted manuscript.

Optional Open Peer-Review

The journal operates optional open peer-review: *Authors are given the option for all review reports and editorial decisions to be published alongside their manuscript. In addition, reviewers can sign their review, i.e., identify themselves in the published review reports.* Authors can alter their choice for open review at any time before publication, but once the paper has been published changes will only be made at the discretion of the *Publisher* and *Editor-in-Chief*. We encourage authors to take advantage of this opportunity as proof of the rigorous process employed in publishing their research. To guarantee impartial refereeing, the names of referees will be revealed only if the referees agree to do so, and after a paper has been accepted for publication.

Editorial Decision and Revision

All the articles, reviews and communications published in MDPI journals go through the peer-review process and receive at least two reviews. The in-house editor will communicate the decision of the academic editor, which will be one of the following:

- *Accept after Minor Revisions*:
  The paper is in principle accepted after revision based on the reviewer's comments. Authors are given five days for minor revisions.
- *Reconsider after Major Revisions*:
  The acceptance of the manuscript would depend on the revisions. The author needs to provide a point by point response or provide a rebuttal if some of the reviewer's comments cannot be revised. A maximum of two rounds of major revision per manuscript is normally provided. Authors will be asked to resubmit the revised paper within a suitable time frame, and the revised version will be returned to the reviewer for further comments. If the required revision time is estimated to be longer than 2 months, we will recommend that authors withdraw their manuscript before resubmitting so as to avoid unnecessary time pressure and to ensure that all manuscripts are sufficiently revised.
- *Reject and Encourage Resubmission*:
  If additional experiments are needed to support the conclusions, the manuscript will be rejected and the authors will be encouraged to re-submit the paper once further experiments have been conducted.
- *Reject*:
  The article has serious flaws, and/or makes no original significant contribution. No offer of resubmission to the journal is provided.

All reviewer comments should be responded to in a point-by-point fashion. Where the authors disagree with a reviewer, they must provide a clear response.

Author Appeals

Authors may appeal a rejection by sending an e-mail to the Editorial Office of the journal. The appeal must provide a detailed justification, including point-by-point responses to the reviewers' and/or Editor's comments using an **appeal form**. Appeals can only be submitted following a "reject and decline resubmission" decision and should be submitted within three months from the decision date. Failure to meet these criteria will result in the appeal not being considered further. The *Managing Editor* will forward the manuscript and related information (including the identities of the referees) to a designated *Editorial Board Member*. The Academic Editor being consulted will be asked to provide an advisory recommendation on the manuscript and may recommend acceptance, further peer-review, or uphold the original rejection decision. This decision will then be validated by the *Editor-in-Chief*. A reject decision at this stage is final and cannot be reversed.

Production and Publication

Once accepted, the manuscript will undergo professional copy-editing, English editing, proofreading by the authors, final corrections, pagination, and, publication on the **www.mdpi.com** website.

Promoting Equity, Diversity, and Inclusiveness within MDPI Journals

Our Managing Editors encourage the Editors-in-Chief and Associate Editors to appoint diverse expert Editorial Boards. This is also reflective in our multi-national and inclusive workplace. We are proud to create equal opportunities without regard to gender, ethnicity, sexual orientation, age, religion, or socio-economic status. There is no place for discrimination in our workplace and editors of MDPI journals are to uphold these principles in high regard.

Resource Identification Initiative

To improve the reproducibility of scientific research, the **Resource Identification Initiative** aims to provide unique persistent identifiers for key biological resources, including antibodies, cell lines, model organisms and tools.

We encourage authors to include unique identifiers - RRIDs- provided by the **Resource Identification Portal** in the dedicated section of the manuscript.

To help authors quickly find the correct identifiers for their materials, there is a single **website** where all resource types can be found and a ‘cite this’ button next to each resource, that contains a proper citation text that should be included in the methods section of the manuscript.

Annexure N

Ethics Approval Letter

Private Bag X1290, Potchefstroom
South Africa 2520

Tel: 086 016 9698
Web: http://www.nwu.ac.za/

North-West University Health Research Ethics Committee (NWU-HREC)

Tel: 018 299-1206
Email: Ethics-HRECApply@nwu.ac.za (for human studies)

23 October 2022

ETHICS APPROVAL LETTER OF STUDY

Based on approval by the North-West University Health Research Ethics Committee (NWU-HREC) on 23/10/2022, the NWU-HREC hereby approves your study as indicated below. This implies that the NWU-HREC grants its permission that, provided the general conditions specified below are met and pending any other authorisation that may be necessary, the study may be initiated, using the ethics number below.

Study title: Self-Double-Emulsifying Drug Delivery Systems: a novel approach to topical fixed-dose anti-tubercular drug delivery
Principal Investigator/Study Supervisor/Researcher: Prof JM Viljoen
Student: D van Staden - 25057146

Ethics number:

N	W	U	-	0	0	1	1	1	-	1	7	-	A	1	-	1	5
Institution				Study Number						Year			Status	Sub-study			

Status: S = Submission; R = Re-Submission; P = Provisional Authorisation; A = Authorisation

Application Type: Sub-study
Commencement date: 23/10/2022
Expiry date: 31/10/2023

Risk: **Minimal**

Approval of the study is provided for a year, after which continuation of the study is dependent on receipt and review of an annual monitoring report and the concomitant issuing of a letter of continuation. A monitoring report is due at the end of October annually until completion of the study.

General conditions:

While this ethics approval is subject to all declarations, undertakings and agreements incorporated and signed in the application form, the following general terms and conditions will apply:

- *The principal investigator/study supervisor/researcher must report in the prescribed format to the NWU-HREC:*
  - *Annually on the monitoring of the study, whereby a letter of continuation will be provided annually, and upon completion of the study; and*
  - *without any delay in case of any adverse event or incident (or any matter that interrupts sound ethical principles) during the course of the study.*
- *The approval applies strictly to the proposal as stipulated in the application form. Should any amendments to the proposal be deemed necessary during the course of the study, the principal investigator/study supervisor/researcher must apply for approval of these amendments at the NWU-HREC, prior to implementation. Should there be any deviations from the study proposal without the necessary approval of such amendments, the ethics approval is immediately and automatically forfeited.*
- *Annually a number of studies may be randomly selected for active monitoring.*
- *The date of approval indicates the first date that the study may be started.*
- *In the interest of ethical responsibility, the NWU-HREC reserves the right to:*
  - *request access to any information or data at any time during the course or after completion of the study;*

- *to ask further questions, seek additional information, require further modification or monitor the conduct of your research or the informed consent process;*
- *withdraw or postpone approval if:*
  - *any unethical principles or practices of the study are revealed or suspected;*
  - *it becomes apparent that any relevant information was withheld from the NWU-HREC or that information has been false or misrepresented;*
  - *submission of the annual monitoring report, the required amendments, or reporting of adverse events or incidents was not done in a timely manner and accurately; and/or*
  - *new institutional rules, national legislation or international conventions deem it necessary.*
- *NWU-HREC can be contacted for further information via Ethics-HRECApply@nwu.ac.za or 018 299 1206*

Special conditions of the research approval due to the COVID-19 pandemic:

Please note: Due to the nature of the study i.e. (laboratory work involving the analysis of previously collected biological samples), this study will be able to proceed during the current alert level, following receipt of the approval letter. No additional COVID-19 restrictions have been placed on the study except that the researcher must ensure that before proceeding with the study that all research team members have reviewed the North-West University COVID-19 Occupational Health and Safety Standard Operating Procedure.

The NWU-HREC would like to remain at your service and wishes you well with your study. Please do not hesitate to contact the NWU-HREC for any further enquiries or requests for assistance.

Yours sincerely,

Chairperson NWU-HREC

Current details:(23239522) G:\My Drive\9. Research and Postgraduate Education\9.1.5.4 Templates\9.1.5.4.2_NWU-HREC_EAL.docm
20 August 2019
File Reference: 9.1.5.4.2

Annexure O

Proof of submission to '*Methods and Protocols*'

[MPs] Manuscript ID: mps-2634536 - Minor Revisions Inbox ×

MPs Editorial Office <mps@mdpi.com> Thu, Oct 19, 6:27 AM (1 day ago)
to Joe, me, Richard, Frank, MPs

Dear Professor Viljoen,

Thank you again for your manuscript submission:

Manuscript ID: mps-2634536
Type of manuscript: Technical Note
Title: Development of a HPLC method for analysis of a combination of clofazimine, isoniazid, pyrazinamide, and rifampicin incorporated into a dermal self-double-emulsifying drug delivery system
Authors: Daniëlle Van Staden, Richard K. Haynes, Frank Van der Kooy, Joe M Viljoen *
Received: 18 Sep 2023
E-mails: dvanstaden711@gmail.com, haynes@ust.hk, frank.vanderkooy@nwu.ac.za, joe.viljoen@nwu.ac.za
Submitted to section: Biochemical and Chemical Analysis & Synthesis, https://www.mdpi.com/journal/mps/sections/biochemical_chemical_analysis_synthesis

Your manuscript has been reviewed by experts in the field. Please find your manuscript with the referee reports at this link:
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(I) Please revise your manuscript according to the referees' comments and upload the revised file within 5 days.
(II) Please use the version of your manuscript found at the above link for your revisions.
(III) Please check that all references are relevant to the contents of the manuscript.
(IV) Any revisions to the manuscript should be highlighted, such that any changes can be easily reviewed by editors and reviewers.

[MPs] Manuscript ID: mps-2634536 - Revised Version Received Inbox ×

MPs Editorial Office <mps@mdpi.com> Wed, Oct 25, 3:38 AM (1 day ago)
to Joe, me, Richard, Frank, MPs

Dear Professor Viljoen,

Thank you very much for providing the revised version of your paper:

Manuscript ID: mps-2634536
Type of manuscript: Technical Note
Title: Development of a HPLC method for analysis of a combination of clofazimine, isoniazid, pyrazinamide, and rifampicin incorporated into a dermal self-double-emulsifying drug delivery system
Authors: Daniëlle Van Staden, Richard K. Haynes, Frank Van der Kooy, Joe M Viljoen *
Received: 18 Sep 2023
E-mails: dvanstaden711@gmail.com, haynes@ust.hk, frank.vanderkooy@nwu.ac.za, joe.viljoen@nwu.ac.za
Submitted to section: Biochemical and Chemical Analysis & Synthesis,
https://www.mdpi.com/journal/mps/sections/biochemical_chemical_analysis_synthesis
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We will continue processing your paper and will keep you informed about the status of your submission.

Kind regards,
--
Best regards,
Ms. Anika Deng
Section Managing Editor
Email: anika.deng@mdpi.com

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