

Establishing three-dimensional cell culture models to measure biotransformation and toxicity

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**B. Sc, Hons. B. Sc (Biochemistry), M. Sc
(Pharmaceutics)**

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

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Graduation May 2018

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**Dedicated to Vasco Botelho Carvalho,
you were taken too soon.**

1989 - 2017

The road not taken

*Two roads diverged in a yellow wood,
And sorry I could not travel both
And be one traveler, long I stood
And looked down one as far as I could
To where it bent in the undergrowth;*

*Then took the other, as just as fair,
And having perhaps the better claim,
Because it was grassy and wanted wear;
Though as for that the passing there
Had worn them really about the same,*

*And both that morning equally lay
In leaves no step had trodden black.
Oh, I kept the first for another day!
Yet knowing how way leads on to way,
I doubted if I should ever come back.*

*I shall be telling this with a sigh
Somewhere ages and ages hence:
Two roads diverged in a wood, and I—
I took the one less traveled by,
And that has made all the difference.*

-Robert Frost-

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LIST OF ABBREVIATIONS

2D	Two-dimensional
3D	Three-dimensional
A	
<i>A. arborescens</i>	<i>Aloe arborescens</i>
A1AT	α_1 -antitrypsin
APAP	Acetaminophen
<i>A. barbadensis</i>	<i>Aloe barbadensis</i>
ADME	Absorption, distribution, metabolism and excretion
AK	Adenylate kinase
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
ASGPR	Asialoglycoprotein receptor
AST	Aspartate aminotransferase
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
<i>A. vera</i>	<i>Aloe vera</i>
B	
BC	Bile canaliculi
Bp	Basis pair
BSA	Bovine serum albumin
BSEP	Bile salt export pump
C	
CAR	Constitutive androstane receptor
CaCl ₂ ·2H ₂ O	Calcium chloride dihydrate
<i>C. angustifolia</i>	<i>Cassia angustifolia</i>
<i>C. senna</i>	<i>Cassia senna</i>
CE	Capillary electrophoresis

cfDNA	Cell-free DNA
CYP450	Cytochrome P450
Cyt c	Cytochrome c
D	
DBV	Divinylbenzene
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
DSIEC	Dietary Supplement Information Expert Committee
E	
<i>E. angustifolia</i>	<i>Echinacea angustifolia</i>
EC ₅₀	Half maximal effective concentration
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EFC	7-ethoxy-4-trifluoromethyl coumarin
EGCG	(-)-epigallocatechin-3-gallate
EHA	2-ethylhexylacrylate
<i>E. pallida</i>	<i>Echinacea pallida</i>
<i>E. purpurea</i>	<i>Echinacea purpurea</i>
F	
F	Female
FCS	Fetal calf serum
GelMA	Gelatin methacryloyl
GGT	γ-Glutamyl transferase
H	
H&E	Haematoxylin and eosin
HCA	Hierarchical cluster analysis
HFC	7-hydroxy-4-trifluoromethyl
HILI	Herb-induced liver injury

I

IC₅₀ Half maximal inhibitory concentration

iPSC Induced pluripotent stem cells

K

K_m Michaelis constant

L

L. tridentate *Larrea tridentate*

LC-MS Liquid chromatography mass spectrometry

LD₅₀ Median lethal dose

LDH Lactate dehydrogenase

LOD Limit of detection

LOQ Limit of quantification

M

M Male

MDR1 Multi-drug resistance protein I

MDR2 Multi-drug resistance protein II

MFO Mixed-function oxidase

MRC Medical Research Council

mRNA Messenger ribonucleic acid

MRP2 Multidrug resistance associated protein 2

MS Mass spectrometry

N

NaCl Sodium chloride

NAFLD Non-alcoholic fatty liver disease

NAPQI N-acetyl-p-benzoquinoneimine

NASA National Aeronautics Space Administration

NCDs Neoclerodane diterpenes

NDGA Non-dihydroguaiaretic acid

NRF National Research Foundation

NWU	North-west University
P	
PBS	Phosphate buffered saline
PC	Polycarbonate
PDMS	Poly(dimethylsilohexane)
PNIPAAm	Poly(N-isopropylacrylamide)
PMMA	Poly-methyl methacrylate
PS	Polystyrene
PSf	Polysulfone
PSf-g-PEG	Polysulfone-g-poly (ethylene glycol)
PXR	Pregnane X receptor
R	
RER	Rough endoplasmic reticulum
RNA	Ribonucleic acid
ROS	Reactive oxygen species
r.p.m	Rotations per minute
R's	Reduce, replace, refine
RSD	Relative standard deviation
RT-PCR	Real-time polymerase chain reaction
RXR	Retinoid-X-receptor
S	
SAPN	Self-assembling peptide nano-scaffold
SAVC	South African Veterinary Council
SC	Sinusoidal channels
SD	Standard deviation
SEM	Scanning electron microscopy
<i>S. repens</i>	<i>Serenoa repens</i>
STY	Styrene

T

TCM Traditional Chinese Medicines

T. chamaedrys *Teucrium chamaedrys*

TG Triglycerides

TJ Tight junctions

TUNNEL Terminal deoxynucleotidyl transferase dUTP Nick-End labeling

U

UGT 5'-diphospho-glucuronosyltransferase

uHPLC Ultra-High-pressure liquid chromatography

V

V. officinalis *Valerian officinalis*

ACKNOWLEDGEMENTS

As I sat in Dr Chrisna Gouws's office in January 2015 at the start of my PhD I was told that I will be embarking on a live changing adventure, that will challenge me, question my sanity but will be one of the most rewarding journeys I could undertake. I was told to make sure I know why I want to do this, as there will come a day that I will ask myself why I did this to myself. It is now November 2017, I only now fully comprehend what she had meant.

The eight chapters of this thesis is the total sum of the most challenging three years of my life, academically and emotionally. I have cried more times than I care to remember, at times I laughed so hard I could not stop and gained so many experiences. I made many friends, lost a great many and I grew, I learned things about myself that I never knew, I pushed myself and reached and crossed boundaries that I previously thought was not possible. Many of my experiences during the last three years were humbling, especially when considering that it takes three years to grow an LS180 spheroid and realising there is so much you do not know. For this reason, I want to thank the following people that has made all this possible.

- To my heavenly Father, all my strength everything that I am lies in You! And nothing in my life up to this point would have been possible if I didn't have You in my life. I have never wanted for anything, even on my darkest day, for this I thank You.
- Steve and Theresa Engelbrecht, -Θupa" and -Θuma"! Wow, what a journey. No amount of words will ever be enough to express my gratitude towards you! You have always been my biggest supporters, motivating me, allowing me to fall apart at times, but never ever letting me give up. Thank you for always making me feel like I am enough. I love you, you are mine!
- Megan Nagel, I do not deserve you! You caught every tantrum and every tear with your bare hands and kept me going on my darkest days, thank you! You are my best friend, my moral compass and my rock! Thank you for all the time and energy that you put into our friendship and this PhD for taking time out of your own busy schedule to read and edit my work. For long distance calls every day for six months and otherwise silly banter just to get me through my days. One day I will try and make it up to you I promise.
- Zenobia Bergh, my friend and my light thank you for your support and insight. For getting excited about cells as only a researcher can and for crying with me when another experiment fails, or my spheroid children succumbs to a technical death. I do not say this enough, I really do appreciate you!
- Dr Clarissa Willers, I have become so fond and dependent on you. You are the yin to my yang, an exceptional lab partner, teacher and friend. You always handle everything,

including myself, like it is easy, and I know this not to be true so thank you!

- Jacques Rossouw and Roan Swanepoel, you will forever be my little ducks! Thank you for all your help, from driving lessons to editing manuscripts and putting together ethical applications. I do not think you realise how fond I have become of the two of you over the last year, your dedication and work ethics are exceptional, and I look forward to seeing what you both will achieve in your future! Thank you so much for everything!
- Caitlin and Cobus Theron and Mea Engelbrecht, thank you for your unfailing love and support you are very dear to my heart.
- Brendan Smit, you met and fell in love with me at my worst. You let me cry over every rat that had to be sacrifice in the name of science, listen intently while I ramble on about my work and you picked me up when I fell apart, or allowed the world to get me down. Thank you for not walking out on me yet, if we could survive this, we can anything. I will now present you with my best.
- Kira Joensen and Søren Damsgaard, my family away from home. Thank you for making me feel welcome and at home while staying in Denmark. Kira for teaching me everything I know about growing spheroids and holding my hand every step of the way while learning, you are a remarkable teacher and a wonderful friend. –Fusind tak!”
- My dearest Vasco Carvalho, you were an exceptional scientist with a very bright future, thank you for all your help and support and allowing me to learn from you.
- Fanie Engelbrecht, thank you for saving my data, many people will not know this, but my PhD will not have been completed without your help. I am forever in your debt.
- Dr Chrisna Gouws, study promotor, mother, friend and confidant. I have grown so much in so many ways because of you. Yes, we do disagree, and yes, I did sometimes disappoint, however I also always tried to give you my best and *vice versa*. Thank you for believing in me and for giving me so many opportunities to grow and learn professionally and personally I appreciate it tremendously!
- Prof Sias Hamman, you are an exceptional study promotor, I have gained so much knowledge from you scientifically and personally and I have the utmost respect for you! Thank you for all your time, energy and commitment to not only me but all your students. We do not say it enough, nevertheless we do appreciate you.
- Prof Jan Steenekamp, my University dad! Thank you for the hours spent listening and giving advice, you have played a critical part in my PhD without even knowing it! Thank you.
- Prof Krzysztof Wrzesinski, I have endless admiration for you. Thank you for being so patient with me, you are truly worth gold to me.
- Prof Steven Fey, thank you for being so enduring always friendly and always helpful. For hospital visits away from home and exceptional writing abilities.
- Mr Cor Bester, Mrs Antionette Fick and Mr Kobus Venter, for your help with the *in vivo* study, I have so much admiration and respect for the work that you do.

- The staff at Pathcare Potchefstroom for your friendly service and help during the *in vivo* study.
- Dr Dewald Steyn, thank you for your input on your subject matter.
- Prof Alvaro Viljoen and staff at the Tswane University of Technology for the characterization of the crude Uzara water extract samples.
- The University of Southern Denmark Odense, for allowing me to learn and work during my stay in Denmark.
- The North-West University for the doctoral, travel and institutional bursary.
- The National Research Foundation (NRF) for the Innovation doctoral scholarship and travel bursary.

ABSTRACT

A great proportion of new chemical entities will be terminated from the clinical drug development pipeline as a result of deficiencies in drug absorption, distribution, biotransformation and elimination as well as potential to cause toxicity. Exposure of the liver (and other organs) to hepatotoxins, may potentially interact with cellular constituents, causing toxicity and various lesions. The pre-clinical assessment of hepatotoxic potential of new drug entities and herbal compounds are investigated on a tissue, cellular and molecular level by employing various *in vitro* and *in vivo* techniques.

The *in vitro* models currently available mainly involve traditional two-dimensional (2D) cell culture techniques; however, these models lack various tissue specific properties found in the *in vivo* environment. As a result, pre-clinical assessment of drug hepatotoxicity and biotransformation still rely predominantly on *in vivo* animal models. To reduce the use of animal models, more reliable and readily available *in vitro* models are needed, capable of bridging the gap between the existing models and the *in vivo* situation. Three-dimensional (3D) spheroid cell cultures offer higher physiological relevance than traditional 2D cell cultures, overcoming many of the shortcomings associated with traditional 2D cell cultures. Specifically, the dynamic micro-tissue 3D spheroid cell culture system produced in micro-gravity bioreactors has attracted attention, although several other types of multi-cellular spheroid systems are also currently under investigation.

This study investigated the potential of the 3D HepG2/C3A spheroid model to evaluate the acute and sub-chronic hepatotoxic potential of a crude aqueous *Xysmalobium undulatum* (Uzara) extract. Acute hepatotoxic effects were investigated in 2D and 3D HepG2/C3A cell cultures at concentrations of 200, 350, 500, and 750 mg/kg. Parameters evaluated included cell proliferation, glucose uptake, intracellular adenosine triphosphate (ATP) levels and adenylate kinase (AK) release. Furthermore, sub-chronic hepatotoxicity of crude Uzara aqueous extract was investigated during a sub-chronic 21-day study in the 3D HepG2/C3A spheroid model as well as in Sprague Dawley rats.

The results from the *in vitro* study clearly indicated hepatotoxic effects and possible liver damage following treatment with valproic acid (the positive control group) as indicated by the growth inhibition observed, the loss of cell viability and the increased cytotoxicity as indicated by the reduced intracellular ATP levels and increased AK levels. The results also indicated that crude Uzara water extract had dose-dependent hepatotoxic potential, although the effects appeared to be exaggerated in the 2D cell cultures compared to the 3D spheroid cultures. The results was also supported by the increased *in vivo* levels of AST, ALT and LDH and the slight increase in triglycerides, following treatment of the Sprague Dawley rats with valproic acid. This is indicative of hepatic cellular damage, possibly resulting in hepatotoxicity. Similarly, following treatment with the crude Uzara aqueous extract, results

obtained from the *in vivo* Sprague Dawley model indicated moderate hepatotoxic potential. The results confirmed the potential of the 3D HepG2/C3A spheroid model to effectively and reliably predict the long-term outcomes of possible hepatotoxicity.

A novel 3D spheroid model for biotransformation applications was also developed, employing the LS180 cell line and micro-gravity bioreactors. The human colon carcinoma cell line, LS180, is often used as a biotransformation model to study inhibition and induction of CYP450 enzymes *in vitro*. The new three-dimensional cell culture model combined the dynamic rotating micro-gravity bioreactor technique with the micro-encapsulation technique, using sodium alginate. These encapsulated LS180 spheroids have the potential to be employed as a novel long-term culturing model for future *in vitro* biotransformation studies.

Key words: Biotransformation, hepatotoxicity, HepG2/C3A, *in vitro* models, LS180, rotating bioreactors, Sprague Dawley, three-dimensional cell culturing.

FOREWORD

Herewith I present the thesis entitled: *Establishing three-dimensional cell culture models to measure biotransformation and toxicity*. The aim of this study was to establish a three-dimensional cell culture model to evaluate the hepatotoxic potential of substances (i.e. *Xysmalobium undulatum* and Valproic acid), which could compare well with *in vivo* models, and to establish a novel three-dimensional bio-transformation model.

This thesis is presented in article format to comply with the necessary guidelines and requirements for the degree Doctor of Philosophy in Pharmaceutics at the North-West University. This thesis includes an introductory chapter, followed by two review manuscripts of which one was published in the peer-reviewed journal *Expert Opinion on Drug Metabolism and Toxicology* and the other review manuscript was prepared for submission to the journal *Toxicology Mechanisms and Methods*. This is followed by three research manuscripts, of which one has been submitted for publication in the peer-reviewed journal *International Journal of Molecular Sciences* and is currently under review, another manuscript was prepared for submission to *Biochemical Pharmacology* and the third paper has been published in *International Journal of Biochemistry and Cell Biology*. I also present a methodology manuscript prepared for submission to the peer-reviewed journal *Journal of Cell Biology*, and I conclude with a chapter advising on future recommendations.

As a PhD candidate I was responsible for all parts of the thesis presented, including experimental design and execution, data collection and processing, interpretation of results and preparation and writing of all manuscripts presented. All supervisors and collaborators involved with the study and manuscripts presented are herewith acknowledged as co-authors.

Co-author contributions:

Dr. Chrisna Gouws

PhD study promotor, is the principle investigator whom was responsible for initiation and design of the study, funding, critical evaluation, data interpretation and co-author.

Prof. Sias Hamman

PhD study co-promotor, responsible for funding, critical evaluation, data interpretation and co-author.

Associate Prof. Krzysztof Wrzesinski

PhD study co-promotor, responsible for funding and the critical evaluation, data interpretation and co-author.

Prof. Stephen John Fey

Editorial input, critical evaluation and co-author.

Prof. Lissinda du Plessis

Editorial input and co-author of the review manuscript published in the journal *–Expert Opinion on Drug Metabolism and Toxicology*".

Dr. Dewald Steyn

Editorial input and co-author of the review manuscript published in the journal *–Expert Opinion on Drug Metabolism and Toxicology*".

Prof. Jan Steenekamp

Editorial input and co-author of the review manuscript published in the journal *–Expert Opinion on Drug Metabolism and Toxicology*".

Dr. Christo Muller

Editorial input and co-author of the review manuscript published in the journal *–Expert Opinion on Drug Metabolism and Toxicology*".

Dr. Janine Aucamp

Shared first authorship of the research article published in the journal *–International Journal of Biochemistry and Cell Biology*". Responsible for data collection and interpretation, critical evaluation, editorial input and co-author.

Dr. Abel Bronkhorst

Responsible for the critical evaluation, data interpretation, editorial input and co-author to the research article published in the journal *–International Journal of Biochemistry and Cell Biology*".

Prof. Piet Pretorius

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Prof. Alvaro Viljoen

Responsible for chemical analysis of the plant material, data interpretation, editorial input and co-author to the research article submitted and under peer-review to the journal *~~International Journal of Molecular Sciences~~*".

Author statement:

Herewith the co-authors verify their involvement with and individual contributions to the study, and grant permission for the inclusion of the relevant manuscripts in the thesis presented.

I herewith declare my role, as stated above, in the manuscripts related to this thesis: *~~Establishing three-dimensional cell culture models to measure biotransformation and toxicity~~*". The PhD candidate Ms. Carlemi Calitz also has my consent to include the manuscripts as part of her thesis presented.

CHAPTER 1

INTRODUCTION AND PROBLEM STATEMENT

1. INTRODUCTION

1.1 Importance of *in vitro* models in drug development

It has been estimated that nearly 40% of new chemical entities are terminated from further clinical drug development due to deficiencies in ADME (i.e. absorption, distribution, metabolism and excretion), while toxicity adds to a further 21% of failures in the clinical drug development process (Tingle & Helsby, 2006). Various *in vitro* screening models and *in vivo* pre-clinical models are available to investigate drug metabolism (or biotransformation) and toxicity properties prior to clinical trials (Tingle & Helsby, 2006; Wrzesinski & Fey, 2013).

Mammalian cell cultures have been employed since the 1940s as *in vitro* models for toxicology studies (Rinaldini, 1952). These models strive to predict the outcomes expected in humans (Wrzesinski & Fey, 2013). However, traditional two-dimensional (2D) *in vitro* models do not effectively fulfil the requirements of predictability of the human situation, and provide only limited information due to a lack of physiological relevance. Ideally, primary human liver-derived cells should be used in metabolism related studies, however, rapid loss of cytochrome P450 (CYP450) enzyme activity, variation between batches, as well as limited availability of donor tissue render it inadequate (Donato *et al.*, 2008). Furthermore, immortal cell lines from hepatic origin in general express relatively low quantities of CYP450 enzymes (Donato *et al.*, 2008). Although recombinant cell lines are genetically engineered to express human drug-metabolising enzymes, they only express a single enzyme with activity profiles different from that of native enzymes *in vivo*, which is a huge disadvantage (Donato *et al.*, 2008).

The current gold standard in toxicology research involves *in vivo* studies, which are not only complex but also costly, time consuming and ethically challenging (Soldatow *et al.*, 2013). *In vivo* animal models have ethical and moral challenges, which led to the development of the three R's principle namely reduce, replace and refine (Wrzesinski & Fey, 2013; Baumans, 2004). Animal studies are also subject to criticism regarding the reliability thereof as they sometimes require high doses exceeding the dosages that humans are exposed to, often resulting in inaccuracies. Standard laboratory animals bred under controlled conditions also do not take into account the genetic variability within the human population (Soldatow *et al.*, 2013). Furthermore, species differences between animals and humans may result in differences in the expression of various membrane transporters and metabolising enzymes

relevant in areas of drug delivery, drug toxicity, as well as drug interactions. Genomic sequence differences between species will also give rise to differences in the tertiary structure of proteins, and thus may affect drug-protein binding (Sabolić *et al.*, 2011).

Three-dimensional (3D) cell culturing systems are being explored as novel models, capable of more closely resembling native tissues and their physiological functions to ensure higher physiological relevance, while at the same time bridging the gap between current *in vitro* and *in vivo* models (Wrzesinski & Fey, 2013; Haycock, 2011; Lin & Chang, 2008).

1.2. Three-dimensional cell culture models

Traditional 2D cell culture models often lack tissue specific properties found within *in vivo* organ systems, since cells within whole organisms (*in vivo*) form part of an intricate structure having interactions with both neighbouring cells, as well as the extracellular matrix (ECM). These interactions between cells and the ECM result in a complex communication network made possible by both biochemical and mechanical signals (Lin & Chang, 2008). Due to the fact that 2D cell models are lacking these advanced physiological functions, cells grown in 2D cannot be seen as equivalent to those present in intact organs (Wrzesinski & Fey, 2013). In an attempt to reduce these differences and to bridge the gap between cell-based experimental approaches, animal models and humans, 3D cell culture models are being developed (Lin & Chang, 2008).

A variety of 3D cell culturing techniques are currently being explored and used, each offering various advantages and disadvantages. Although tissue explants (i.e. small pieces of excised tissue with dimensions of millimetres) that are dissected from animal models and maintained *in vitro* are currently being exploited in biomedical research fields, they are subject to strict ethical considerations, making the ease of obtaining specimens difficult (Lin & Chang, 2008; Antoni *et al.*, 2015). The multi-cellular 3D spheroid cell culture system has the potential to overcome the difficulties presented by both animal and 2D cell culture models (Lin & Chang, 2008; Wrzesinski & Fey, 2013). Different multi-cellular spheroid systems are currently under investigation, including hanging drop cultures, non-adhesive surfaces, spinner flasks, National Aeronautics Space Administration (NASA) rotary system, micro-moulding, 3D scaffolds, poly(N-isopropylacrylamide) (PNIPAAm) cell sheets, pimarid dishes, galactosylated substrates, pellet cultures, monoclonal growth and external force enhancement (Lin & Chang, 2008; Soldatow *et al.*, 2013).

The 3D cell spheroid model that has been established in this study was grown in microgravity ProtoTissue™ bioreactors developed by Wrzesinski and colleagues, as depicted in Figure 1 (Wrzesinski & Fey, 2013). These spheroids have multi-cellular arrangements that mimic the 3D architecture of tissues, with sizable cell-cell interactions such as tight junctions and diffusion limits mimicking *in vivo* physiological barriers found during drug transport (Metha *et al.*, 2012:2). The microgravity bioreactor, capable of producing 3D spheroids, is revolutionising mainstream *in vitro* cell culture work by providing better *in vivo* mimicking properties than the traditional 2D cell culturing models and, although a novel concept, its application in herb-drug interaction studies has great potential.

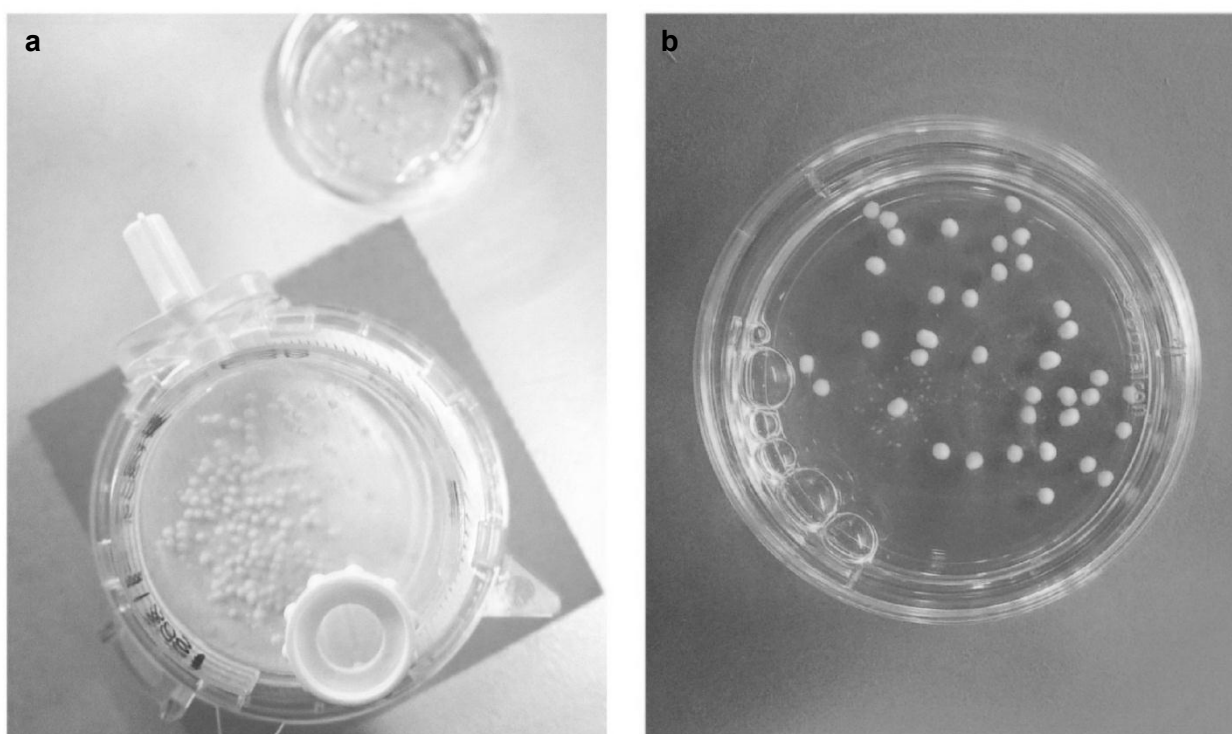


Figure 1: Images of multiple HepG2/C3A spheroids in a ProtoTissue™ bioreactor (a) and 39-day old HepG2/C3A spheroids prepared for microscopy (b).

Although there are many different multi-cellular spheroid systems available, this specific rotating bioreactor based spheroid model was chosen for this study, since it has numerous advantages for the specific applications of this study. A comparison of the most common multi-cellular spheroid systems available is presented in Table 1.

Table 1:**Overview of various three-dimensional cell culture models (Calitz *et al.*, 2018)**

Sandwich culture	Micro-chip	Scaffolds	Hollow Fibre	Suspension culture	Hanging drop culture	Rotating bioreactors
Culture time of one week	Culture time less than one week	Two week culture time	Four week culture time	Three week culture time	Three week culture time	Six weeks culture time
Good short-term functionality with lesser long-term functionality	Good short-term functionality with lesser long-term functionality	Excellent short-term functionality with lesser long-term functionality	Excellent short and long-term functionality	Short- and long-term functionality both little	Excellent short-term functionality with lesser long-term functionality	Excellent short- and long-term functionality
No recovery of <i>in vivo</i> physiology	No recovery of <i>in vivo</i> physiology	Little to no recovery of <i>in vivo</i> physiology	Moderate recovery of <i>in vivo</i> physiology	No recovery of <i>in vivo</i> physiology	Moderate recovery of <i>in vivo</i> physiology	Advanced recovery of <i>in vivo</i> physiology
High-throughput. Easy to use, little skill required, however little versatility in sampling	Very high-throughput. Very easy to use, little skill required, however little versatility in sampling	Very high-throughput. Very easy to use, little skill required, however little versatility in sampling	Low throughput. Difficult to use, skill required, with little versatility in sampling	Moderate throughput. Very easy to use, little skill required, excellent versatility in sampling	High-throughput. Easy to use, little skill required, moderate versatility in sampling	High-throughput. Easy to use, little skill required, excellent versatility in sampling
Expensive	Expensive	Expensive	Very cost effective	Cost effective	Expensive	Expensive

1.3. Hepatotoxicity

Xenobiotics entering the human body as part of medicinal or herbal products or dietary supplements pose a potential risk of damaging the liver or causing liver dysfunction, leading

to hepatotoxicity. Not only does hepatotoxicity occur with high dosage regimens, but it may also occur when certain substances are taken within the therapeutic range (Singh *et al.*, 2011). The liver serves as the major site for drug bio-transformation and detoxification of xenobiotic compounds, and this makes the liver a target for chemical toxicological effects (Schwarz & Watkins, 2008; Singh *et al.*, 2011). Toxic substances may cause chemically induced liver injury, which may present as one or more of the following diseases: steatosis, porphyria, veno-occlusive disease, cholestasis, hepatitis, granuloma, vascular lesions, neoplasm and necrosis or apoptosis (Schwarz & Watkins, 2008; Singh *et al.*, 2011).

Plants have been employed since ancient times for the treatment of various ailments, however, the risk of liver injury associated with the use of these herbal medicines remain eminent. This is because herbal medicines are generally poorly characterised in terms of cultivation, administration, dosage, with little regulation and uncharacterised hepatotoxic effects (Teschke & Eickhoff, 2015). Herbal hepatotoxicity is reviewed in detail in the published manuscript presented in Chapter 2.

1.3.1. *Xysmalobium undulatum* as model herbal medicine

Xysmalobium undulatum (L.) W.T. Aiton (Apocynaceae), also known as Uzara, milk bush, milkwort (Eng.); melkbos, bitterwortel (Afr.); iyeza elimhlophe, iShongwane (Xhosa); iShinga (Zulu); is a traditional herbal medicine indigenous to sub-Saharan Africa (Kenya, Malawi, Namibia, Angola Botswana, Zimbabwe, Tanzania, Zambia, Lesotho, Mozambique, Swaziland and South Africa) (Bester, 2009; Schmelzer & Gurib-Fakim, 2013). Uzara is the most widely used traditional herbal remedy in Southern Africa (Schmelzer & Gurib-Fakim, 2013). This robust geophyte herb grows approximately 0.5 - 2.0 m in height and blooms during October until December. This plant has large, hairy and heart shaped leaves, is almost stalkless with prominent veins and a rounded base. The plant produces cream-green to yellowish flowers growing in small clusters around the stem (Bester, 2009; Vermaak *et al.*, 2014; Schmelzer & Gurib-Fakim, 2013). A characteristic trait of this plant is the tips of the flowers that are covered in short white hair. Large fruits are also present and covered with long curly hairs that aid as a "parachute", improving seed dispersion (Bester, 2009; Vermaak *et al.*, 2014). The roots are fleshy, and have an almost carrot-like appearance with a nauseating smell (Schmelzer & Gurib-Fakim, 2013). The roots are used in traditional remedies and the leaves are ingested as spinach supplement, while the stems are poisonous (Reid *et al.*, 2006).

Traditional uses of the roots of Uzara includes the treatment of indigestion and stomach aches, diarrhoea, dysentery, malaria, colic, headaches, sores, wounds and abscesses,

afterbirth cramps, hysteria as well as food poisoning (Reid *et al.*, 2006; Vermaak *et al.*, 2014; Van Wyk, 2011; Steenkamp *et al.*, 2004). Active constituents identified in Uzara root include the cardenolide cardiac glycosides uzarin (the main active constituent) and xysmalorin, and their isomers allouzarin and alloxysmalorin. Minor constituents are the cardenolide aglycones uzarigenin and xysmalogenin, as well as allouzarigenin, alloxysmalogenin, ascleposide, coroglaucigenin, corogluaucigenin, alloxysmalogenin, ascleposide, coroglaucigenin, corogluacigenin-3-O-glucoside, pachygenol, pachygenol-3 β -O-glucoside, desglucouzarin, smalogenin, desglucoxysmalorin, uzaroside, pregnenolone and β -sitosterol (Vermaak *et al.*, 2014). No reports or studies related to Uzara-induced hepatotoxicity were found, although reports did indicate a toxic digitalis-like action on the heart (Vermaak *et al.*, 2014).

1.3.2 Valproic acid as positive control for hepatotoxicity studies

Valproic acid is an anti-convulsing drug, administered for the treatment of seizure disorders, epilepsy, mania, and prophylactic treatment of migraine headaches. Valproic acid, or valproate, is a branched chain organic acid that is a well-known cause of several distinctive forms of acute and chronic liver injury (Lee *et al.*, 2008; Vitins *et al.*, 2014; PubChem, 2017). Clinical and experimental studies have shown that treatment with valproic acid result in biochemical abnormalities of the liver, which include inhibition of β -oxidation, synthesis of fatty acids, inhibition of gluconeogenesis, synthesis urea and oxidative phosphorylation (Lee *et al.*, 2008).

Dosages of valproic acid administered to rodents in various chronic *in vivo* studies range from 11, 21, 42, 84, 100, 168, 337, 500 to 674 mg/kg, with an oral LD₅₀ value of 1098 mg/kg in mice and 670 mg/kg in rats (Tong *et al.*, 2005a; Lee *et al.*, 2008; Vitins *et al.*, 2014; Drugbank, 2017). 200 to 600 mg/kg was shown to result in micro-vesicular steatosis of the liver (Lee *et al.*, 2008; Tong *et al.*, 2003).

Ahmed and Siddiqi reported in their 2006 review on anti-epileptic drugs that hepatic biotransformation is the main route of elimination of valproic acid, and involves glucuronidation, β -oxidation and ω -oxidation. Some patients (10-15%) on valproic acid experience a transient elevation of liver aminotransferases. It was also reported that the levels of other liver enzymes, including alkaline phosphatase (ALP), lactate dehydrogenase (LDH) and γ -glutamyl transferase (GGT) may also rise in serum and that treatment may continue if this rise in enzyme levels is moderate (two to three times the baseline levels), as long as the patient remains asymptomatic. When hepatic effects are clinically symptomatic, it is

recommended that the drug is discontinued immediately. It was also noted that valproic acid therapy may be associated with hyperammonemia in the presence of normal aspartate aminotransferase (AST), alanine aminotransferase (ALT) and ALP levels. Idiosyncratic hepatic toxicity because of valproic acid occurs within two to three months of therapy and presents with reduced alertness, vomiting, haemorrhage, increased seizures, anorexia, jaundice, oedema and ascites. Most frequently necrosis and steatosis are reported as hepatic histopathological findings.

A single dosage of valproic acid in rats resulted in dosage dependant elevated levels of lipid peroxidation in the plasma and liver, and is associated with oxidative stress and mitochondrial dysfunction (Pourahmad *et al.*, 2012). Two types of valproic acid hepatotoxicity exist, type I is associated with dosage dependant changes in serum liver enzyme levels and low plasma fibrinogen levels, while type II valproic acid mediated hepatotoxicity is characterized by microvesicular steatosis accompanied by necrosis (Tong *et al.*, 2005a).

Lee *et al.* (2008) conducted a study on the sub-chronic effects of valproic acid in mouse livers, as idiosyncratic microvesicular steatosis can develop in the early weeks of therapy. Valproic acid was administered at either 100 mg/kg or 500 mg/kg to male ICR mice aged 5 weeks, and valproic acid was administered over a period of 28 days by means of oral gavage. Livers were harvested at weeks 1, 2 and 4 after initial treatment, and serum ALT, AST activities and triglyceride levels (TG) were measured. It was found that there was a significant increase in the TG concentration after two weeks.

1.4. Drug bio-transformation

The principal site of metabolism for the majority of drugs is the liver, with the CYP450 enzyme system accounting for 30% of the hepatic metabolic activity and more than 70% of the intestinal metabolism (Hellum & Nilsen, 2008:466; Pal & Mitra, 2006:2136). The mucosa of the gastrointestinal tract remains the most significant extra-hepatic site for CYP450 bio-transformation (Paine *et al.*, 2006:880). Various chemical reactions in the liver are responsible for the biotransformation of drugs, which include oxidation, reduction, hydrolysis and conjugation as a two-phased system (Liska, 1998:190; Shargel *et al.*, 2005:320). Drug biotransformation may be influenced by either induction and/or inhibition of the CYP enzyme system resulting in either decreased or increased drug plasma concentrations (Bibi, 2008; Pelkonen, 2009; Wilkinson, 2005). During induction of CYP3A4, the pregnane X receptor (PXR), as well as the constitutive androstane receptor (CAR), are activated. Activation

causes both CAR and PXR to homo-dimerise with the retinoid-X-receptor (RXR), forming a heterodimer that binds with the response elements located on target genes. This prompts an increase in the transcription of the specific gene, thus, increasing messenger ribonucleic acid (mRNA), which in turn results in increased enzyme production and consequently an increase in the enzyme activity (Pal & Mitra, 2006; Pelkonen, 2009; Wilkinson, 2005). This up-regulation of CYP enzymes by certain drug modulators may have a detrimental effect on co-administered drug substrates in terms of their bioavailability and efficacy (Wilkinson, 2005). Conversely, inhibition of the CYP enzyme system results in the increased bioavailability of orally administered drugs that are CYP substrates, in some instances causing heightened adverse effects and drug toxicity (Wilkinson, 2005).

1.4.1. The need for novel biotransformation models

Withdrawal of drug candidates being developed has been estimated at 40% due to pharmacokinetic deficiencies and disparities in biotransformation that indirectly result in toxicity (Tingle & Helsby, 2006). Discrepancies in drug or herbal biotransformation processes may result in the production of hepatotoxins that elicit interactions with cellular constituents, including lipid and protein synthesis as well as ribonucleic acid (RNA) and deoxyribonucleic acid (DNA), resulting in hepatotoxicity (Guillouzo, 1998, Singh *et al.*, 2011). Consequently, liver toxicity due to pharmaceuticals and xenobiotics remain a concern and is in many instances associated with histopathological and clinical phenotypes, namely steatosis, cholestasis and hepatitis (Driessen *et al.*, 2013; Sirenko *et al.*, 2016). Current pre-clinical models used in the assessment of drug biotransformation cannot always accurately predict *in vivo* biotransformation and elimination (Brandon *et al.*, 2006). Preliminary biotransformation studies rely strongly on the extrapolation of data obtained from *in vitro* cell culture models and animal models (Nakamura *et al.*, 2011; Sirenko *et al.*, 2016). Cells cultured in suspension or on solid flat surfaces in two-dimensions have long been employed in drug discovery and, although convenient, these systems are plagued with disadvantages resulting from discrepancies in cellular communication and culturing time (Antoni *et al.*, 2015; Fang & Eglén, 2017). Therefore, the development of complex systems that can identify and effectively test potential bio-transformation remain an area of active investigation (Sirenko *et al.*, 2016). Novel three-dimensional cell culturing techniques attempt to overcome the disadvantages of current *in vitro* models by providing a cellular environment more closely related to the *in vivo* state, with the ability to more effectively predict drug efficacy, biotransformation and toxicity prior to clinical trials (Antoni *et al.*, 2015; Wrzesinski & Fey, 2015; Fang & Eglén, 2017).

2. PROBLEM STATEMENT

Various 2D cell cultures have long been used as *in vitro* models, while animal models have been utilised for pre-clinical *in vivo* research. However, both these types of models are afflicted by various shortcomings and restrictions. Conventional cell culture models that are used to investigate pharmacokinetic interactions and hepatotoxicity can only provide limited information due to a lack of physiological relevance, while the use of animal models in scientific research causes an ethical dilemma. In addition, experimental data obtained from animal models are not always successfully correlated to humans, and certain side effects may not even be detectable. It is therefore clear that a need exists for new or alternative *in vitro* biotransformation and toxicity screening models such as 3D cell culture systems, which can more closely resemble the *in vivo* environment.

3. AIMS AND OBJECTIVES

The aims of this study are firstly, to establish a 3D spheroid cell culture model to evaluate the hepatotoxic properties of substances and to compare the results from this model with results from an *in vivo* animal study in order to identify the predictive value of the 3D cell culture model. Secondly, to investigate the potential hepatotoxic effects of acute and chronic administration of a crude aqueous extract of *Xysmalobium undulatum* *in vitro* and *in vivo*. Thirdly, to develop a 3D spheroid culture based LS180 cells that can be used as an *in vitro* model for biotransformation studies.

The specific objectives are:

- To establish the HepG2/C3A cell line as 3D spheroid cell culture by means of the microgravity bioreactor technique, that can serve as an *in vitro* model for hepatotoxicity studies.
- To prepare and chemically characterise extracts from *X. undulatum* by means of ultra-high-pressure liquid chromatography (uHPLC) linked to mass spectrometry (MS).
- To conduct acute *in vitro* hepatotoxicity studies in HepG2/C3A cells cultured as a traditional two-dimensional culture, with *X. undulatum* crude water extract as model compound.
- To conduct acute *in vitro* hepatotoxicity studies in the HepG2/C3A spheroid culture model, with *X. undulatum* crude water extract as model compound.
- To conduct chronic *in vitro* hepatotoxicity studies in the HepG2/C3A spheroid culture model, with *X. undulatum* crude water extract and valproic acid as model compounds.

- To conduct chronic *in vivo* hepatotoxicity studies in the Sprague Dawley rat model, treated with valproic acid and *X. undulatum* crude water extract as model compounds.
- To compare all results from the acute *in vitro* traditional 2D HepG2/C3A cell culture hepatotoxicity study with results obtained from the acute *in vitro* hepatotoxicity study done in the HepG2/C3A 3D spheroid culture model.
- To compare all results from the chronic *in vitro* HepG2/C3A spheroid culture hepatotoxicity study with results obtained from the chronic *in vivo* hepatotoxicity study.
- To establish the LS180 cell line as 3D spheroid cell culture model by means of the microgravity bioreactor technique.

4. STUDY OUTLINE AND STRUCTURE OF THESIS

This thesis is presented in article format to comply with the necessary guidelines and requirements for the degree Doctor of Philosophy in Pharmaceutics at the North-West University. A graphical representation of the project and thesis layout is depicted in Figure 2. The review manuscripts presented in Chapters 2 and 3 serve as the literature overview for this study. Chapter 2 presents an article entitled: “*Herbal hepatotoxicity: current status, examples, and challenges in the peer-reviewed journal*”, which was published in the peer-reviewed journal *Expert Opinion on Drug Metabolism and Toxicology*.

Chapter 3 presents a manuscript entitled: *Recent advances in three-dimensional cell culturing to assess liver function and dysfunction: From a drug biotransformation and toxicity perspective*, published in the journal *Toxicology Mechanisms and Methods*. For the research manuscripts (Chapters 4, 5 and 6), a crude *Xysmalobium undulatum* (Uzara) aqueous extract was prepared and characterized by means of uHPLC-MS, which was used in the acute and chronic toxicity studies. The results from the acute studies performed in two- and three-dimensional HepG2/C3A cell cultures is presented in the manuscript titled: *Toxicity and anti-proliferative properties of Xysmalobium undulatum water extract during short term exposure to two-dimensional and three-dimensional spheroid cell cultures* (Chapter 4), for submission to the peer-reviewed journal *Toxicology Mechanisms and Methods*.

Chapter 5 presents the results of the sub-chronic 28-day toxicity study on the crude Uzara aqueous extract, in the three-dimensional HepG2/C3A cell model and the *in vivo* Sprague Dawley rat model, compiled as a research manuscript prepared for submission to the journal *Biochemical Pharmacology*. Ethical approval was obtained for the chronic *in vivo* toxicity

study in the rat model, and details regarding this are supplied in Appendix H and J. The results from a preliminary study in the three-dimensional HepG2/C3A model, conducted in collaboration with the department of Biochemistry at the North-West University, was published in the peer-reviewed journal *International Journal of Biochemistry and Cell Biology*. It is titled *Cell-free DNA in a three-dimensional spheroid cell culture model: A preliminary study*, as shown in Chapter 6.

Chapter 7 is presented in the form of a methodology manuscript prepared for submission to the journal *Journal of Cell Biology*, and this manuscript presents the method to establish the LS180 cell line as a novel 3D spheroid cell culture model. Finally, Chapter 8 consists of the final conclusions from the study and recommendations for future endeavours.

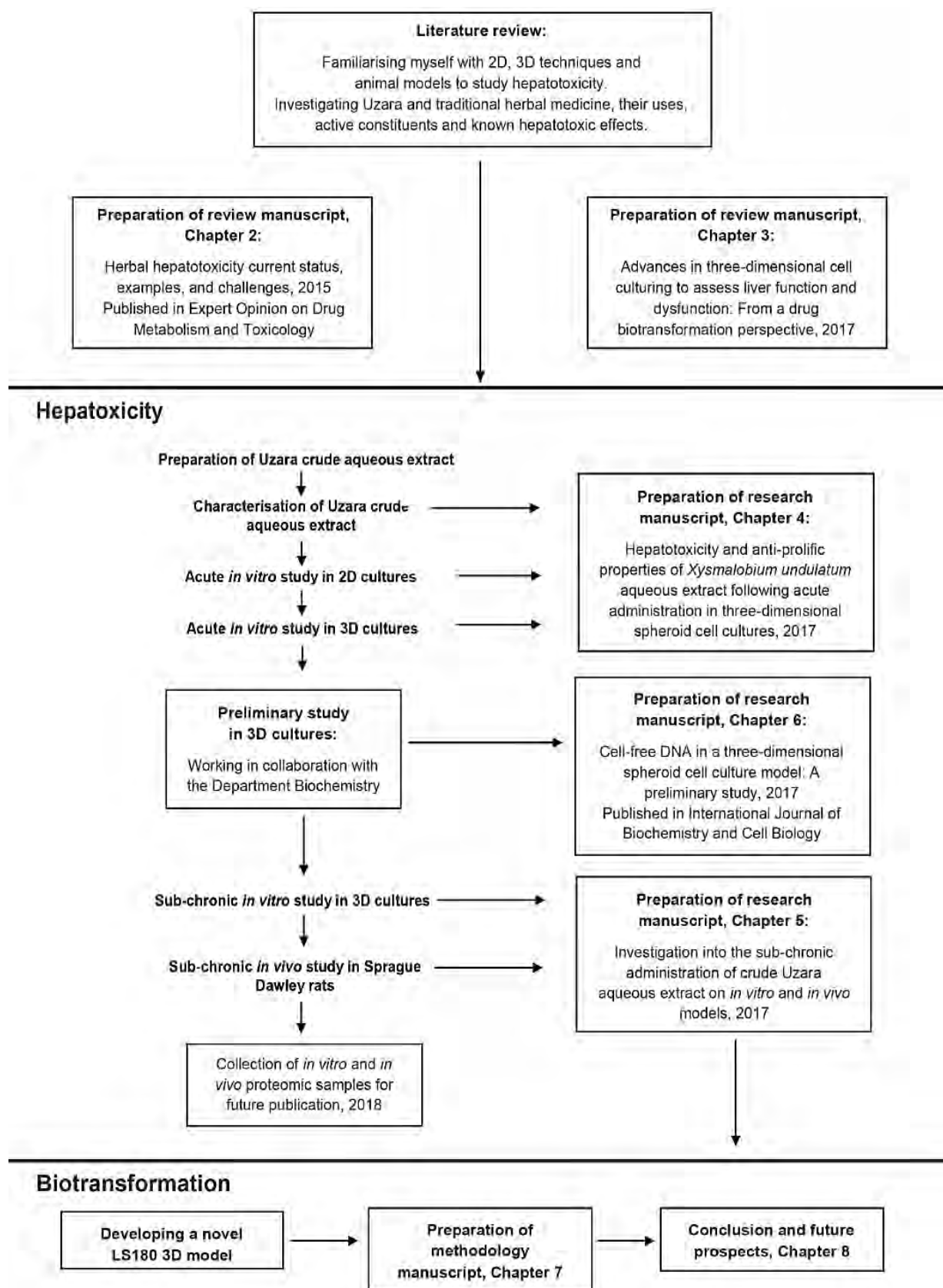


Figure 2: Schematic depiction of study design and layout of the thesis

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CHAPTER 2**ARTICLE PUBLISHED IN EXPERT OPINION ON DRUG
METABOLISM AND TOXICOLOGY**

Chapter 2 is presented in the form of a review article published in the journal *Expert Opinion on Drug Metabolism and Toxicology* in 2015 (doi: 10.1517/17425255.2015.1064110). The complete guide for authors for this journal is provided in Appendix A, it is written in U.S. English as per the journal style.

EXPERT OPINION

1. Introduction
2. Herbal products linked to hepatotoxicity
3. Conclusion
4. Expert opinion

Herbal hepatotoxicity: current status, examples, and challenges

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Introduction: Herbal medicines have commonly been considered safe by the general public due to their natural origin and long history of traditional uses. In contrast to this belief, many plants produce toxic substances as secondary metabolites that are sometimes not easily distinguishable from the pharmacological active compounds. Some herbal medicines have been associated with adverse effects and toxic effects, including hepatotoxicity, which have been reversed upon discontinuation of the herbal medicine by the patient.

Areas covered: This review reflects on selected herbal medicines that are associated with hepatotoxic effects including a description of the phytochemicals that have been linked to liver injury where available. Although case studies are discussed where patients presented with hepatotoxicity due to use of herbal medicines, results from both *in vitro* and *in vivo* studies that have been undertaken to confirm liver injury are also included.

Expert opinion: Increasing evidence of herbal hepatotoxicity has become available through case reports; however, several factors contribute to challenges associated with causality assessment and pre-emptive testing as well as diagnosis of herb-induced liver injury.

Keywords: complementary and alternative medicine, herb induced liver injury, herbal medicine, phytochemical, traditional herbal medicine

Expert Opin. Drug Metab. Toxicol. [Early Online]

1. Introduction

The liver is the main metabolizing organ involved in the biotransformation of xenobiotics and is responsible for conversion of foreign chemical substances into metabolites that are readily excreted in the bile and urine. Due to the liver's anatomical location, physiological, and biochemical functions, it is prone to pathologies and diseases as a result of changes in cellular homeostasis and gene expression [1]. It is a common belief that complementary and alternative medicines from natural origin such as herbs are non-toxic [2]. However, many plants produce secondary metabolites that are toxic as part of their natural defense mechanisms. The toxic substances of some medicinally relevant plant species are sometimes not distinguishable from their biologically active ingredients, for example, in plants such as *Digitalis purpurea*, *Hyoscyamus niger*, and *Atropa belladonna* [3].

Among the most serious adverse effects that can be caused by herbal products is chemically induced liver injury. Liver injury can be classified as predictable (i.e., dose-related high incidence liver injury) or unpredictable (i.e., low incidence that may be dose-related or not). Unpredictable liver injuries include idiosyncratic reactions that are rare and without allergic characteristics, but this type of liver injury also includes immune-mediated hypersensitivity reactions that are associated with symptoms such as fever, rash, and eosinophilia [4,5]. Herb-induced liver injury may range from mild asymptomatic liver enzyme elevation to more serious

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Article highlights.

- A common, but erroneous belief exists that medicines from natural origin such as herbs are in general non-toxic and therefore safe.
- Although the problem of herbal hepatotoxicity seems to expand, it remains poorly defined and conflicting results are sometimes published.
- Current causality assessment relies primarily on retrospective analysis of case reports.
- Better models are needed for pre-emptive testing of potential hepatotoxic properties of herbs.

This box summarizes key points contained in the article.

conditions such as cirrhosis, liver failure, acute and/or chronic cholangitis, macro- and micro-vesicular steatosis, and vascular lesions [6]. Moreover, plant constituents with toxic effects might induce liver injury manifesting as accumulation of fat in hepatocytes (steatosis), disturbances of porphyrin metabolism (porphyria), occlusion of central and sub-lobular veins (veno-occlusive disease), inhibition of bile flow (cholestasis), as well as inflammation in the liver (hepatitis) and cell death (necrosis or apoptosis). The hepatotoxicity of certain plant species has been associated with the presence of specific phytochemicals such as pyrrolizidine alkaloids [7], triterpene saponins [8], anthraquinones [9], and ephedrine alkaloids [2].

There are a number of factors that contribute to the increasing incidence of herb-induced liver injury such as long-term self-medication without disclosure to medical practitioners, misidentification of the plant or mislabeling of the product, poor quality, adulteration, contamination, and inadequate storage [5,6]. Although the problem of herbal hepatotoxicity seems to expand, it remains poorly defined and conflicting results are sometimes published [9]. Causality assessment of herb-induced liver injury (HILI) has been the subject of extensive reviews [10-12], but a distinct lack of standardized pre-clinical and clinical safety assessment of herbs in terms of liver toxicity exists in the scientific literature. This is clearly visible in the following discussion of selected herbal products associated with human liver injury for which case reports are available, but very little information is available on pre-clinical evaluations of these products.

2. Herbal products linked to hepatotoxicity

2.1 Aloe (*Aloe vera*)

Aloe (commonly known as aloes) is a genus that contains over 500 species of flowering plants with succulent leaves arranged in a rosette. Another noticeable characteristic of all aloes is their tall, candle-like inflorescences. Aloes are mainly native to tropical and southern Africa, Madagascar, Arabia, and eastern Indian Ocean Islands [13-15]. The natural products derived from aloes are mainly the leaf exudates and whole-leaf extracts or leaf mesophyll (more commonly known as aloe leaf gel or pulp) [14,15]. The leaf gel is mainly used in cosmetics, wound,

and burn healing [16], but other applications such as anti-inflammatory, anti-diabetic [17], anti-septic [18], anti-ulcer [19], and anti-tumor [20] activities have also been described. The aloe leaf gel mainly consists of polysaccharides, amino acids, trace elements, minerals, phenolic compounds, and organic acids [15,21]. The leaf exudate is also known as bitter sap, juice, or latex and contains C-glycosides, anthrones, and anthraquinones namely aloin, aloe-emodin, aloesin, and aromatic compounds including aldehydes and ketones [22,23] and is used medicinally as laxative due to the main ingredients aloin and aloe-emodin [15,23].

Various patient case studies have reported on the acute inflammation of the liver (hepatitis) after orally ingesting *A. barbadensis* also known as *A. vera*. In 2005, a female patient presented with severe acute hepatitis after ingestion of 500 mg of *A. vera* extract for a period of about 4 weeks [24]. In another case, a male patient presented with acute hepatitis after ingesting *A. vera* extract for 3 weeks [25]. A case also reported on a female patient who suffered from hepatitis after using *A. vera* as a laxative [26]. An article by Yang *et al.* reports on three female patients, all with acute hepatitis due to ingestion of aloe products. They ingested a combination of 250 mg *A. arborescens* extract and 28.5 mg *A. vera* extract for 6 months, 420 mg *A. vera* extract for 3 months, and aloe extracts (species not specified) for 5 months, respectively. All three patients presented with similar clinical symptoms [27]. One case reported that a female patient presented with acute hepatitis after ingesting an *A. vera* gel containing preparation for 4 weeks [28]. All patients presented with acute (hepatocellular-type) liver injury and in most cases, the causality assessment were probable. In all of these cases, the patients recovered after discontinuation of the herbal products, and in one case the patient was re-challenged with the aloe product, whereafter the liver inflammation returned. This provided additional evidence for the aloe gel material-containing products being the main cause of hepatitis.

There are only a limited number of reports on animal hepatotoxicity studies involving aloes. In an animal study where the toxicity of *A. vera* was evaluated in male and female mice, the mice were administered orally with aloe gel up to a dose of 15,000 mg/kg and no significant toxic effects were observed. No liver damage was seen; however, significant changes in kidney weight were observed in male mice. The upper intake level for humans, based on the animal's results, was calculated at 24 mg/kg/d for a 60 kg adult [29]. A sub-chronic toxicity study of aloesin for use as a potential food ingredient revealed no effects on the livers of rats and the no-observed-adverse-effect level was set at 1000 mg/kg body weight/d [30]. However, aloesin may not share the same toxicity profiles as the other anthraquinones.

Conflicting results regarding aloes are available in the scientific literature with various studies reporting on the hepatic protective effects of aloes in diabetic rats [31], in chronic alcohol-induced hepatotoxicity in mice and rats [32,33], carbon tetrachloride-induced hepatotoxicity in mice and rats [34,35],

Herbal hepatotoxicity: current status, examples, and challenges

and 1,4-naphthoquinone-induced hepatotoxicity [36]. These effects were in most cases attributed to the anti-oxidant effects of aloe components [31-33] or maintenance of intracellular thiols [36]. Up to date, there are few studies on the ratio of efficacy versus toxicity of aloe products, there are few reports on the monitoring of side effects due to aloe products, and there are no results on the mechanism of action of aloe or aloe constituents.

2.2 Black cohosh (*Actaea racemosa*)

Black cohosh (*Actaea racemosa* previously known as *Cimicifuga racemosa*) also known as black snakeroot is a perennial plant, indigenous in eastern North America [37]. Nearly all commercially available material is supplied from wild-harvested plant material from the Appalachian forests in the US [38,39]. The rhizomes and roots of this plant have been used to alleviate peri-menopausal and post-menopausal symptoms such as hot flushes, sweating, vaginal dryness, and sleep disturbances [40-42]. Besides the aforementioned uses, other reported uses include treatment of rheumatoid arthritis, muscle pain, and dysmenorrhea. Recent work has illustrated that black cohosh might be of value in the prevention and treatment of breast cancer [41].

Black cohosh is administered either as dried root or liquid extract in doses of 40 – 200 mg daily or 40 mg daily for the dry root and extract, respectively [37,42]. This plant contains a complex mixture of pharmacologically active ingredients. Identified compounds include triterpene glycosides (actein, 23-epi-26-deoxyactein, cimicifugoside), phenolic acids (isoferulic acid, fukinolic acid), flavonoids, volatile oils, and tannins [37,40]. The principal active ingredients are *N*- ω -methylserotonin and the triterpene glycosides, actein, 23-epi-26-deoxy-actein, and cimicifugoside [38].

Adverse effects of black cohosh are generally rare, mild, and reversible with gastrointestinal symptoms, and rashes are the most common [40,43]. Sporadic case reports have been published since the turn of the century that highlighted a possible causality between black cohosh use and hepatotoxicity [44,45]. However, consensus on black cohosh consumption as the cause of hepatotoxicity has not been reached and information is conflicting. The liver damage in suspected black cohosh cases is usually characterized by histopathological features mimicking autoimmune hepatitis [44,46]. According to a recent review on the suspected hepatotoxicity of black cohosh in 69 cases, there exist serious doubts on the initial claims of the causality between black cohosh and hepatotoxicity. These doubts are based on several confounding factors such as clear evidence of poor case data quality, uncertainty of the black cohosh product, the quality thereof, insufficient adverse event definition, missing or inadequate evaluation of alcohol use, co-medication, co-morbidity, and alternative diagnoses [47]. However, in studying two cases involving hepatotoxicity related to black cohosh use, it was concluded that the liver damage following the consumption of black cohosh in both cases is identical to trolox necrosis, seen with auto-immune

hepatitis. The mechanism of toxicity by black cohosh is idiosyncratic, like autoimmune hepatitis and presents as gradual distraction of liver parenchyma secondary to immunologic synapses between hepatocytes and lymphocytes. The interaction is irreversible. It is important to note that fast recovery was seen when black cohosh consumption was halted [46].

2.3 Chaparral (*Larrea tridentata*)

Larrea tridentata also known as creosote bush or chaparral, is a common shrub found in semiarid grassland deserts and is currently dominating vast areas of land, which were previously classified as desert grasslands [48,49]. Chaparral has been reported to be abundant in the Mexican States of San Luis Potosi, Coahuila, Chihuahua, Durango, Sonora, Zacatecas, Baja California Norte, and Sur. It is also commonly found in the Southwest of the US in Arizona, California, Nevada, Texas, and New Mexico [48]. Chaparral is an evergreen shrub with glossy leaves of approximately 1 cm in length which are covered in a thick resinous coating. The shrub is branched and knotty by nature and may grow to heights of between 1 – 3 m. The flowers consist of five clawed petals which are yellow in color, while the fruit is a roundish capsule shape which are densely covered in white hairs [50].

Traditionally, the leaves, twigs, and roots of chaparral were used to make aqueous or alcohol-based liquid extracts for oral administration and in some cases also for poultice, fomentation, salves, and excoriations. Tablets and capsules have also been manufactured for oral use [51,52]. Chaparral has been used to treat a wide variety of ailments such as gallbladder and kidney stones, liver disease, allergies, arthritis, bowel cramps, bruises, chicken pox, colds, diabetes, diarrhea, dysmenorrhea, dyspepsia, emesis, heartburn, menstrual cramps, skin disorders, and tuberculosis [53-55]. There is, however, no conclusive scientific evidence to support the successful treatment claims of the above medical conditions with chaparral-based treatments.

At least 16 cases have been documented where regular ingestion of chaparral has caused cholestatic hepatitis. In four of the cases, there was progression to cirrhosis and in two other cases, the damage was so extensive that it has led to fulminant liver failure which required liver transplantation. The duration of chaparral use in the above cases ranged from 3 to 52 weeks [54].

Based on the current evidence, the primary polyphenolic compound of *L. tridentata* is nor-dihydroguaiaretic acid (NDGA), which is the most likely compound responsible for the reported hepatotoxicity. *In vitro* and *in vivo* studies have shown that NDGA inhibits the induction of ornithine decarboxylase. It has also been postulated that NDGA may block cellular respiration and promote anti-oxidative effects [56,57]. NDGA inhibits collagen and adenosinediphosphate-mediated platelet aggregation [58,59] has shown that NDGA inhibits both lipooxygenase and cytochrome P-450 mono-oxygenase activity in rat epidermal and hepatic microsomes [60,61]. At NDGA concentrations of 100 mM or more, it was found

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that protein syntheses are inhibited and that Golgi complexes are altered irreversibly [62]. Cellular ATP are reduced by NDGA via inhibition of electron flux in the respiratory chain and cellular volume regulation is inhibited due to an inhibition of taurine channels at concentrations ranging between 50 and 150 mM [63,64].

2.4 Comfrey (*Symphytum officinale*)

Comfrey belongs to the Boraginaceae family and is found worldwide as a common garden plant. Although there are several species belonging to the *Symphytum* genus, this review will only report on the most commonly used species, *Symphytum officinale*. This evergreen perennial plant has large, hairy leaves with narrowing ends and small purple flowers [65-67].

Since ancient times, comfrey has been consumed and used as a medicinal herb and tea in several countries [66-68]. The leaves are generally used fresh or dried, while the roots are generally dried, and it is applied in the form of infusions, decoctions, extracts, ointments, and compress paste [66]. Its medicinal uses include treatment of gastritis, ulcers of the gastrointestinal tract, lung congestion, bone fractures, wounds, gout, hematomas, thrombophlebitis, distortions, joint inflammation, and as an oral or pharyngeal gargle [65,69].

Some of the active components of comfrey include allantoin, flavones, rosmarinic acid, triterapene saponins, tannins, mucopolysaccharides, hydro-xy-cinnamonic acid derivatives, antioxidant vitamins, and vitamin B12 [66,69]. It has become evident that comfrey also contains hepatotoxic pyrrolizidine alkaloids (including intermedine, acetylintermedine, lycopsamine, lasiocarpine, acetyllycopsamine, anadoline, asperumine, symphytine, echimidine, echinatine and symviridine, myoscorpine, symlandine and uplandicine), raising concern regarding its chronic use and causing various countries to restrict comfrey distribution and use in products [66,69-72]. Pyrrolizidine alkaloids are present in much higher concentrations in the roots than in the leaves of comfrey [65,67,68]. The pyrrolizidine alkaloid content of different comfrey preparations can vary significantly (as much as 30 – 1150 mg/kg), making it hard to judge toxic potential and measure total ingestion [65,72].

Hepatotoxicity as a result of comfrey ingestion is well established. Although some countries have started to restrict the use of comfrey, it is still prescribed widely by herbalists and ingested as a food, showing that more science-based data are needed to establish guidelines, more specifically regarding the dose-effect relationship [65].

2.5 Greater celandine (*Chelidonium majus*)

Greater celandine (*Chelidonium majus*) contains alkaloid-rich orange colored latex with spasmolytic, anti-inflammatory, anti-viral, anti-microbial, anti-tumor, analgesic, and antifungal activities used in Western and Chinese traditional medicine and in phytotherapy [73,74]. Traditional uses include treatment of gastric ulcers, oral infections, liver diseases, skin eruptions, tuberculosis, and pain, while the juice of the plant

is applied to remove cornea opacities [74]. Isoquinoline alkaloids present in celandine known to be effective include protopine, chelerythrine, sanguinarine, coptisine, and berberine, and the main alkaloid present in the plants' aerial parts is chelidone [74,75]. A carcinostatic agent prepared from this alkaloid has been patented [75]. Chelidocystatin, a cysteine proteinase inhibitor, was also isolated from the green parts of mature greater celandine [73].

Greater celandine have been reported to result in acute cholestatic hepatitis, with typical symptoms such as jaundice, itching and fatigue presenting after approximately 2 months or more of oral ingestion. The clinical profile of the toxic hepatitis consists of frequent hepatomegaly, above normal bilirubin and serum transaminases levels, cell necrosis, and possible fibrosis [76]. Patients recovered from the hepatotoxicity rapidly after they terminated use of the plant [74]. One reported case involved a 65-year-old male consuming 4 – 5 teaspoons dried leaves boiled in 150 ml water daily for more than a month to treat pyrosis. The person developed sudden onset asthenia, dyspepsia, and jaundice and was diagnosed with acute cholestatic hepatitis [76]. Consumption of greater celandine was ceased and liver parameters returned to normal within 2 months. Two cases were reported where women were treated with Panchelidon® (containing greater celandine) capsules. Both women developed hepatotoxic reactions after taking the product for > 4 months [77]. In a clinical review [78], a total of 69 cases of celandine-induced hepatotoxicity were reviewed, and they determined that 16 cases had highly probable or probable causality of hepatotoxicity due to celandine ingestion. An animal study where a hydro-alcoholic celandine extract was administered to Wistar rats did not show hepatotoxicity even at doses 50 – 100 times higher than those humans commonly use [74,79]. The authors did note possible oxidative stress induction after prolonged ingestion and suggested that greater celandine should not be used chronically.

Following reports of 40 cases of liver damage in Germany, oral use of Greater celandine has been prohibited in several European countries, including Germany, while the Australian Complementary Medicines Evaluation Committee recommended that a warning label should appear on oral products containing greater celandine [74]. The use of greater celandine in food supplements and herbal remedies has been prohibited in Italy, and in England it can only be used under pharmacist supervision [76]. Greater celandine products are, however, still widely available and can be purchased from internet sites [79].

2.6 Green tea (*Camellia sinensis*)

Making tea from the leaves of *Camellia Sinensis* L., Family *Theaceae* was discovered in China > 2000 years ago as liquid refreshment. Today, it is one of the most popular beverages consumed worldwide. In the West, black or fermented tea is preferred, while green tea is popular in China and Japan [80]. The polyphenolic content is approximately three or four times higher in green than in fermented black tea [81]. The

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polyphenol constituents include several flavanols, flavonols and phenolic acids previously reviewed [82]. Green tea is further characterized by a high content of water-soluble catechins. These include (-)-epigallocatechin-3-gallate (EGCG), (-)-epigallocatechin, (-)-epicatechin-3-gallate, and (-)-epicatechin [80]. The beneficial biological activities (e.g., modulation of glucose and lipid metabolism) of green tea are mainly attributed to these catechins, particularly EGCG [83].

The consumption of green tea is generally regarded as safe and healthy. Ever-increasing quantities of green tea are being consumed for health promotion, which is mostly attributed to anti-oxidant, metabolic, and immune-modulating activities. In rats and mice, green tea extract prevented hepatotoxin-induced formation of lipid peroxides in the liver [84-86]. The popularity of green tea extracts as weight-loss supplements has increased dramatically. The weight-loss activity of green tea extracts are attributed to EGCG's inhibition of lipases and stimulation of thermogenesis [87,88], and is claimed to act as an appetite suppressant.

Although anecdotal evidence strongly suggests that green tea extract consumption up to the equivalent of 24 cups of green tea per day is safe, the uncontrolled consumption of green tea extracts as supplements and as adjunctive therapies with current chronic medications has been shown to be hepatotoxic to a small percentage of individuals. The cause(s) of the observed toxicity is mostly attributed to high levels of catechins, particularly EGCG [89]. In Denmark, a case of hepatotoxicity was reported after the consumption of four to six cups of green tea per day for 6 months [90]. In 2008, the US Pharmacopeia Dietary Supplement Information Expert Committee (DSIEC) reviewed case reports related to green tea toxicity from various databases during the period 1966 – 2007. A total of 216 case reports were reviewed with 34 reports of hepatotoxicity. Of these, the causality in 27 cases was categorized as 'possible' and 7 as 'probable' [91]. The precise cause of the hepatotoxicity of green tea is unknown; however, *in vitro* studies suggest that EGCG at increased concentrations acts as a pro-oxidant-inducing oxidative damage in the liver [92]. The increased hepatotoxicity of EGCG appears to be related to the gallic acid moiety [79]. Furthermore green tea-attributed hepatotoxicity affects more women than men. This is partially related to the use of green tea extracts as slimming supplements. In 2003, a weight-loss supplement containing high concentrations of EGCG (i.e., Exolise[®]) was withdrawn from the market in France and later in Spain due to hepatotoxicity [79,93]. Another popular weight-loss supplement (i.e., Hydroxycut[®], which contains 117 mg EGCG) was voluntarily withdrawn from the market in 2009 following 23 reported cases of severe hepatotoxicity to the US FDA [94]. In 2012, a case study linked severe hepatotoxicity of a herbal weight-loss product containing green tea extract (i.e., SlimQuick[™], which contains 135 mg of EGCG) in a young woman to her alpha-1 antitrypsin MZ phenotype deficiency [93]. Dieting or restricting food-intake is another confounding factor related to green tea (EGCG)

weight-loss supplementation as the risk of EGCG-induced hepatotoxicity is increased if taken during periods of fasting [95].

2.7 Echinacea (*Echinacea purpurea*)

The *Echinacea* spp. (Asteraceae) also known as the common purple coneflower belong to the perennial prairie wildflowers with its native origin in the central grasslands of mid-western North America [67,96,97]. *Echinacea purpurea*, *E. angustifolia*, and *E. pallida* have been in use for medicinal purposes [98,99]. *E. purpurea* is a plant characterized by erect stems of about 2 meters in height with long stalks and alternate leaves, solitary spiny, reddish-orange flowers surrounded by purplish bracts and coarse hairs [96]. Traditionally, *Echinacea* spp. were used in the treatment of snake bites, septic wounds, scarlet fever, syphilis, diphtheria, typhus, dysentery as well as cancer [100]. Today, preparations of *E. purpurea* are used in the treatment of common colds, influenza, upper respiratory tract infections, and inflammation of the mouth and pharynx and also as an immunostimulant [100-102]. The major active constituents of *Echinacea* spp. include the polysaccharides arabinogalactan and acidic arabinorhamnogalactan, cichoric acid (a caffeoyl phenol), echinoside, the pyrrolizide alkaloids isotussilagine and tussilagine and isobutylamides that make up the unsaturated lipophilic components [100,103]. *Echinacea* hepatotoxicity usually occurs with long-term use (> 8 weeks) and concomitant use of drugs such as anabolic steroids, amiodarone, methotrexate, and ketoconazole [103,104].

A case of a 45-year-old male patient presenting with fatigue and jaundice was reported. The patient was treating his flu symptoms with *Echinacea* root extract (1500 mg/day). There had been no history of other medication. Tests for biochemical parameters revealed elevated levels of alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, γ -glutamyl transferase, total bilirubin, albumin, and gamma globulin. A liver biopsy revealed hepatitis, cholestasis, and portal lymphoplasmocyte and eosinophilic granulocyte infiltration. Upon cessation of the *Echinacea* product, the biochemical parameters returned to normal within a month [105].

2.8 Kava kava (*Piper methysticum*)

Piper methysticum also known as intoxicating pepper, kava pepper, kavaroote, or kava kava (also commonly referred to as kava) is a perennial tropical shrub indigenous to the regions of Melanesia, Micronesia, Hawaii, Fiji, and Polynesia of the South Pacific Islands [67,106]. The rhizomes and roots are traditionally extracted with water or coconut milk and used as both a recreational and medicinal drug in various countries [106,107]. In the US and Europe, it is employed as a sedative for the treatment of anxiety, stress as well as restlessness. Kava is also traditionally used as diuretic, muscle relaxant as well as a general dietary supplement [107-109].

Kava contains approximately 18 active compounds referred to as kavalactones. Six of these account for up to 95% of the pharmacological activity namely kavain,

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yangonin, methysticin, dihydrokavain, desmethoxyyangonin, and dihydromethysticin [106,110]. Kavalactones are found within the roots, root stems as well as the rhizomes of the plant, while the aerial parts are not traditionally consumed because they contain a toxic alkaloid, pipermethystine. Other constituents include amino acids, minerals, and the chalcones flavokavins A, B, and C [106]. Kava was initially seen as a safe alternative for serotonin re-uptake inhibitors; however, this was questioned when reports of kava-induced liver disease started to emerge [106,111]. To illustrate the hepatotoxicity of kava, six case reports will be discussed briefly, although many more have been reported in the literature.

One of the first cases of hepatotoxicity associated with kava was reported in 1998. A 39-year-old female was admitted for the recurring acute hepatitis. She had taken a herbal remedy containing kava (60 mg/day). Liver function tests revealed elevated γ -glutamyltransferase concentrations, while autoimmune, viral, or metabolic causes of the hepatitis were excluded. Liver biopsy revealed acute necrotizing hepatitis. Upon cessation of the use of the herbal preparation, liver function test levels returned to normal [111,112]. A case of kava hepatotoxicity in a 33-year-old female was reported. The patient consumed a herbal product (Laitan[®]) containing 210 mg kava for a period of 3 weeks, while no other drugs except for a homeopathic preparation (Exsept[®]) had been taken during this time. Within 8 weeks after discontinuing the use of Laitan[®], liver enzyme levels returned to normal [113]. A case of hepatitis associated with kava was reported in a 50-year-old male. The patient presented with jaundice, dark urine, and fatigue. The patient had been taking four kava capsules daily equivalent to 210 – 280 mg kava lactones with no history of alcohol or drug abuse. Liver function test revealed elevated levels of alkaline phosphatase, γ -glutamyltransferase, lactate dehydrogenase, total bilirubin, and conjugated bilirubin. Hepatitis A, B, C, and E as well as HIV, cytomegalovirus, and Epstein-Barr were excluded by means of blood tests. Within 48 h, the patient's condition deteriorated and developed into Stage IV encephalopathy. A liver transplant was performed within 2 days and the patient recovered successfully. A liver biopsy revealed extensive and severe hepatocellular necrosis as well as lobular and portal infiltration of lymphocytes and eosinophils [114].

In 2003, liver failure in a 60-year-old female patient taking 1200 mg kava per day for a duration of 12 months was reported. The patient presented with progressive liver failure, concomitant renal failure, as well as progressive encephalopathy. Viral, autoimmune, and metabolic causes were excluded by means of serological tests. A liver biopsy revealed extensive hepatocellular necrosis and intrahepatic cholestasis. Due to deterioration in the patient's condition, she received a liver transplant 11 days after admission and made a full recovery [111,115]. Acute hepatitis of a 14-year-old female was reported who presented with jaundice, nausea, and continuous vomiting for 10 days prior to admittance. Liver function tests revealed elevated total bilirubin, conjugated bilirubin,

aspartate aminotransferase, alanine aminotransferase, and γ -glutamyltransferase. The patient had been taking two Tension Tamers[®] capsules daily over a period of 4 months containing 100 mg Kava extract together with other minerals, vitamins, and herbal extracts. A urine screening test tested positive for rantidine, ibuprofen, and caffeine. Liver biopsy showed 70% hepatocellular necrosis. After 8 days of admission, the patient underwent orthotopic liver transplantation with the removal of the left lateral segment. The patient responded well to the transplant and liver function returned to normal within 7 months [109]. Toxic hepatitis was reported in a 42-year-old male after the consumption of a traditional kava preparation. The patient had no history of liver disease but consumed alcohol on a daily basis, never exceeding one drink. The patient presented with weakness, loss of appetite, and jaundice after consumption of a cumulative volume of 2 to 3 l of a traditional Kava preparation. Liver function tests revealed elevated levels of aspartate aminotransferase, alanine aminotransferase, γ -glutamyl transpeptidase, alkaline phosphatase, lactate dehydrogenase, and total bilirubin. Viral, metabolic, and autoimmune causes were excluded. A liver biopsy revealed toxic liver injury with infiltration of the portal fields by lymphocytes, eosinophilic granulocytes, necrosis of hepatocytes, and swollen Kupffer cells. Symptomatic treatment ensued and the patient recovered within 36 days [116].

2.9 Saw palmetto (*Serenoa repens*)

Saw palmetto (*Serenoa repens*) is a dwarf palm tree indigenous to the south-eastern US. The dried, ripe fruit of this plant is popular in the treatment of benign prostate hyperplasia and lower urinary tract symptoms [37,117]. Recent work has also highlighted the possible benefit of saw palmetto extract in the prevention and treatment of erectile dysfunction [118].

Saw palmetto is used as a dried fruit or as extract with doses ranging from 0.5 to 1.0 g as a decoction three times daily for the dried fruit and 320 mg daily as lipophilic extract [37]. The pharmacologically active components in saw palmetto consist of fatty acids (i.e., myristate, palmitate, stearate, oleate, and linoleate) and phytosterols (i.e., primarily β -sitosterol, campesterol, and stigmasterol) [37,119,120].

Data regarding the safety and toxicity of saw palmetto is limited and it appears to be relatively safe and well tolerated [121]. A detailed safety assessment of a saw palmetto extract administered in a randomized clinical trial to 225 men over 1 year has shown no serious clinical adverse effects [122,123]. In one study, however, cholestatic hepatitis has been attributed to the use of a commercial saw palmetto-containing product (Prostata[®]) [124]. In contrast to this study, no liver toxicity was found after the administration of a saw palmetto extract that was prepared in-house, to rats for up to 4 weeks. In fact, the results of this study suggested that saw palmetto might have a hepato-protective effect as its use was associated with a decrease in alanine aminotransferase and gamma-glutamyl transferase activities. The contradictory results between this study and the earlier study could in

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part be related to the fact that the commercial preparation that was associated with the cholestatic hepatitis did not only contain saw palmetto but also other ingredients that could contribute to the cholestatic hepatitis either as single ingredients or in combination. Contaminants in the product could have been responsible or contributed to the adverse effect [119].

A 58-year-old Caucasian male was admitted to hospital because of severe pain in the right hypochondrium and asthenia. Acute liver injury was diagnosed in the patient after a physical examination and blood tests were conducted. The patient had taken a commercially available product of *S. repens* during the last week before admittance to the hospital to treat the symptoms of benign prostatic hyperplasia. Upon discontinuation of the *S. repens* product, the symptoms disappeared and the biomarkers in the patient's blood returned to within the normal range. Chemical analysis (high performance liquid chromatography-mass spectrometry) of the product ruled out the presence of synthetic drugs or chemical contaminants as well as heavy metals [125].

2.10 Senna (*Cassia senna* and *Cassia angustifolia*)

Cassia is a diverse genus with over 600 known species [126]. *Cassia senna* or *Cassia acutifolia* is also known as Alexandrian senna, Aden senna, or Nubian senna and *Cassia angustifolia* is known as Indian senna, Tinnevely senna, or Mecca senna [127,128]. *Cassia senna* is indigenous to tropical Africa, Egypt, the Middle East, as well as Sinai and is cultivated in Sudan, whereas *Cassia angustifolia* is indigenous to Somalia and Southern Arabia and is cultivated in areas of northwest Pakistan, Southern India, California, and China [127,128]. Both the seeds and pods of these annual herbaceous subshrubs are traditionally used [128] for treatment of leprosy, ringworm, eczema, scabies, flatulence, colic, dyspepsia, constipation, cough, bronchitis, and cardiac disorders [127]. *Cassia* seeds are also roasted and used as a coffee substitute while the powder is employed in the pet-food industry [129]. Active constituents of *C. senna* and *C. angustifolia* include Senoside A, B, C, and D as well as aloe-emodin diathrone diglucoside [128]. Anthraquinone derivatives are contained in the pods of *C. senna* (i.e., gluco aloe-emodin, rhein-8-monoglucoside, and aglycone sennidin) and *C. angustifolia* (i.e., aloe emodin, chrysophanol, rhein and rhein glucosides, emodin anthranoids, senoside A, B, and senoside A₁) [126].

Of the aforementioned senna species, *C. angustifolia* has been associated with hepatotoxicity. A case of hepatitis after chronic abuse of senna was reported. A 26-year-old female ingested 10 g senna leaves infused as a tea twice a week and supplemented her treatment with 100 g senoside B. Liver function tests revealed elevated serum transaminase levels and histologically there had been moderate portal and lobular infiltration of lymphocytes and histocytes as well as extensive necrosis of the central veins. Upon discontinuing the use of senna and senoside B, her liver function test returned to normal within a week [130]. In 2005, the acute liver failure as well

as renal impairment in a 52-year-old female taking senna anthraquinone glycosides was reported. The patient presented with weakness, loss of appetite, and was stuporous and confused. Liver function tests revealed elevated levels of creatinine, uric acid, urea, phosphorus, aspartate aminotransferase, alanine aminotransferase, γ -glutamyl transferase, and bilirubin. Metabolic, viral, and auto-immune causes of hepatitis were excluded and the patient had no record of alcohol or drug abuse. She had, however, been taking 1 L herbal tea daily containing 70 g dry senna of *C. angustifolia* fruits. The use of senna had been discontinued and kidney as well as liver function test results returned to normal within 1 month [131]. A case of portal vein thrombosis related to *C. angustifolia* in a 42-year-old female was reported. The patient presented with epigastric pain, anorexia, vomiting, and fever with no history of alcohol or drug abuse. She had been taking 200 ml boiled dried senna leaves daily for 2 years. Abdominal examination resulted in generalized tenderness, the liver and spleen were not palpable or tender, and there were no indication of ascites. Liver function tests revealed mild abnormalities with increased alanine aminotransferase, and aspartate aminotransferase levels. Serological tests excluded viral, metabolic, and autoimmune afflictions as causes of hepatitis. Ultrasound screenings showed the liver and spleen to be normal in size with no ascites. The lumen was obstructed with no blood flow causing acute phase portal vein thrombosis. The patient was treated and discontinued the use of senna, but during a 2-month monitoring thereafter, no improvement in the obstruction of the portal vein ensued [132].

2.11 Traditional Chinese Medicines

Although the concept of Traditional Chinese Medicines (TCM) may include a variety of alternative treatment techniques as well as components from plant origin, animal origin, and minerals, the discussion in this paper will specifically focus on herbal medicines. It is estimated that > 25,000 TCM herbal formulations are prepared from 11,000 plant species [133]. It is well known that TCM can cause adverse effects and even Chinese herbal medicines that are used for treatment of liver diseases have been reported to cause hepatotoxicity [134].

Chinese herbal medicines usually consist of mixtures of different herbs. One of the herbs (the sovereign) will contain the main pharmacologically active ingredients, while the other herbs enhance the effect by means of synergism or additive effects or by facilitating delivery of the pharmacologically active ingredients. The aim of using complex mixtures of multiple biologically active compounds is to hit multiple targets and thereby achieving synergistic pharmacological effects at lower concentrations with less toxicity. This is in direct contrast with modern Western medicines where a single purified compound with well-defined pharmacological effects are aimed at specific targets to treat a disease [133,135].

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2.11.1 Cinnabar containing Chinese herbal medicines (An-Gong-Niu-Huang-Wan and Zhusha-Anshen-Wan)

An-Gong-Niu-Huang Wan is a patented TCM that contains 10% cinnabar, which has been used for acute and chronic brain diseases. Despite the fact that cinnabar contains 96% mercuric sulfide (HgS), it was shown in an *in vivo* study in mice with histopathology that common mercury salts (i.e., MeHg and HCl₂) induced much more severe liver damage than An-Gong-Niu-Huang Wan. Serum aminotransferase levels were only increased by the mercury salts, while long-term exposure to An-Gong-Niu-Huang Wan could potentially cause Hg–drug interactions [136].

Zhusha-Anshen-Wan is a TCM that contains cinnabar together with four herbs (i.e., *Coptis chinensis*, *Angelica sinensis*, *Rehmannia glutinosa*, *Glycyrrhiza uralensis*). Zhusha-Anshen-Wan is widely used for its sedative and calming effects. It was shown in an *in vivo* study in rats that these four herbs exhibited a protection effect against the toxicity of cinnabar [137].

2.11.2 Cang-Er-Zi

The fruit of *Xanthium strumarium* (known in Chinese as Cang-Er-Zi) has been used in Traditional Chinese Medicine for the treatment of nasal sinusitis, headache, urticaria, and arthritis. An *in vivo* study in rats showed that the major constituents of Cang-Er-Zi that cause hepatotoxicity are atractyloside, carboxyatractyloside and 4'-desulfate-atractyloside. Their hepatotoxic effects were associated with mitochondrial function inhibition and influencing of fatty acid metabolism [138].

2.11.3 He-Shou-Wu

He-Shou-Wu is a TCM obtained from the roots of *Polygonum multiflorum* and is used in the raw state (natural root) or in the processed form (boiled in black-bean liquid according to a traditional process). The raw form is used as an antioxidant and as a laxative, while the processed form is used as a tonic. It was shown that the hepatotoxicity of He-Shou-Wu is reduced by processing of the plant material. Furthermore, toxicity in general does not depend on the content of anthranoid derivatives, but rather the presence of tetrahydroxystilbene glucosides [139]. In another study, where the major phytoconstituents of He-Shou-Wu (i.e., 2, 3, 5, 4'-tetrahydroxy-stilbene-2-O-β-D-glucoside, physcion, and emodin) were investigated for cytotoxicity on the human liver cell line L-02, only emodin exhibited severe liver enzyme secretion [140].

In a systematic review of the literature on the hepatotoxicity of He-Shou-Wu preparations, a total of 450 case reports were found in 76 articles. From these case studies, it is clear that He-Shou-Wu causes liver damage to different degrees and liver injury is often associated with long-term use and overdose. The liver injury caused by He-Shou-Wu is reversible with improvement of liver function after discontinuation [141].

2.11.4 Rheum palmatum

Rheum palmatum (rhubarb) is a Chinese herbal medicine used in the treatment of liver diseases; however, many studies on the anthraquinone constituents of rhubarb have revealed conflicting results between this herbal medicine causing liver protection and liver injury. In an *in vivo* study on normal and carbon tetrachloride-treated rats, the rats were exposed to four different doses of rhubarb extract. It was clearly shown that the two lower doses exerted a curative effect in the carbon tetrachloride-treated rats, while the two higher doses caused liver fibrosis in both the normal and treated rats [9].

2.11.5 Xiao-chai-hu-tang

Xiao-chai-hu-tang is a very popular traditional Chinese herbal medicine, which consists of a blend of seven herbs namely *Bupleurum falcatum* root, *Pinellia ternata* tuber, *Scutellaria baicalensis* root (*Radix Scutellaria*), *Ziziphus jujuba* fruit, *Panax ginseng* root, *Glycyrrhiza glabra* root, and *Zingiber officinale* (ginger) rhizome. It was initially used to treat respiratory, gastrointestinal, and hepatobiliary diseases and has been reported to have anti-inflammatory and anti-cancer activities. In modern times, it is more used for the treatment of liver diseases. In contrast to its current use of curing liver diseases, at least five case studies have been reported where Xiao-chai-hu-tang has been responsible for HILI [142]. Saikosaponin D was isolated from *Bupleurum falcatum* root, which is a triterpene saponin with a steroid-like structure that may cause liver injury. In a mechanistic *in vitro* study on human LO2 hepatocytes, it was shown that saikosaponin D is capable of reducing cell viability, decreasing mitochondrial membrane potential, changing cell morphology, and stimulating hepatocyte apoptosis [8].

2.12 Valerian (*Valeriana officinalis*)

Valeriana species are part of the Caprifoliaceae family and can be described as perennial flowering plants native to Europe and parts of Asia, although it has also been found in Central and North America. Three species are commonly used for medicinal purposes including *Valeriana officinalis*, *Valeriana edulis* (Mexican valerian), and *Valeriana wallichii* (Indian valerian) [143]. *V. officinalis* is more commonly known as valerian, garden valerian, all-heal, or garden heliotrope. Traditionally, the roots and rhizomes of the plants are soaked in mixtures of water and/or ethanol or methanol and then the mixture is dried to concentrate the plant constituents. There are variations in the extraction techniques and extraction solvent (ethanol or aqueous) that relates to variations in the chemical constituents in the extracts [144,145]. More than 100 – 150 compounds have been found in the essential oil of *Valeriana* species; however, their concentrations vary due to seasonal variations [145,146]. The chemical composition of *Valeriana* species includes sesquiterpenes (valeric acid), iridoids (valepotriates), alkaloids, and several free amino acids. The sesquiterpenes are mostly associated with the biological or

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pharmacological effects of valerian [147]. *V. officinalis* roots and rhizomes contain 0.2 – 2.8% essential oils, which mostly consist of sesquiterpenoids [146]. Valerian is medicinally used as a sleep aid to treat mild anxiety and reduce muscle tension [144,147-150]. The typical therapeutic dose as a sleep aid is 300 – 600 mg valerian daily. Valerian is often used in combination with other herbs as sleep aids and was shown to be an effective and safe alternative to zolpidem [151].

In a systematic review of clinical trials with various types of valerian extracts, it was reported that no side effects occurred, including any changes in liver function or other hepatic symptoms [144]. Case studies on hepatic impairment have, however, been reported for valerian. Over-the-counter products that aid with sleep disorders that contain valerian have shown evidence of hepatotoxicity in four patients [152]. Hepatic inflammation was reported in a 27-year-old female that took valerian extract for 3 months. Discontinuation of the herbal preparation led to a full recovery with no permanent effects [153]. Valerian is often used in combination with other herbal remedies leading to hepatitis in various cases [152,154,155].

A case study reported on a 50-year-old woman with altered liver biochemistry after consuming tea with valerian as well as over-the-counter tablets containing 125 mg dry valerian. A follow-up investigation revealed further changes in liver biochemistry after 1 month since discontinuation of the herb; however, a gradual recovery was observed 2 months later [156]. Various clinical studies have proved the effectiveness of valerian (as noted above), but only a few reported on adverse effects associated with the liver. In a study involving 23 patients taking 'Sleep-Qik' with 75 mg dry valerian extract, no clinical evidence of acute hepatitis was observed [157]. Another study in 39 patients reported improvement of insomnia by valerian-containing products with only mild adverse events [151].

A study investigated the effect of valerian on rat liver (*in vivo*) and on cultured human hepatoma cells (*in vitro*). The rats received a single dose of valerian (acute) or daily doses for 28 days (chronic) whereafter the rat livers were removed for histological investigations. The results indicated that liver enzyme activity, sinusoidal histology, and bile flow were unaffected after the acute and chronic treatments. The *in vitro* study exhibited a high cell death at the highest concentration (20 mg/ml) of valerian tested. This dose is, however, 2 – 3 orders in magnitude higher than is recommended for therapeutic use in humans [158].

2.13 Wild germander (*Teucrium chamaedrys*)

Wild germander or Wall germander (*Teucrium chamaedrys*) is a member of the Lamiaceae (Labiata, Mint) family, which primarily inhabits the northern temperate and subtropical regions of the eastern hemisphere. It abounds in southern Europe and the Mediterranean and in the UK as well as the US [134,159].

The blossoming aerial parts of wild germander have been used in traditional medicine for many years. The aerial parts

are used to prepare herbal teas (1 g per bag) or alcohol-based extracts (75 – 150 mg per 100 ml) which are mixed with various other herbs. *T. chamaedrys* capsules have also been manufactured, each containing 200 – 250 mg of the powdered aerial parts [160]. The extracts, tea, and dried plant materials are used to treat depression, asthma, bronchitis, fever, digestive problems, and jaundice. It is also used as an antiseptic, diuretic, and tonic. In France, it has been used since 1991 as an adjuvant to weight control diets in spite of the fact that no published scientific proof of efficacy exists [160].

In a literature review, it was reported that a total of 52 cases of hepatitis were documented where remedies containing *Teucrium* species were implicated to be the causative agent. In most cases, hepatotoxicity became evident at 2 to 3 months after ingestion of decoctions containing wild germander constituents. Varying degrees of hepatotoxicity has been reported ranging from fulminant hepatitis to chronic hepatitis and also cirrhosis [160-162]. In the majority of cases, the hepatitis was transient in nature followed by complete recovery although one fatality, due to fulminant hepatic necrosis, has been documented [162]. Typical symptoms of hepatocellular hepatic adverse reactions such as anorexia, nausea, vomiting, abdominal pain, and jaundice were usually present in the majority of reported cases of hepatotoxicity [134].

The aerial parts of wild germander contain a number of constituents such as furanic neoclerodane diterpenes (NCDs), saponins, and glycosides [160,163,164]. Wild germander-induced hepatotoxicity is primarily mediated by the biotransformation of NCDs by CYP3A4 that leads to the formation of electrophilic metabolites which deplete cellular thiols [165]. The NCD fraction is comprised of, among others, teucriin A and teuchamaedryn A, which are considered to be the major toxic substances. NCDs contain a furan ring which is readily oxidized by CYP3A4 at the double bond of the furan moiety to form reactive epoxides and/or unsaturated aldehydes. These epoxides initiate chemical reactions with proteins such as CYP3A and epoxide hydrolase [166]. It has been proposed that rearrangement of the putative epoxide intermediates may form reactive metabolic products such as 1,4-enedials [167]. Studies performed in rat hepatocytes have shown that reactive epoxides caused mitochondrial permeability transition, caspase activation, and apoptosis [166]. Teucriin A, which is covalently bound to rat hepatocyte proteins, and the furan diterpenoid fraction were also reported to be responsible for a reduction in cell glutathione and cytoskeleton-associated protein thiols, but elevated cytosolic levels of Ca²⁺ [165,166].

A combination of the above events is considered to be chiefly responsible for the formation of membrane blebs, DNA fragmentation, and cell apoptosis which are evident in hepatocytes following exposure to wild germander constituents [165-167].

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Table 1. Herbs linked to a high probability of causing hepatotoxicity.

Herbal product	Ref.
Chaparral (<i>Larrea tridentata</i>)	[54,168]
Comfrey (<i>Symphytum officinale</i>)	[65-72]
Greater celandine (<i>Chelidonium majus</i>)	[10,74,76,78]
Kava kava (<i>Piper methysticum</i>)	[106,109,111,114,115,168]
Senna (<i>Cassia acutifolia</i>)	[168]
Traditional Chinese Medicines (<i>He-Shou-Wu</i>)	[141,168]
Wild germander (<i>Teucrium chamaedrys</i>)	[160-162,168]

3. Conclusion

Although hepatotoxicity has been linked to certain herbal products, causality has not necessarily been proven and contradictory reports exist for some. From the selected herbs discussed in this review, a summary is given in Table 1 of herbal products that can be deemed highly probable to be hepatotoxic. These herbs are best avoided due to the risk related to potential hepatotoxicity.

4. Expert opinion

The liver has numerous vital functions; one of paramount importance is the detoxification of compounds. Hepatocytes possess high concentrations of xenobiotic-metabolizing enzymes that are able to transform potentially harmful compounds to harmless water-soluble metabolites. The liver is therefore a target for toxic effects of plant constituents. Plant chemistry is variable and plants from different regions can contain different compounds. One plant can also contain multiple compounds that act together to result in a therapeutic (or toxic) effect. Although active compounds in herbal medicines are frequently isolated and characterized, they are rarely studied in depth with regard to potential toxicity.

Causality assessment is currently the gold standard for identification of herb-induced hepatic injury, although the methods used for this assessment are not without weaknesses and shortcomings [10-12]. The Council for the International Organization of Medical Sciences has developed the most widely used herbal hepatotoxicity causality diagnostic tool, but its performance is hindered by poor reporting, including fragmentary herbal identification, incomplete case data, failure to state duration of use, and a lack of liver enzyme evaluations. The best way to prove hepatotoxicity of a specific herbal medicine is to re-challenge the patient, but the ethical aspects related to this method remains problematic. All causality

assessments are retrospective and do little to prevent similar occurrences due to the indiscriminate use of herbal medicines as the case reports rarely allow for characterization of general hepatotoxicity for the specific herbal product. There is some development with respect to the use of analytical methods and 'omics' technologies to help identify intrinsic hepatotoxicity in patients, but this is still retro-active with limited use for prediction of potential hepatotoxicity.

The lack of requirements for extensive pre-clinical and/or clinical studies and safety standards for marketing of herbal products in comparison with conventional medicine is troubling. Several factors contribute to challenges in producing reliable data in this regard including the complexity of the chemical composition of herbal products, unregulated production of herbal products causing inconsistent content, and variation in phytochemical composition due to preparation method, time of harvesting (i.e., seasonal differences), storage, geographical area, and different parts of the plants used. Another challenge for herbal hepatotoxicity research is that herb-induced liver damage often appears to be via idiosyncratic immunological reactions, which can be very unpredictable. Therefore, more studies are needed to confirm the mechanisms of hepatotoxicity.

Although some scientists indicated that basic experimental studies and use of hepatocyte cell cultures are of limited use in the prediction of toxicity due to the unpredictable nature of herbal hepatotoxicity, not all herbal injury cases are idiosyncratic and intrinsic hepatotoxicity can be investigated in cell-based models, especially with newer 3D culturing techniques which enable long-term exposure to potential toxins. These models may also be useful in the search for biomarkers of herbal hepatotoxicity, to identify dose-dependent patterns as well as to clarify efficacy of herbal products and isolated phytochemicals. Such studies will invariably need to be based on standardized, quality-controlled herbal products to be of value and *in vitro-in vivo* correlations will have to be determined. The contribution of herb-drug interactions to potentiation of hepatotoxicity is another area that needs to be investigated in future.

Declaration of interests

The financial assistance of the National Research Foundation (NRF) of South Africa is hereby acknowledged. Opinions expressed and conclusions arrived at, are those of the author and are not necessarily to be attributed to the NRF. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript.

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CHAPTER 3**MANUSCRIPT PUBLISHED IN TOXICOLOGY MECHANISMS AND
METHODS**

Chapter 3 is presented in the form of a review manuscript published in the journal *Toxicology Mechanisms and Methods* in 2018 (doi: 10.1080/15376516.2017.1422580). The complete guide for authors for this journal is provided in Appendix B, stating the manuscript should be written in Times New Roman font according to the Microsoft Word template file provided.

Recent advances in three-dimensional cell culturing to assess liver function and dysfunction: from a drug biotransformation and toxicity perspective

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ABSTRACT

The liver is a vital organ fulfilling a central role in over 500 major metabolic functions, including serving as the most essential site for drug biotransformation. Dysfunction of the drug biotransformation processes may result in the exposure of the liver (and other organs) to hepatotoxins, potentially interacting with cellular constituents and causing toxicity and various lesions. Hepatotoxicity can be investigated on a tissue, cellular and molecular level by employing various *in vivo* and *in vitro* techniques, including novel three-dimensional (3D) cell culturing methods. This paper reflects on the liver and its myriad of functions and the influence of drug biotransformation on liver dysfunction. Current *in vivo* and *in vitro* models used to study liver function and dysfunction is outlined, emphasizing their advantages and disadvantages. The advantages of novel *in vitro* 3D cell culture models are discussed and the possibility of novel models to bridge the gap between *in vitro* and *in vivo* models is explained. Progression made in the field of cell culturing methods such as 3D cell culturing techniques over the last decade promises to reduce the use of *in vivo* animal models in biotransformation and toxicological studies of the liver.

ARTICLE HISTORY

Received 23 November 2017

Revised 21 December 2017

Accepted 25 December 2017





KEYWORDS

3D models; drug biotransformation; drug toxicity; *in vitro* models; liver dysfunction

1. Introduction

The liver, a powerhouse of metabolic processes, is a complex three-dimensional vital organ with a multitude of interrelated physiological and biochemical functions (Guillouzo 1998; Wolf 1999; Van Zijl and Mikulits 2010; Singh et al. 2011). Due to the liver's involvement in various metabolic functions, in particular biotransformation of xenobiotics, it has become apparent that studying the physiological and pathophysiological condition of the liver is key to drug development (Van Zijl and Mikulits 2010). On the microscopic level, a myriad of cells within the liver is responsible for this maintenance of normal physiology and biochemistry (Nakamura et al. 2011; Wong et al. 2011). Hepatocytes, comprising 70–80% of the cytoplasmic liver mass, are the chief functional cells of the liver. These versatile somatic cells have a remarkable ability to regenerate and play a central role in the dynamic homeostasis of the liver (Vekemans and Braet 2005; Holt and Smith 2007; Ramadori et al. 2008; Nakamura et al. 2011; Wong et al. 2011). Functions performed by hepatocytes, and essentially the liver, include exocrine and endocrine functions, protein synthesis and storage, synthesis of cholesterol, bile salts and phospholipids, metabolism of carbohydrates and lipids as well as biotransformation of various endogenous and exogenous compounds (Holt and Smith 2007; Ramadori et al. 2008). The liver sinusoid, a capillary lined

with sinusoidal endothelial cells and surrounded by hepatocytes, is the most basic functional unit of the liver. The endothelial cells are separated from the hepatocytes by a small space referred to as the space of Disse. Small channels referred to as bile canaliculi are formed between adjacent hepatocytes. The bile secreted by hepatocytes are collected into bile ducts and transported to the gall bladder for storage until it is needed in the intestines (Kang et al. 2013). Other non-parenchymal cells contribute to the remaining 20–30% of the liver mass, these include the stellate cells that help maintain the extracellular matrix (ECM), Kupffer cells that act like macrophages, natural killer cells and fibroblasts (Vekemans and Braet 2005; Kang et al. 2013). These various hepatic cell types working in a coordinated manner constitute the basic building blocks of the liver as a tissue. During dysfunction of the liver the 'normal' behavior of these cells is altered influencing growth, differentiation, marker secretion, invasion, migration or even death. It is these attributes that are helpful in the investigation of drug biotransformation and hepatotoxicity during drug development as well as in the assessment of normal and disease liver states (Gupta et al. 2016). However, current models to study biotransformation and hepatotoxicity hinder the elucidation of complex mechanistic liver functions, thus the development of novel experimental tools remain essential (Van Zijl and Mikulits 2010).

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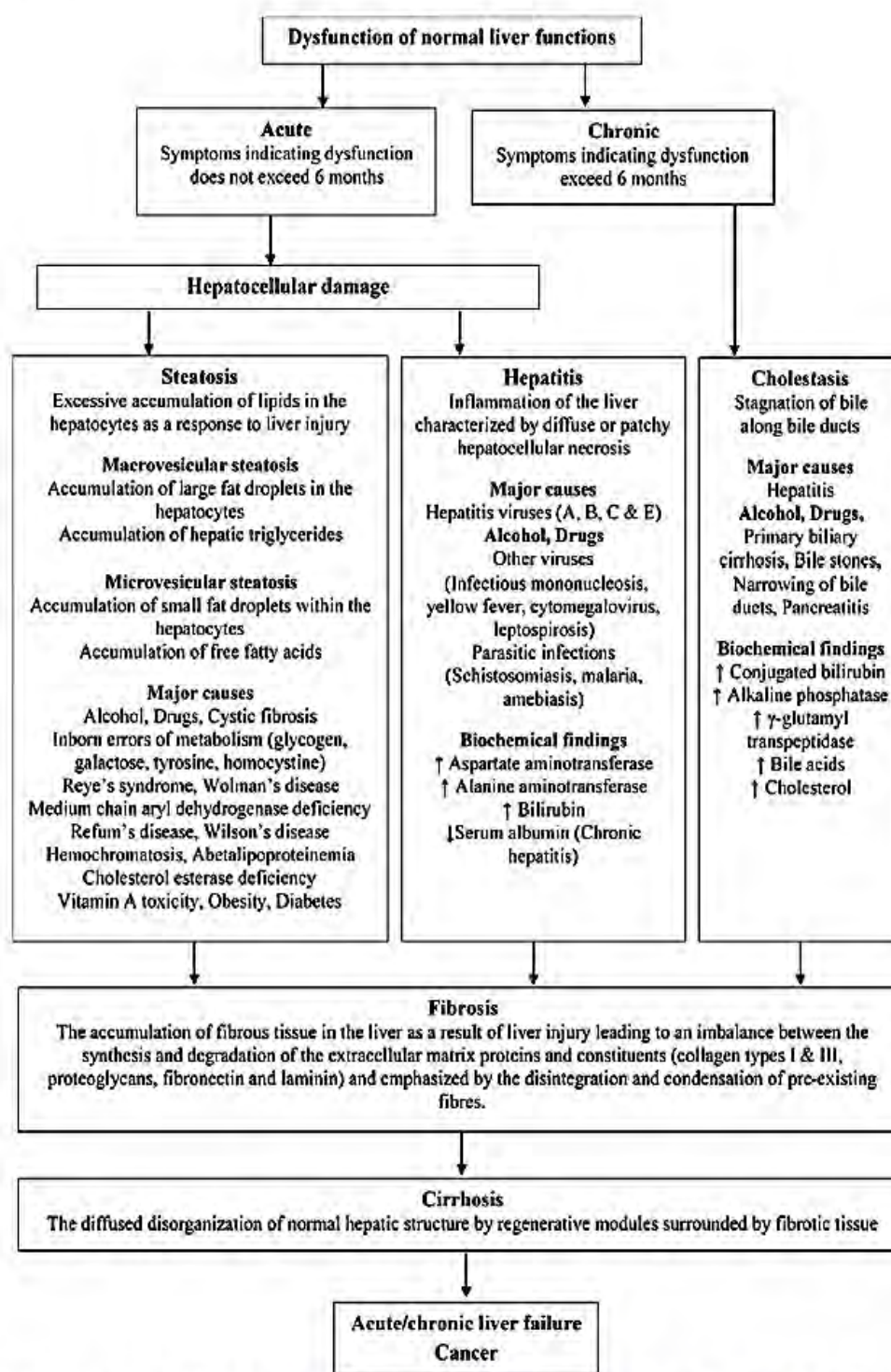


Figure 1. The progression from liver damage to liver disease as a result of liver dysfunction (Adapted from Wolf 1999; Heidelbaugh and Bruderly 2006; Featherstone 2007; Bernal et al. 2010; Hirschfeld et al. 2010; Merrel and Cherrington 2011; Panqueva 2014; Privitera et al. 2014).

2. Liver dysfunction

Dysfunction and disease states of the liver results in devastating and often lethal consequences, as illustrated in Figure 1. Liver dysfunction presents as either hepatocellular damage and/or cholestasis and is classified as acute or chronic depending on the time of onset (Wolf 1999; Featherstone

2007; Privitera et al. 2014). Dysfunction in the form of hepatocellular damage occur due to various inflammatory responses including steatosis, hepatitis and/or cell death (necrosis), ultimately resulting in fibrosis and/or cirrhosis depending on the duration of the assault (Featherstone 2007). Steatosis (fatty liver or nonalcoholic fatty liver disease

(NAFLD)) is defined as the infiltration of hepatic triglycerides exceeding 5% by weight in the liver that occurs as a result of dysfunctional lipid metabolism. This accumulation of lipids may either be microvascular, macrovascular or both (Featherstone 2007; Merrel and Cherrington 2011). Microvascular steatosis is influenced by multiple factors and is also the result of drug toxicity from tetracyclines, while the development of macrovascular steatosis is attributed to alcoholic steatosis. NAFLD is the most common liver disorder in developed countries, is often related to the metabolic syndrome, insulin resistance and obesity. Persons suffering from NAFLD often show few symptoms apart from fatigue or general discomfort and so are often diagnosed only during routine blood tests. Since there is currently no approved treatment, acute liver failure due to NAFLD remains problematic (Featherstone 2007). Hepatitis or inflammation of hepatocytes due to cellular damage is attributed to the dysfunction of drug metabolism, alcohol metabolism, lipid metabolism and autoimmune diseases (Heidelbaugh and Bruderly 2006; Featherstone 2007). The most common cause of chronic hepatitis, however, remains viral infection (hepatitis C and B virus) (Featherstone 2007; Ramadori et al. 2008; Bernal et al. 2010).

During cholestasis there is an elevation of substances excreted by the bile and liver enzymes associated with the biliary tract as seen in Figure 1 (Featherstone 2007; Panqueva 2014). Accumulation of bile acids due to obstruction of the bile duct may also damage hepatocytes leading to the onset of fibrosis and cirrhosis if left untreated (Featherstone 2007; Hirschfield et al. 2010; Panqueva 2014). Cholestasis impedes drug biotransformation and metabolism due to sluggish or stagnant bile flow that impairs the biliary excretion of drugs causing a decrease in the solubility and absorption of fat soluble vitamins (Featherstone 2007).

The liver has remarkable regenerative capabilities and if disease states are addressed at an early stage, most of the hepatocellular damage may be reversed. However, if disease states are left untreated, then liver scar tissue can form during the restorative processes, which is a result of an imbalance between fibrogenesis and fibrolysis. This may lead to liver fibrosis, which disrupts blood flow and delivery of many essential substances (Featherstone 2007; Ramadori et al. 2008). Fibrosis is also the endpoint for most disease states resulting in chronic liver injury (Ramadori et al. 2008). Continued exposure to harmful substances, unhealthy lifestyle habits and disease states can ultimately lead to liver cirrhosis and liver failure or cancer (Heidelbaugh and Bruderly 2006; Featherstone 2007; Ramadori et al. 2008; Bernal et al. 2010; Merrel and Cherrington 2011). Thus, addressing possible liver dysfunction at an early stage may prove to enhance the life of a great many people.

3. Drug bio-transformation in the liver and its effect on liver dysfunction

The liver is seen quantitatively and qualitatively as the most essential site for drug biotransformation due to its ability to metabolize an almost endless selection of substrates

(Wilkinson 2005; Liddle and Stedman 2006). A lack of biotransformation may result in an increase in the bioavailability and pharmacological activity of most drugs (by reducing the rate of their removal) (Tingle and Helsby 2006). Some drugs are inactive ('prodrugs') and need to undergo bio-activation to form the pharmacologically active metabolites. Hepatic drug biotransformation is governed by various factors including hepatic blood flow, plasma albumin binding, hepatocyte drug uptake, the functional integrity of hepatocytes as well as influences of the hepatobiliary system (George 1995; Ahmed and Siddiqi 2006). The biotransformation process can result in the production of hepatotoxins which may elicit interactions with cellular constituents, including proteins, lipids, ribonucleic acid (RNA) and deoxyribonucleic acid (DNA), which eventually will cause hepatotoxicity and various liver lesions, most frequently steatosis (Guillouzo 1998; Singh et al. 2011). These interactions can result in a series of events in the liver, with a subsequent reaction from the liver as a result of injury. Liver toxicity due to pharmaceuticals and xenobiotics remain a concern as this is associated with distinct histopathological and clinical phenotypes, namely steatosis, cholestasis and hepatitis as illustrated in Figure 1 (Driessen et al. 2013; Sirenko et al. 2016). Currently, preliminary hepatotoxicity studies rely strongly on the extrapolation of data obtained from *in vivo* animal models and available *in vitro* cell culture models (Nakamura et al. 2011; Sirenko et al. 2016). Therefore, developing complex systems that can easily identify as well as effectively test potential hepatotoxicity remain an area of active investigation (Sirenko et al. 2016).

4. Current models used to study drug biotransformation and dysfunction in the liver

Due to the critical role of drug biotransformation in the development of new drug entities, as well as the potentially serious effects liver dysfunction may have on drug plasma levels and overall well-being of patients, numerous models and techniques have been developed to study dysfunction of the liver and its impact on drug biotransformation pathways. All these models and techniques have advantages and disadvantages, and the most suitable model must be selected for each application. The models and techniques frequently used for these various applications is briefly summarized in Table 1, along with some advantages and disadvantages. Animal models and the ethical considerations they involve, as well as the recent advances in three-dimensional cell culture techniques are discussed in more detail below.

4.1. Animal models: Ethical considerations and advances

In vivo animal models are able to take into account the combined effect of all pharmacokinetic parameters and pharmacological effects (i.e. multifactorial), while *in vitro* models can only measure a limited number of features (Zhang et al. 2012). The relevance of animal models may be questionable due to species differences, but the importance of these models in the development and testing of medicines, especially toxicity, is irrefutable. Historically, approximately 70% of

Table 1. Models used to study drug metabolism and dysfunction in the liver, with some of their advantages and disadvantages.

Model	Advantages	Disadvantages	References
<i>In vitro</i>			
Recombinant enzymes	<ul style="list-style-type: none"> • Effective and fast means to identify single CYP enzymes involved in drug metabolism • Various CYP enzymes are commercially available • Simplistic, well established, cost effective and high throughput screening assays • Identification of possible biotransformation pathways, CYP enzymes involved and mechanisms • Identification of substrates/inhibitors of metabolizing enzymes • Generating metabolites of interest 	<ul style="list-style-type: none"> • Highly simplified specific assays which do not fully resemble the <i>in vivo</i> environment • Extrapolation process of data is complicated • Metabolic rates differ from those seen <i>in vivo</i> and in tissue • Phase I with phase II sequential metabolism is not addressed 	(Ekins et al. 2000; Tingle and Helsby 2006; Lipscomb and Poet 2008; Zhang et al. 2012).
Subcellular fractions			
• Liver cytosol	<ul style="list-style-type: none"> • Drug enzyme activities preserved • High concentrations of N-acetyl transferase, sulfotransferase and glutathione S-transferase, present • Well established, affordable and simplistic method • Most widely used subcellular fraction • Inter-individual variation can be studied • Drug enzyme activities preserved • Qualitative determination of metabolic identity • CYP reaction phenotyping • Provides information regarding drug-drug interaction and/or herb-drug interactions • Provides information regarding metabolic stability of the drug • Production of metabolites • Maintains higher levels of metabolizing enzyme activity than hepatocytes • Isolation of groups of enzymes possible • Easy to prepare and reproducible • Potential for long term storage • Offers a more complete representation of the metabolic profile • Contains both phase I and II enzyme activity 	<ul style="list-style-type: none"> • Short term studies • Only soluble phase II enzymes present • Full metabolic pathways cannot be investigated • Short term studies • Lack cell plasma membranes and drug transporters • Unstable for quantitative measurements • Incomplete representation of <i>in vivo</i> situation • Do not maintain the balance between phase I and II enzymes • No cytosolic phase II enzyme reactions only CYP and UGT enzymes • Difficulty in studying multi-step metabolic processes • Co-factors required for activity 	(Guillouzo 1998; Brandon et al. 2003).
• Liver microsomes	<ul style="list-style-type: none"> • Provides information regarding metabolic stability of the drug • Production of metabolites • Maintains higher levels of metabolizing enzyme activity than hepatocytes • Isolation of groups of enzymes possible • Easy to prepare and reproducible • Potential for long term storage • Offers a more complete representation of the metabolic profile • Contains both phase I and II enzyme activity 	<ul style="list-style-type: none"> • Short term studies • Lack cell plasma membranes and drug transporters • Unstable for quantitative measurements • Incomplete representation of <i>in vivo</i> situation • Do not maintain the balance between phase I and II enzymes • No cytosolic phase II enzyme reactions only CYP and UGT enzymes • Difficulty in studying multi-step metabolic processes • Co-factors required for activity 	(Guillouzo 1998; Brandon et al. 2003; Tingle and Helsby 2006; Lipscomb and Poet 2008; Alqahtani et al. 2013).
• S9 fractions	<ul style="list-style-type: none"> • Provides information regarding metabolic stability of the drug • Production of metabolites • Maintains higher levels of metabolizing enzyme activity than hepatocytes • Isolation of groups of enzymes possible • Easy to prepare and reproducible • Potential for long term storage • Offers a more complete representation of the metabolic profile • Contains both phase I and II enzyme activity 	<ul style="list-style-type: none"> • Overall lower enzyme activity compared to liver microsomes and cytosol fractions • Requires co-factors for activity 	(Brandon et al. 2003; Lipscomb and Poet 2008).
Hepatocytes			
• Isolated hepatocytes	<ul style="list-style-type: none"> • Obtained from whole livers or wedge biopsies • Functions close to those of <i>in vivo</i> hepatocytes • Studies possible on several compounds at different concentrations • Interspecies studies possible • Representative of different lobular sub-populations • Drug transporters are present and functional • Sequential examination of Phase I and II pathways possible • Cryopreservation and preservation of phase I and II enzymes possible • Conservation of physiological co-factors • Well established and well characterized <i>in vitro</i> model • Viable for 4 weeks • May be incubated in suspension culture or plated • Study of mediators and enzyme inducers possible • Commercially available • Sequential examination of Phase I and II pathways possible • Partially differentiated cells, all enzymes and co-factors were initially present at physiological levels when the hepatocytes were isolated • Functions expressed for several days in certain conditions • Induction and inhibition of drug metabolizing enzymes possible • Interspecies studies possible • Sandwich culture and gel-immobilized techniques can be performed 	<ul style="list-style-type: none"> • No bile canaliculi • Difficulty in obtaining fresh human cultures • Prolonged culture conditions result in complex data interpretation • Isolation process can be complicated and damage cells altering enzyme activity • Cellular interactions more difficult to study due to lack of non-hepatocyte liver cells • Inter-individual variation in CYP expression • Pathological status of donor and donor availability • Rapid decline in CYP activities (within hours or days): current supplier recommendations state that they should be used within 2 h of thawing. 	(Guillouzo 1998; Brandon et al. 2003; Tingle and Helsby 2006; Lipscomb and Poet 2008; Alqahtani et al. 2013).
• Primary hepatocyte cultures	<ul style="list-style-type: none"> • Sequential examination of Phase I and II pathways possible • Partially differentiated cells, all enzymes and co-factors were initially present at physiological levels when the hepatocytes were isolated • Functions expressed for several days in certain conditions • Induction and inhibition of drug metabolizing enzymes possible • Interspecies studies possible • Sandwich culture and gel-immobilized techniques can be performed 	<ul style="list-style-type: none"> • Early phenotypic changes • Altered bile canaliculi • Rapid decline in CYP activities 	(Guillouzo 1998; Ekins et al. 2000; Brandon et al. 2003; Tingle and Helsby 2006; Alqahtani et al. 2013; Kanuri and Bergheim 2013; Ohkura et al. 2014).

(continued)

Table 1. Continued

Model	Advantages	Disadvantages	References
Liver cell lines HepG2; Huh7; HepG2/C3A HepaRG Fa2N-4 Transgenic cell lines	<ul style="list-style-type: none"> • Unlimited cell number • High availability • Low variability between experiments • Some functions preserved (and some can be recovered by 3D culture in systems which mimic <i>in vivo</i> conditions) • Co-culture with additional liver cell types (e.g. fibroblasts, endothelial, Kupffer cells or biliary-endothelial cells can induce additional functionality) • Available in larger amounts than primary hepatocytes • Throughput depends on the application • Relatively unlimited supply • Defined phenotype 	<ul style="list-style-type: none"> • Various drug enzyme activities lost or decreased • Genotype instability • Lack phenotypical and functional characteristics of liver tissue 	(Guillouzo 1998; Fey and Wrzesinski 2012b; 2013; Soldatow et al. 2013; Kanuri and Bergheim, 2013).
Stem cells	<ul style="list-style-type: none"> • Available in larger amounts than primary hepatocytes • Throughput depends on the application • Relatively unlimited supply • Defined phenotype • Allows studies of inter-individual variability • Qualitative and quantitative data • Intact cellular architecture and lobular structure preserved • All enzyme and cell types preserved • Maintains zone-specific CYP activity • Cell-cell contact • Study of mechanisms of toxicity possible • Co-factors maintained close to physiological concentrations • Morphological studies possible • Selective intra-lobular effects detectable • Studies on human liver possible • Can be performed on various species making comparisons easy – standard procedure • Good <i>in vivo</i> - <i>in vitro</i> correlation • Studies possible on several compounds at different concentrations • Easy to prepare and avoids the use of harmful proteases or other enzymes 	<ul style="list-style-type: none"> • Currently, conditions are not known which can induce complete differentiation: the best can reach early fetal stages. • Ethical concerns • Highly variable functionality within cell populations • May be useful for short term culture only (2-4 days) • Requires special media • Low expression levels of liver specific metabolism enzymes • Complex 'reprogramming' steps • Low expression levels of liver specific metabolism genes • May be useful for short term culture only (2-4 days) • Variability among preparations • Limited literature for toxicology • Limited viability (6h to 5 days) • Necrosis starts to develop after 48-72 h • No collection of bile possible • Poor correlation between intrinsic clearance rates and K_m values • Donor variability • Inter-assay variability (not all cells preserved similarly, damaged cells on outer edges and inadequate penetration of medium) • Need for specialized equipment and further development • CYP cannot be induced • Slight decrease in Phase II enzymes, albumin production and gluconeogenesis after 96 h • Metabolic enzyme levels decreases after 72h • False prediction of metabolic rates • Uptake and metabolism lower in liver slices than hepatocytes • Cryopreservation has been demonstrated, needs optimization 	(Soldatow et al. 2013; Ranga et al. 2014; Shearier et al. 2016).
Induced pluripotent stem cells	<ul style="list-style-type: none"> • Throughput depends on the application • Not as controversial source of cells as embryonic stem cells • Relatively unlimited supply • Defined phenotype • Allows studies of inter-individual variability • Qualitative and quantitative data • Intact cellular architecture and lobular structure preserved • All enzyme and cell types preserved • Maintains zone-specific CYP activity • Cell-cell contact • Study of mechanisms of toxicity possible • Co-factors maintained close to physiological concentrations • Morphological studies possible • Selective intra-lobular effects detectable • Studies on human liver possible • Can be performed on various species making comparisons easy – standard procedure • Good <i>in vivo</i> - <i>in vitro</i> correlation • Studies possible on several compounds at different concentrations • Easy to prepare and avoids the use of harmful proteases or other enzymes 	<ul style="list-style-type: none"> • Currently, conditions are not known which can induce complete differentiation: the best can reach early fetal stages. • Ethical concerns • Highly variable functionality within cell populations • May be useful for short term culture only (2-4 days) • Requires special media • Low expression levels of liver specific metabolism enzymes • Complex 'reprogramming' steps • Low expression levels of liver specific metabolism genes • May be useful for short term culture only (2-4 days) • Variability among preparations • Limited literature for toxicology • Limited viability (6h to 5 days) • Necrosis starts to develop after 48-72 h • No collection of bile possible • Poor correlation between intrinsic clearance rates and K_m values • Donor variability • Inter-assay variability (not all cells preserved similarly, damaged cells on outer edges and inadequate penetration of medium) • Need for specialized equipment and further development • CYP cannot be induced • Slight decrease in Phase II enzymes, albumin production and gluconeogenesis after 96 h • Metabolic enzyme levels decreases after 72h • False prediction of metabolic rates • Uptake and metabolism lower in liver slices than hepatocytes • Cryopreservation has been demonstrated, needs optimization 	(Soldatow et al. 2013; Zhang et al. 2014; Rookmaaker et al. 2015).
Liver slices	<ul style="list-style-type: none"> • Qualitative and quantitative data • Intact cellular architecture and lobular structure preserved • All enzyme and cell types preserved • Maintains zone-specific CYP activity • Cell-cell contact • Study of mechanisms of toxicity possible • Co-factors maintained close to physiological concentrations • Morphological studies possible • Selective intra-lobular effects detectable • Studies on human liver possible • Can be performed on various species making comparisons easy – standard procedure • Good <i>in vivo</i> - <i>in vitro</i> correlation • Studies possible on several compounds at different concentrations • Easy to prepare and avoids the use of harmful proteases or other enzymes 	<ul style="list-style-type: none"> • Limited viability (6h to 5 days) • Necrosis starts to develop after 48-72 h • No collection of bile possible • Poor correlation between intrinsic clearance rates and K_m values • Donor variability • Inter-assay variability (not all cells preserved similarly, damaged cells on outer edges and inadequate penetration of medium) • Need for specialized equipment and further development • CYP cannot be induced • Slight decrease in Phase II enzymes, albumin production and gluconeogenesis after 96 h • Metabolic enzyme levels decreases after 72h • False prediction of metabolic rates • Uptake and metabolism lower in liver slices than hepatocytes • Cryopreservation has been demonstrated, needs optimization 	(Guillouzo 1998; Ekins et al. 2000; Brandon et al. 2003; Tingle and Helsby 2006; Lipscomb and Poet 2008; Aiqahani et al. 2013; Soldatow et al. 2013).
Isolated perfused liver	<ul style="list-style-type: none"> • Best representation of <i>in vivo</i> system (all enzyme functions preserved) • Three-dimensional architecture and lobular structure preserved • All liver cell types present, biotransformation studies of non-hepatocytes possible • Functional bile canaliculi • Collection of bile possible • Short term kinetic studies 	<ul style="list-style-type: none"> • Short term viability (2-3 h) • Delicate model and difficult to handle • Study of one or a few compounds only • Bile excretion decreased after 1 to 3 h • Suitable only for livers of relatively small animals • No significant reduction in the number of animals needed • Poor reproducibility 	(Guillouzo 1998; Brandon et al. 2003).
<i>In vivo</i> Animal models: Rhesus monkey Macaque monkey Opossum Mini pig Rabbits Dogs Various rat and mice species Hepatitis C virus mouse models Zebrafish Nematode (<i>Caenorhabditis elegans</i>)	<ul style="list-style-type: none"> • Ability to mimic human liver disease states (chemically or by means of doming) • More complex and gives a holistic approach to elucidating drug metabolism and mechanisms 	<ul style="list-style-type: none"> • Ethical considerations • Inter-species differences in drug metabolism and toxicology (worsening with evolutionary distance to man) • Labor intensive • Expensive • Time consuming • Requires skilled personnel (e.g. animal technicians, veterinarian) • Requires highly specialized facilities and equipment • Requires cloning in certain instances 	(Rahman and Hodgson 2000; Tingle and Helsby 2006; Guinness et al. 2013; He Bergheim 2013; Kanuri and Bergheim 2013; Liu et al. 2013; Vercauteren et al. 2014).

human drug toxicities have been identified through animal testing; however, the development of better models for the prediction of human hepatotoxicity and liver dysfunction remains critically important (Davila et al. 2007; Freires et al. 2017). *In vivo* studies involving various animal species still serve as the gold standard in toxicology research, although a question raised more often than not is the moral legitimacy of animal experimentation (Paixão and Schramm 1999; Soldatow et al. 2013; Freires et al. 2017). From an ethical point of view, a strong need exists for the development of long term *in vitro* screening models, enabling the reduction of the number of animal subjects currently used in drug development in an attempt to abide by the three R's principle (i.e. reduce, replace and refine) (Baumans 2004; Hewitt et al. 2007; Freires et al. 2017). Furthermore, species differences between animals and humans can result in variances such as different levels of expression of various membrane transporters and metabolizing enzymes, relevant in areas of drug delivery, drug toxicity as well as drug interactions (Sabolić et al. 2011). The most important drawback relating to species differences between mouse, rat, dog, monkey and human CYP-mediated drug metabolism, inhibition and induction have been extensively reviewed by Martignoni et al. (2006). The search for alternative models to address these shortcomings has increased in recent years, and the Zebrafish, a lower invertebrate *in vivo* model, has been found to be a valuable model for *in vivo* hepatotoxicity testing and drug discovery, overcoming many of the limitations mentioned (Driessen et al. 2013; Freires et al. 2017).

The Zebrafish (*Danio rerio*) is a tropical freshwater fish of the Cyprinidae family with an approximate length of 5 cm (Driessen et al. 2013; Freires et al. 2017). This *in vivo* model is well characterized, offering various advantages such as a completely sequenced genome with 71% of its genes homologous to humans', a short life cycle, accessibility and availability. Furthermore, the transparency of the zebrafish during larval stage allows for direct assessment of drug toxicity in a 96-well plate format, while the generation of high numbers of test subjects facilitates high throughput testing (McGrath and Li 2008; Driessen et al. 2013; He et al. 2013; Mesens et al. 2015; Freires et al. 2017). The assessment of drug induced hepatotoxicity in the Zebrafish is possible because of the similarities in the means by which the Zebrafish reacts to xenobiotic chemicals, including phase I and II biotransformation, exhibiting mechanisms of enzyme induction as well as oxidative stress when compared to these mechanisms in mammals (McGrath and Li 2008; Mesens et al. 2015). Zebrafish have been used successfully in enzyme reporter assays, cytochrome P450 assays, the visual assessment of liver necrosis and in evaluating histopathology (McGrath and Li 2008). Although this model still requires ethical approval it does address the three R's, since fewer animals are needed and because this is a lower invertebrate that can replace vertebrates (Redfern et al. 2008; Freires et al. 2017). However, there are still various limitations involved such as the need for specialized equipment, facilities and maintenance staff. To determine the effective compound concentration in the Zebrafish seems to be problematic, as it poses limitations when correlated and extrapolated to dosages administered

to humans and rodents (Diekmann and Hill 2013; Freires et al. 2017). Other limitations include difficulty in achieving accurate oral dosages as most dosages are absorbed through the skin, and hepatotoxicity assessment is currently limited to acute studies (Hill et al. 2005; Redfern et al. 2008; Diekmann and Hill 2013; Flemming and Alderton 2013).

4.2. Three-dimensional cell culture models: Current advances in hepatotoxicity and biotransformation screening

In recent years, the efficacy of current two-dimensional *in vitro* cell culture models has increasingly come into question (Antoni et al. 2015). Efforts to improve the existing *in vitro* cell-based methods, used for pharmacokinetic and toxicity investigations as well as liver dysfunction, has shown that the physiological relevance of the system should be taken into consideration (Donato et al. 2008; Antoni et al. 2015). It is important to remember that the liver is a three-dimensional organ, established by cells *in vivo* that are continuously interacting with neighboring cells, as well as the extracellular matrix. These interactions by means of biochemical and mechanical signals are of utmost importance in normal cell and organ physiology (Lin and Chang 2008; Bell et al. 2016; Brajša et al. 2016). Some critical tissue-specific properties are absent in traditional two-dimensional (2D) *in vitro* cell culture models, far removed from the natural *in vivo* state (Lin and Chang 2008; Antoni et al. 2015). Some of the important differences between cells cultured in 2D and 3D are presented in Table 2.

Three-dimensional (3D) cell culturing systems are being explored in an attempt to establish novel *in vitro* models, capable of resembling native tissue and their normal functions more closely to ensure higher physiological relevance (See Table 3), while at the same time bridging the gap between current *in vitro* and *in vivo* models (Lin and Chang 2008; Wrzesinski and Fey 2013; Bell et al. 2016). In the last two decades, numerous advances have been made to produce high fidelity 3D *in vitro* models using systems capable of long term maintenance, resulting in more accurate determination of drug activity, biotransformation and toxicology (Antoni et al. 2015). All the systems discussed offer various advantages and disadvantages which should be assessed individually before deciding on an appropriate method for a specific application.

4.2.1. Spheroid and organoid models

Spheroids are cell aggregates that are cultured in dynamic or static systems. Spheroid culture is seen as the best characterized model for 3D culturing. Spheroid cultures as a model offers reproducibility, simplicity, as well as distinct similarities to the *in vivo* situation when compared to other models, and are also seen as a primary tool in drug discovery initiatives (Hirschhaeuser et al. 2010; Tung et al. 2010). The main difference between spheroids and other 3D culturing techniques, such as scaffolds and hydrogels, is that spheroid assembly mimics the natural processes occurring during embryogenesis, morphogenesis and organogenesis with cell-cell

Table 2. Main differences between two-dimensional and three-dimensional cultured cells.

Two-dimensional (2D) cell culturing	Three-dimensional (3D) cell culturing
Cells can only be cultured for a limited period (days to one week) without intervention, allowing only acute toxicity studies.	Cells can be cultured for longer time periods (weeks to months) without intervention, allowing both acute and chronic exposure during toxicity studies.
Standardised and established assays are available to measure bio-transformation and toxicity.	Methods and assays to determine bio-transformation and toxicity are not standardized and are still being developed.
Altered cellular differentiation as cells are committed to cell growth, rather than differentiation.	Greater amount of cellular differentiation as cells within 3D constructs are at various stages of the cell cycle, resulting in <i>in vivo</i> tissue-like structures within aggregates.
Cells cultured in 2D present with unnatural cell shape, geometry and morphology, altering cellular communication and the cytoskeleton.	Cells cultured in 3D present with a more natural cell shape, geometry and morphology, better facilitating cell-cell communication, regulatory mechanisms and signaling networks.
Physiological relevance is questionable as most cells <i>in vivo</i> grow as aggregates rather than monolayers. Cells in the 2D environment adapt to the unnatural flat and ridged surfaces, which results in constructs with altered cellular metabolism, biochemical features and cell cycle kinetics.	More physiologically relevant as most organ and tissue structures <i>in vivo</i> consist of cell aggregates. Cells within 3D constructs adopt tissue-like structures with more physiologically relevant cellular metabolism, biochemical features and cell cycle kinetics.
Data collected from 2D experiments lack clinical relevance, since it provides misleading data in many instances, not representative of the <i>in vivo</i> response because of altered cellular responses	Provides clinically relevant data, more representative of the <i>in vivo</i> condition and, in many instances, comparable with animal studies.

Adapted from Khoruzhenko 2011; Breslin and O'Driscoll 2013; Wrzesinski et al. 2014; Edmondson et al. 2014; Antoni et al. 2015; Brajša 2016; Fang and Eglén 2017.

interactions dominating over cell-scaffold interactions (Hirschhaeuser et al. 2010; Tung et al. 2010; Achilli et al. 2012; Fang and Eglén 2017). This model effectively mimics avascular tumors through the formation of molecular gradients, namely oxygen, nutrient and growth gradients (Hirschhaeuser et al. 2010; Van Zijl and Mikulits 2010; Achilli et al. 2012; Fang and Eglén 2017). Spheroids can be cultured using hanging drop systems, rotating micro-gravity bioreactors, spinner flasks, ultra-low attachment plates, self-aggregation systems, microcarrier beads and pellet cultures (Lin and Chang 2008; Achilli et al. 2012; Breslin and O'Driscoll 2013; Page et al. 2013; Soldatow et al. 2013; Wrzesinski and Fey 2015; Fang and Eglén 2017).

4.2.1.1. Hanging drop cultures. Hanging drop spheroid cultures are the result of suspended cells at various seeding densities, which assemble through self-aggregation by means of gravitational forces at the air liquid interface of specifically designed 96-well plates (Messner et al. 2013; Kim et al. 2015). Messner et al. (2013) developed multi-cell liver spheroids for hepatotoxicity testing by co-culturing primary human hepatocytes with the non-parenchymal Kupffer and endothelial cells, to be used in inflammation mediated hepatotoxicity testing. Spheroids were allowed to form for three days after being seeded onto a 96-well hanging drop culture platform (GravityPLUS™) and were subsequently transferred to non-adhesive spheroid-specific GravityTRAP™ 96-well plates. Spheroids were maintained for a duration of 5 weeks. Cell viability was measured by means of an ATP assay and morphological characterization was done by immunohistochemistry. Results obtained from the ATP assay indicated that the spheroids remained stable and functional over the 5-week period, with Kupffer and endothelial cells distributed throughout the spheroids.

Immunohistochemistry also indicated the presence of the broad specificity efflux pump (multi-drug resistance protein 1, MDR1) and bile salt export pump (BSEP). During hepatotoxicity testing over 14 days with acetaminophen and diclofenac, spheroids treated with acetaminophen presented with

concentration dependant cell death, with a half maximal inhibitory concentration (IC₅₀) value of 754.2 μM, while spheroids treated with diclofenac presented with increased sensitivity and an IC₅₀ value of 178.6 μM. Results indicated that this model allowed for the routine testing of compounds as well as determining chronic and inflammation mediated toxicity (Messner et al. 2013).

Gunness et al. (2013) explored HepaRG spheroids by means of the static hanging drop system and maintaining them in culture for a period of 3 weeks. Functionality was accessed by determining phase I enzyme and transporter activities as well as the expression of liver-specific proteins. To access if the model can be employed to predict hepatotoxicity and bio-transformation the model drugs acetaminophen, troglitazone and rosiglitazone were administered. All results where possible were compared to classic 2D cell culturing techniques. Results indicated that the spheroid cultures maintained liver-specific functionality while expressing liver-specific markers such as albumin, CYP3A4, 2E1 and MRP-2 during the 3-week culturing period. With significantly higher production of albumin and CYP2E1 activity in the 3D cultures compared to the classic 2D cell cultures. Toxicity assessment indicated that the half maximal effective concentration (EC₅₀) value of 2.7 mM obtained for acetaminophen in 3D cultures correlated with published *in vivo* data. Indicating that spheroids constructed from HepaRG cells can serve as a valuable *in vitro* tool for the assessment of incidences of acute and possibly chronic hepatotoxicity.

4.2.1.2. Microgravity cultures. Wrzesinski et al. developed and characterized a microgravity bioreactor spheroid cell culturing system using the immortal hepatic HepG2/C3A cell line, a subclone of the widely used HepG2 cell line (Fey and Wrzesinski 2012a; Wrzesinski and Fey 2013; Wrzesinski et al. 2013; 2014). In 2012, Fey and Wrzesinski investigated the median lethal dose (LD₅₀) of six drugs (acetaminophen, amiodarone, diclofenac, metformin and valproic acid), commonly used in toxicity studies, by means of microgravity bioreactors and HepG2/C3A cell spheroids while comparing data from to

Table 3. Three-dimensional (3D) culture methods for liver cells designed to retain, recover or induce liver functionality *in vitro*.

Method	Advantages	Disadvantages	References
Microfluidic device	<ul style="list-style-type: none"> • Described as suitable for high throughput testing • Possibilities for multiple chambers with different cells in each 	<ul style="list-style-type: none"> • Specialized equipment required • Expensive • Further analysis of 3D cultures produced may be difficult • Closed systems prone to blocking or air bubble problems • Lipophilic compounds tend to attach to devices • Reproducibility needs to be improved • Needs further toxicological validation • Variability in cell size and shape if not a fixed cell number per well • Plate-coating is relatively labor intensive 	(Breslin and O'Driscoll 2013; Benam et al. 2015; Maschmeyer et al. 2015; Materne et al. 2015; Wrzesinski and Fey 2015).
Forced floating	<ul style="list-style-type: none"> • Relatively simple • Inexpensive • Suitable for high-throughput testing • Spheroids produced are easily accessible 	<ul style="list-style-type: none"> • Variability in cell size and shape if not a fixed cell number per well • Plate-coating is relatively labor intensive 	(Breslin and O'Driscoll et al. 2014; Guo et al. 2014).
Hanging drop	<ul style="list-style-type: none"> • Inexpensive and simple to perform if using standard 96-well plate • Well-controlled homogenous spheroid size suitable for high-throughput testing • Fast spheroid formation and easily accessible • Co-culturing of different cell types possible • Easy to trace spheroid assembly 	<ul style="list-style-type: none"> • Labor intensive if preparing plates in-house • More expensive if using specialized plates • Small culture volume makes medium exchange, without disturbing cells, difficult (proposed easier handling with commercially available formats) • Small spheroids (up to ~0.3mm) 	(Lin and Chang 2008; Takahashi 2015; Wrzesinski and Fey 2015; Breslin and O'Driscoll 2016).
Non-adhesive surface (liquid overlay)	<ul style="list-style-type: none"> • Inexpensive • Simple to perform • Easy to scale up 	<ul style="list-style-type: none"> • Variation in size/cell number/shape 	(Lin and Chang 2008).
Ultra-low attachment plates	<ul style="list-style-type: none"> • Inexpensive in 96-well format • Simple to perform • Easy to scale up • Well-controlled spheroid size • Suitable for high throughput testing • Microscopic and HCA evaluation possible 	<ul style="list-style-type: none"> • Part of spheroid has reduced access to media • Limited material for assay • Medium-sized spheroids (up to ~0.6mm) 	(Ivanov 2014; Takahashi 2015; Raghavan et al. 2016).
Micro-moulding and printing	<ul style="list-style-type: none"> • Well-controlled spheroid size • Designed aggregate geometry • Co-culture of different cell types 	<ul style="list-style-type: none"> • Require specialized facilities • Spatial resolution is currently limiting • Needs further validation before widespread toxicological application 	(Lin and Chang 2008; Zarowna-Dabrowska et al. 2012; Murphy and Atala 2014).
Galactosylated substrates	<ul style="list-style-type: none"> • High preservation of viability and functions of primary hepatocytes 	<ul style="list-style-type: none"> • Only suitable for cells expressing galactose receptors 	(Lin and Chang 2008).
Pellet culture	<ul style="list-style-type: none"> • Simple to perform • Rapid aggregation of large number of cells 	<ul style="list-style-type: none"> • Shear force • Mass production difficult • Small spheroids (up to ~0.3mm) 	(Lin and Chang 2008; Zannoni et al. 2016).
Monoclonal growth	<ul style="list-style-type: none"> • Little work involved • Some are useful morphogenesis models 	<ul style="list-style-type: none"> • Only occurs in certain cell types • Relatively long incubation periods • Require extra procedures to harvest the multicellular spheroids 	(Lin and Chang 2008).
External force enhancement	<ul style="list-style-type: none"> • Rapid cell aggregation • Advantageous for working with stem cells 	<ul style="list-style-type: none"> • Potentially undefined effects to cells • Require specialized equipment and culture conditions 	(Lin and Chang 2008; Antonchuk 2013).
Agitation based approaches Spinner flasks	<ul style="list-style-type: none"> • Simple to culture cells • Mass production relatively easily achievable • Large spheroids possible (up to ~1 mm) • Long term culture (months) • Dynamic control of culture conditions • Co-culture of different cell types • Motion of culture assists nutrient transport • Spheroids produced are easily accessible 	<ul style="list-style-type: none"> • Requires specialized equipment • Variation in size/cell number of spheroids (can be overcome by additional culture step; see forced floating methods) • Time consuming due to extra step required for homogenous spheroids • High shear force in spinner flask (may be problematic for sensitive cells) 	(Lin and Chang 2008; Jiang et al. 2010; Miranda et al. 2010; Breslin and O'Driscoll 2013).

(continued)

Table 3. Continued

Method	Advantages	Disadvantages	References
Microgravity culture	<ul style="list-style-type: none"> • Simple to perform • Mass production possible • Large spheroids possible (up to ~1 mm) • Long-term culture (months) • Dynamic control of culture conditions • Better cell differentiation • Co-culture of different cell types possible • Recovery of some <i>in vivo</i> physiology • Good correlation with <i>in vivo</i> toxicity • Provide 3D support that mimics <i>in vivo</i> • Some incorporate growth factors 	<ul style="list-style-type: none"> • Requires specialized equipment • Variation in size/cell number (Well-controlled spheroid size can be achieved by the use of AggreWell™ plates to initiate spheroids) • Cells require 18 days to recover from trypsination 	(Lin and Chang 2008; Fey and Wrzesinski 2012a; 2013; Wrzesinski and Fey 2013; Wrzesinski et al. 2014; Zanolini et al. 2016; Aucamp et al. 2017).
Matrices and scaffolds	<ul style="list-style-type: none"> • Provide 3D extracellular support 	<ul style="list-style-type: none"> • Can be expensive for large-scale production • Can have difficulty in retrieving cells following 3D culture formation • Can limit cell-cell interactions 	(Nakamura et al. 2011; Schutte et al. 2011; Breslin and O'Driscoll 2013; Ravi et al. 2015).
3-D scaffolds	<ul style="list-style-type: none"> • Provide 3D extracellular support 	<ul style="list-style-type: none"> • Require specialized equipment for scaffold fabrication • Have not been tested on many cell types 	(Lin and Chang 2008; Heffernan et al. 2015).
Collagen, hydrogel or Matrigel™ supports	<ul style="list-style-type: none"> • Often used in sandwich or raft cultures • Depending on application, presence of exogenous extracellular matrix material can enhance or exacerbate <i>in vivo</i> functionality • Prevent anoikis-induced cell death • Well-mixed co-culture • Well preservation of viability and functions of primary hepatocytes 	<ul style="list-style-type: none"> • Massive production difficult • Can be expensive • Potential batch to batch variation 	(Miranda et al. 2010; Totok et al. 2011; Yip and Cho 2013; Wrzesinski and Fey 2015).
Supported cell sheet	<ul style="list-style-type: none"> • Prevent anoikis-induced cell death • Well-mixed co-culture • Well preservation of viability and functions of primary hepatocytes 	<ul style="list-style-type: none"> • Labor intensive and time consuming • Mass production difficult 	(Lin and Chang 2008; Torok et al. 2011).
Primaria dishes	<ul style="list-style-type: none"> • Moderate throughput • Counter-directional flow • Small cell numbers and media volumes • Microscopic evaluation is easy 	<ul style="list-style-type: none"> • Primarily used for hepatic-linkage cells 	(Lin and Chang 2008).
Hollow-fiber reactor	<ul style="list-style-type: none"> • High throughput • Cells from 3D tissue constructs • Sustained liver-like cell functionality • Physiological shear stress • Good correlation with <i>in vivo</i> clearance rates • Ability for microscopic examination 	<ul style="list-style-type: none"> • A complex system • Lack of physiological gradients 	(Soldatow et al. 2013).
Single-well and multi-well perfused bioreactor	<ul style="list-style-type: none"> • High throughput • Cells from 3D tissue constructs • Sustained liver-like cell functionality • Physiological shear stress • Good correlation with <i>in vivo</i> clearance rates • Ability for microscopic examination 	<ul style="list-style-type: none"> • Uses greater cell numbers and larger media volumes • Has been validated with rat and human hepatocytes 	(Soldatow et al. 2013).
Ex vivo models Bio-artificial livers	<ul style="list-style-type: none"> • Low throughput • Microenvironment most similar to <i>in vivo</i> tissue • Allows for studies of functional heterogeneity • Ability to evaluate hepatotoxicity using human blood 	<ul style="list-style-type: none"> • Requires large numbers of cells • Currently not in use with other cell types • Complex membranes needed for proper use • Does not maintain viability or functionality of hepatocytes longer than other methods 	(Soldatow et al. 2013).

2D cell cultures and available *in vivo* observations. To circumvent uncertainty experienced regarding cell numbers and population size, the spheroid data was normalized to amount of protein (μg) present within the spheroids. The latter allowed for dosages administered to spheroids to correlate with dosages administered to animal models during *in vivo* toxicity studies (mg/Kg). Spheroids were prepared using AggreWell[®]400 plates and cultured within resealable bioreactors (Fey and Wrzesinski 2012b patent). Microscopy, planimetry and protein content were evaluated, and they found the comparison of planimetric area and protein content of the spheroids to demonstrate a clear correlation with a relative standard deviation (RSD) of 21%. Intracellular ATP content was measured to determine cell viability for each of the drug treatments. Data from the later correlated better with published *in vivo* data than with LD_{50} values obtained using either primary hepatocytes or with cell lines in 2D culture (correlation coefficient of 86% compared to either 75% or 55%, respectively), indicating the usefulness of this *in vitro* technique for determination of LD_{50} values (Fey and Wrzesinski 2012a).

Wrzesinski and Fey (2013) then went on to determine that 18 days of culture is needed for HepG2/C3A spheroids to reestablish physiological functions and ultra-structural traits after trypsination. Trypsination at regular intervals such as is needed for continuous 2D cell culturing will result in a disruption of advanced cellular functions, signal transduction, gene expression as well as influencing ECM repair processes and natural cell structure (Page et al. 2013; Wrzesinski and Fey 2013). Wrzesinski and Fey found that cells recover after trypsination in 3D cultures. However, in 2D cultures cells must be trypsinised every 5 days, preventing them from recovering, placing 2D cells in a continuous 'wound healing' cycle, whereas, with the 3D spheroids this recovery continues up until 15–18 days, as suggested by changes in adenylate kinase, ATP, urea and cholesterol production. The latter corresponded well with published literature as several other cell lines, namely Caco-2, HT 29, MDCK, MCF-10A and HepG2, are reported to have similar recovery, needing between 15 and 21 days. Wrzesinski and Fey thus proposed that this is a pervasive recovery process rather than differentiation, which may explain the physiological capabilities that more closely resemble *in vivo* conditions within 3D spheroid cultures.

In 2013, Wrzesinski et al. continued to determine that the HepG2/C3A 3D spheroids exhibited stable physiological functionality for a duration of at least 24 days after this recovery from trypsination. They found that 3D spheroid culture provides a metabolically competent homeostatic cell model that reaches equilibrium within the culturing environment for a period of at least 24 days. Such a stable system permits determination of drug toxicity and mode of drug action, evaluation of biomarkers as well as the study of system biology, all of which requires metabolic functions to be stable over a long-term period (Justice et al. 2009; Wrzesinski et al. 2013; Antoni et al. 2015).

They further strengthened their argument by investigating the proteome of both exponentially growing 2D cells and the 3D spheroids at dynamic equilibrium. They concluded

that there are significant changes within every aspect of cellular metabolism that serves as the foundation of architectural, functional and physiological differences within cells. Cells grown in 3D constructs such as spheroids at dynamic equilibrium are focused on functionality, effectively mimicking the *in vivo* condition (Page et al. 2013; Wrzesinski et al. 2014; Antoni et al. 2015).

4.2.1.3. Spinner flasks. The human HepaRG cell line is a well characterized cellular model used to study the incidence and prediction of drug-induced hepatotoxicity (Leite et al. 2012; Szabo et al. 2013; Nelson et al. 2017). Monolayer cultures show phenotypic characteristics similar to primary human hepatocytes, which include the expression of phase I, II and III liver enzymes, sensitivity towards prototypical inducers as well as the possibility for several weeks of culturing with a stable phenotype (Leite et al. 2012; Nelson et al. 2017). Leite and associates (2012) investigated the long-term 3D culturing of HepaRG employing spinner flasks. These HepaGR spheroids maintained liver-specific functions mimicking *in vivo* liver morphology for seven weeks, as well as a dose-dependent effect to acetaminophen exposure.

4.2.1.4. Ultra-low attachment plates. Janorkar et al. (2011) created a 3D steatosis model using elastine-like polypeptide-polyethyleneimine co-polymer coated plates, resulting in the spontaneous aggregation and spheroid formation of a H35 rat hepatoma cell line that mimics the *in vivo* liver architecture and provides information on transcription regulation in fatty liver disease. The latter is seen in the ability of the model to promote the reuptake of fatty acids, accumulation of triglycerides, decrease in proliferation, depressed liver-specific functions and the accumulation of reactive oxygen species. This model thus provides a platform for the elucidation of relationships that exist during nonalcoholic fatty liver disease available in a 3D model (Janorkar et al. 2011).

Wong et al. (2011) created sized controlled self-aggregating static spheroids termed hepatospheres, and co-cultured heterospheres from primary hepatocytes and hepatic stellate cells – both isolated from adult Sprague-Dawley rats. As a means to circumvent and address the challenge of producing large amounts of uniform sized hepatospheres and heterospheres, the spheroids were cultured by constructing concave microwell arrays with diameters between 300 μm and 500 μm , from poly(dimethylsilohexane) (PDMS) membranes and treating the membranes with 3% (w/v) bovine serum albumin (BSA) to prevent cell attachment. Both monoculture hepatospheres and co-cultured heterospheres were cultured for a period of 12 days. The cell viability and morphology of the outer and inner structures, namely bile canaliculi and cell-cell contacts, were determined on day three of culturing using scanning electron microscopy and transmission electron microscopy. Functional assessment relating to urea and albumin secretion, as well as CYP3A4 activity of both hepatospheres and heterospheres were conducted on days 1, 3, 5, 7 and 9 after culture initiation. Results indicated that hepatic stellate cells play an important role in the control and

organization of spheroid aggregates and in establishing cell to cell communication. The functional assessment showed an increase in albumin secretion of 30%, as well as an increase in CYP3A4 production in heterospheres compared to hepatospheres on day 8 and 9, respectively. Urea secretion of both heterospheres and hepatospheres remained unchanged. Based upon these findings, Wong et al. (2011) proposed that these heterospheres be used to create an artificial 3D hepatic tissue construct to assess liver hepatotoxicity, regeneration and failure.

Bell et al. (2016) developed and characterized an easily scalable 3D spheroid system. Primary human hepatocytes were allowed to self-aggregate within low binding 96-well plates, creating spheroid within 7 days. Spheroid phenotypes and molecular signatures were assessed and compared to 2D cells by means of proteomic analysis. This self-aggregating static system was shown to be functional for a period of 35 days according to haematoxylin and eosin (H&E) scanning, immunohistochemical staining and physiological parameters assessed. This spheroid system also proved to be an excellent model to study the incidence of drug induced liver injury, as five known hepatotoxins (amiodarone, bosentan, diclofenac, fialuridine and tolcapone) were evaluated and half maximal effective concentration (EC_{50}) results approached clinically observed concentrations. Co-culture with non-parenchymal cells is also possible in this system, to even more closely resemble the *in vivo* situation. By being able to chemically induce liver dysfunction in this system, it is a promising *in vitro* model to study liver dysfunction and disease (Bell et al. 2016).

Induced pluripotent stem cells (iPSC)-derived hepatocytes offers a hepatic model that closely represents primary hepatocytes due to comparable functionality and phenotypes, while overcoming batch to batch variability and limitations found within the latter (Sirenko et al. 2016). Sirenko et al. created a static three-dimensional spheroid model from iPSC-derived hepatocytes within a matrix suspension, cultured in 96-well GravityTRAP ultra-low attachment spheroid plates for a period of 72 hours. They attempted to develop and characterize confocal high-content imaging as a high-throughput screening technique, to establish drug induced hepatotoxicity of 50 known hepatotoxic drugs and comparing this to the HepG2 cell line and 2D cell culturing. By employing fluorophores with the capacity to determine cell viability, DNA binding, incidences of apoptosis and mitochondrial markers in combination with high content screening, one can overcome the use of classic disruptive spheroid analysis techniques in high-throughput screening. The assays employed enabled characterization of hepatotoxicity by evaluation of spheroid size, cell number and spatial disruption, viability, nuclear characterization, apoptosis and mitochondrial potential. Their results indicated significant differences across the two cell types (iPSC and HepG2) and at different culturing conditions (2D and 3D spheroids), with regard to the pharmacological effects expressed by the model compounds. The latter indicated the potential of using spheroids from iPSC-derived hepatocytes in combination with confocal imaging techniques in high-throughput screening to determine of drug induced hepatotoxicity (Sirenko et al. 2016).

4.2.2. Hydrogel and scaffold supports

Hydrogels and scaffold supports are attractive methods for growing cells in three dimensions. This group of models refers to synthetic three-dimensional constructs produced from various materials, offering differences in porosities, permeability, surface chemistry and mechanical attributes (Hayward et al. 2013; Fang and Eglén 2017). The extra cellular matrix (ECM) plays an important role in the growth, differentiation, polarization, maintenance and signal transduction of cells growing *in vivo*, and typically the ECM is composed of various proteins, namely laminin, collagens, elastin, glycoproteins and proteoglycans (Hughes et al. 2010; Verhulsel et al. 2014). To mimic this *in vivo*-like environment *in vitro*, a variety of hydrogels and scaffolds can be used to create an artificial ECM. Hydrogels and scaffolds are biomaterials that can be grouped into two main categories, namely natural and synthetic (Hoffman 2012; Verhulsel et al. 2014; Fang and Eglén 2017). Natural hydrogels and scaffolds can be simple compounds, consisting of purified mixed proteins such as collagen, gelatin, fibrin and laminin; or they can be obtained from living cells such as the commercialized matrix Matrigel™, consisting primarily of laminin, collagen IV and enactin (Hughes et al. 2010; Hoffman 2012; Verhulsel et al. 2014; Fang and Eglén 2017). Other natural hydrogels or scaffolds include chitosan, alginate, agarose and silk fibers that are derived from natural materials. Conversely, synthetic hydrogels and scaffolds are produced from synthetic, non-biodegradable, porous polymers that are inert, reproducible and versatile (Hayward et al. 2013). The latter includes poly(ethylene-glycol), diacrylate, poly(acryl-amide) and poly(vinyl-alcohol) (Verhulsel et al. 2014). Hydrogels and scaffolds have been explored as biomaterial for many years offering a hydrophilic character, biocompatibility, chemical stability as well as being biodegradable in certain instances. These attributes make them valuable to the fields of three-dimensional cell culturing and tissue engineering (Hoffman 2012).

The two-week cultivation of HepG2 liver cells in 3D constructs by encapsulating cells within two different sodium alginate-based hydrogels, SLM100 and SLG100 was also investigated and made possible. To understand cell-matrix interactions and systemic behavior on a micro-scale, cell viability, morphology and drug metabolism were quantitatively and qualitatively studied. All results, where possible, were directly compared to classic 2D cell cultures. The results obtained by Lan et al. (2010) indicated that encapsulated HepG2 cells showed high cellular viability, albeit slight proliferation within 14 days of cultivation. The production of CYP1A1 and CYP3A4 liver-specific enzymes, as well as phase II glutathione production over the 14-day cultivation period indicated viability as well as functionality of the encapsulated cells. The encapsulated cells were also capable of the linear bio-transformation of the pro-drug EFC (7-ethoxy-4-trifluoromethyl coumarin) to HFC (7-hydroxy-4-trifluoromethyl).

To improve commercially available polystyrene-based scaffolds modifying surface properties was explored. Polystyrene-based scaffolds are produced by means of poly-HIPE technology (porous polymers derived from high internal

phase emulsions) and have the advantages of being highly porous, with controllable morphology and suitable mechanical properties. Hayward et al. (2013) attempted to overcome one of the major limitations of these polystyrene based scaffolds, namely surface chemistry. The extracellular matrix of cells *in vivo* allows for cell anchorage, cell-cell communication and normal functionality of cells. While carbohydrates and proteins provide biochemical cues to aid in the regulation of normal cellular function, the aforementioned are not provided by polystyrene-based scaffolds. The aim was to mimic these surface interactions by surface functionalization with galactose, a carbohydrate that is known to bind to hepatocytes by means of the asialoglycoprotein receptor (ASGPR). A parent formulation, SDE-polyHIPE, was constructed from the monomers styrene (STY), divinylbenzene (DBV) and 2-ethylhexylacrylate (EHA). A 26% pentafluorophenyl acrylate in a SDE-polyHIPE mixture was further modified by coupling either 2'-aminoethyl- β -D-glucopyranoside or 2'-aminoethyl- β -D-galactopyranoside to create Glu-SDE-polyHIPE and Gal-SDE-polyHIPE, respectively. HepG2 cells and cryopreserved primary hepatocytes derived from Sprague-Dawley rats were then seeded onto the three constructs and allowed to grow under normal culture conditions. The morphology of the 26PFPA-SDE-polyHIPE was characterized using scanning electron microscopy (SEM) and, using the HepG2 cell culture, histological analysis was performed. The results indicated that after 5 days of growth cells anchored and formed tissue-like layers in a similar fashion as commercially available Alvetex scaffolds that served as control. Image analyses indicated that cells appeared healthy and viable, with no signs of necrosis although less penetrations of the cells are noticed compared to the control due to the smaller void area of the 26PFPA-SDE-polyHIPE construct. After functionalization of the 26PFPA-SDE-polyHIPE with aminoethyl glycosides, the surface carbohydrates were characterized, primary rat hepatocytes were cultured onto the Glu-SDE-polyHIPE and Gal-SDE-polyHIPE constructs and albumin secretion was assessed. The results indicated that the addition of galactose to polystyrene based scaffolds proved to be advantageous in adhesion of hepatocytes to these scaffolds and improved cellular functionality.

4.2.3. Microfluidic, organs on chips and bioartificial liver models

Microfluidic technology provides an adaptable platform for biological applications, offering various advantages. It is a system of microscale dimensions corresponding to the cellular organization found in the *in vivo* situation, with chemical gradients able to mimic the dynamic micro-environment (Kim et al. 2015; Gupta et al. 2016). It is of the utmost importance that the micro-environment within 3D cell cultures should mimic the *in vivo* physiological conditions. Microfluidic technology has the potential to replicate realistic fluid retention times and liquid-to-cell ratios (Kim et al. 2015). This multifaceted technology allows for the handling of several processes at once during cellular growth, in that it supplies nutrition, liquid flow, oxygen as well as removal of degradation products. The construction material of

microfluidic devices is often permeable to oxygen (enhancing growth and proliferation), and as sample and reagent volumes are so small it also provides a very cost-effective platform. Typical substances used in the construction of microfluidic devices are glass, silicon, polymers (polydimethylsiloxane (PDMS), polycarbonate (PC), polystyrene (PS) and poly-methyl methacrylate (PMMA)) and chromatographic paper. Cells are grown either with the support of hydrogels or in gel-free systems (Gupta et al. 2016).

4.2.3.1. Microfluidics. A dynamic microfluidic bioreactor as alternative *in vitro* hepatotoxicity testing method was created using the transparent polymer polydimethylsiloxane (PDMS). In this system, hepatic HepG2/C3A cells were seeded onto micro bioreactors coated with fibronectin. Viability experiments were conducted over 96 hours and all experiments, where possible, were related to static 2D culturing conditions. Viability was investigated by means of lactate dehydrogenase, and bio-transformation by monitoring glucose, glutamine, ammonia and albumin concentrations as well as the expression of CYP1A1. Their findings indicated that cell proliferation was dependent on cell seeding density and flow rate, and the increased proliferation and metabolism within the dynamic conditions compared to the static conditions indicated the importance of a dynamic microenvironment for optimal cell metabolic activity (Baudoin et al. 2011).

Prot et al. (2012) then incorporated the use of integrated systems biology with microfluidics and biochip data for the use in the transcriptomic, proteomic and metabolomic profiles assessment during hepatotoxicity of HepG2/C3A cells exposed to 1 mM acetaminophen (APAP). Biochips were fabricated from PDMS and coated with fibronectin before seeding cells onto the biochips as described (Baudoin et al. 2011). All experiments were compared to normal 2D cell culture, and untreated cells showed adaptive cellular responses to the microfluidic environment. However, cells treated with APAP resulted in perturbation of calcium homeostasis, lipid peroxidation and apoptosis due to the reactive metabolite N-acetyl-p-benzoquinoneimine (NAPQI) produced from APAP. Reported biomarkers of hepatotoxicity from APAP ingestion and glutathione depletion namely 2-hydroxybutyrate and 3-hydroxybutyrate, and the consumption of methionine, cysteine and histidine were observed in treated biochips. The later correlated well with literature and resulted in a more complete reconstruction of the APAP injury pathways, demonstrating a potential new approach to predictive toxicology (Prot et al. 2012).

4.2.3.2. Organs on chips. Organs on chips is an emerging and promising platform to study bio-transformation and drug toxicity, with the potential to study bio-threats and even chemical warfare. Bhise (2016) reported on a directly accessible liver on chip platform that can be employed for long term 3D culture of human HepG2/C3A spheroids for four weeks, as a means of investigating drug toxicity comparable to *in vivo* conditions. Perfusable bioreactors were fabricated via a bioprinting approach, using polydimethylsiloxane (PDMS) and poly-(methyl methacrylate) (PMMA).

These bioreactors were then loaded with self-aggregating HepG2/C3A spheroids, encapsulated in photocrosslinkable gelatin methacryloyl (GelMA) hydrogel. The latter was incubated under continuous perfusion at $200\mu\text{l h}^{-1}$ for 30 days, allowing evaluation of cellular functionality and response to an acute dosage of 15 mM acetaminophen (APAP). Secretion of the hepatic biomarkers namely: albumin, α_1 -antitrypsin (A1AT), transferrin and ceruloplasmin, were used to determine cellular functionality. Immunostaining was used to assess the non-secreted cellular proteins cytokeratin 18, multidrug resistance-associated protein II (MRP2) bile canalicular transport proteins and tight junction protein ZO-1. Both the secreted and non-secreted proteins remained fully functional and stable for 30 days within the bio-printed constructs. However, this metabolic activity significantly decreased within the bio-printed constructs during the course of treatment with APAP, correlating well with data previously published by Fey and Wrzesinski (2012b).

As the human body is composed of interrelated tissue systems, microfluidic devices capable of mimicking the *in vivo* situation by simultaneously culturing various cell lines will be advantageous (Huh et al. 2011). Mateme et al. (2015) have attempted just that by the 14-day long-term simultaneous culturing of NT2 neurospheres and co-cultured HepaRG and human hepatic stellate liver spheroids on a microfluidic organ on chip model. The microfluidic system was molded from apolydimethylsiloxane (PDMS) bonded to a glass microscope slide, consisting of two compartments for spheroid culturing and three pump membranes. Liver spheroids and neurospheres, created by means of the hanging-drop model, were loaded into the culturing compartments in HepaRG growth medium. Growth medium samples were taken daily to determine lactate dehydrogenase (LDH), glucose and lactate analyses, while drug biotransformation and toxicity of 2,5-hexanedione (16 mM and 32 mM) were assessed from day 6 by means of terminal deoxynucleotidyl transferase dUTP Nick-end labeling (TUNNEL) assay and physiological parameters. Results indicated that cells were viable for a period of 14 days, showing tissue specific expression of markers such as CYP3A4 and MDR2, and the ability to respond to the toxic onslaught of 2,5-hexanedione (Materne et al. 2015).

4.2.3.3. Hollow-fiber reactor. Shen et al. (2010) developed a polysulfone-g-poly (ethylene glycol) (PSf-g-PEG) hollow fiber to circumvent the instances of adsorption of hydrophobic drugs onto these hollow fiber systems. Two hollow fibers were produced and characterized, polysulfone (PSf) and polysulfone-g-poly (ethylene glycol) (PSf-g-PEG). Freshly isolated hepatocytes were imbedded in a type I collagen gel and then loaded into the hollow fibers by injection, cut into 2 cm sections and cultivated for 48 hours. To create a cylindrical gel, hepatocytes in collagen gel were loaded into hollow fibers and extruded after 10 minutes. After 48 hours of cultivation in various conditions, CYP1A2, 3A and 2E1 activity and drug hepatotoxicity of model drugs (tetracycline, azathioprine, acetaminophen, salicylate, clozapine, rifampicin, chloroquine and aminodarone) were measured within PSf, PSf-g-PEG and cylindrical gels. The culturing medium of

cell-free hollow fibers were also analyzed to determine drug adsorption to these model drugs after 48 hours. The results indicated that cells within the hollow fibers showed aggregate formation after 48 hours, compared to cells within the cylindrical gels which remained dispersed. Also, the activity of CYP enzymes were increased within the hollow fibers, with the PSf-g-PEG hollow fiber producing slightly higher CYP3A and 2E1 activity. Higher liver-specific functions were also noted for cells within hollow fibers compared to the cylindrical gel. The cells within the PSf-g-PEG hollow fibers also showed increased hepatotoxicity to the model drugs administered compared to the cylindrical gels. Furthermore, the PSf-g-PEG hollow fibers outperformed the PSf hollow fibers in terms of drug adsorption and accumulation greatly, reducing the number of drugs and proteins adsorbing to these surfaces indicating a promising tool for drug investigation *in vitro* (Shen et al. 2010).

4.2.3.4. Single-well and multi-well perfused bioreactors. It is well known that the incidence of nonalcoholic fatty liver disease (NAFLD) influences the efficacy of drug bio-transformation and ultimately drug toxicity. Current 2D cell culturing techniques give limited information regarding molecular mechanisms of disease progression, and as there are major discrepancies between results obtained from humans and animal models of NAFLD it is necessary to investigate the possibility of more complex *in vitro* tissue organization systems. Thus, the contribution of Kozrzewski et al. (2017) *in vitro* 3D perfused human NAFLD model is a step in the right direction. Cryopreserved human hepatocytes were seeded onto a multi-well collagen coated Liverchip[®] platform. Cells were cultured in lean or fat culturing medium containing physiological quantities of insulin (2 nmol/L) and glucose (5.5 mmol/L) with fat media containing 600 $\mu\text{mol/L}$ free fatty acids. Cells were cultured in these conditions for a period of 14 days without the incidence of hepatotoxicity. Cells cultured in fat loaded medium, however, presented with changes in transcriptomics, proteomics as well as metabolic functionality changes such as reduced CYP3A4 and 2C9 activity. Also, cells cultured within the fat loaded medium were reactive to metformin, a known anti-steatotic drug serving as proof of concept that this could serve as an excellent steatotic liver model.

Creation of a bioartificial liver system was made possible by culturing primary rat hepatocytes from male Sprague-Dawley rats, using a 0.5% naturally self-assembling peptide nano-scaffold (SAPN) from PuraMatrix[™] in 6-well and 24-well bioreactors, for a duration of 35 days. Gene expression was analyzed every 10 days employing semi-quantitative real-time polymerase chain reaction (RT-PCR), while liver functions (albumin secretion, urea metabolism, ammonia detoxification) and cell membrane stability from lactate dehydrogenase were determined on days 5, 15, 25 and 35. Mitochondrial structural status was determined using confocal microscopy and the liver specific markers Albumin and CYP 3A1 were analyzed by means of immunofluorescence. Drug biotransformation was investigated with the model compound Diazepam and its metabolites in a two-compartmental

model, and all results were compared with traditional 2D cell cultures. The results indicated a physiologically stable 3D system for a period of 4 weeks, capable of serving as an alternative to animal experiments and transforming current pre-clinical drug screening and drug development protocols with the ability to mimic native liver regeneration time. The results also indicated stable liver gene expression and biochemical functions, including drug biotransformation and detoxification, which were significantly better than traditional 2D models employed during this study (Giri et al. 2012).

5. Conclusions

It is important to reflect on the current means of studying drug biotransformation and toxicity in both functional and dysfunctional liver. Animal models and primary hepatocytes still remain the golden standard in drug biotransformation and toxicity studies, but costs and ethical considerations make these methods increasingly problematic. Currently, scientists are challenged to reproduce human hepatic function sufficiently to study drug biotransformation and toxicity at multiple levels. Several challenges are facing *in vitro* models, such as the inability to maintain fully functional primary hepatocytes in culture for long periods of time. The recovery of the majority of liver functions in stem cell-derived hepatocytes still has to be demonstrated, while most established cultured cell lines are known to lack physiologically relevant levels of function. A renewable source of human non-parenchymal cells is lacking, and various cell types cannot yet be built into liver-like structures. It is important to note that, although 2D cell cultures will always have its place, there are numerous advantages to culturing cells in 3D. However, a general lack of understanding to this regard hinders the development and implementation of this technology to its full potential.

Despite these challenges, rapid progress is made at many levels. While intrinsically obvious, the importance of cultivating cells in a 3D environment, similar to the one from whence they came, in order to obtain liver-mimetic tissue has only been realized during the last 10 years. Converting this realization to reality is the focus of much research in the field today. Diversification in this research illustrates that currently no one approach holds all the advantages. Furthermore, 3D spheroids in low attachment plates will permit high throughput analysis of drug-drug or drug-compound interactions. Microgravity cultures allow for multifunctional and long-term repeated analysis in *in vivo* like conditions. Inclusion of additional cell types like Kupffer cells adds the 'immunological' and other angles for drug induced liver injury. Rapid transdifferentiation of adult cells (via induced pluripotent stem cells) into hepatocytes holds the promise to approach the human diversity of drug response and an approach to idiosyncratic drug response. Finally, the addition of other organs in chip-based systems, make progress towards simulating whole body toxicology. When all of these features coalesce, we will be standing with a powerful tool which will allow us to dispel the ethical shadow of using animals in toxicological studies and

understand the biochemical and metabolic wizardry that is occurring every second in our livers. But there is still much to do.

Acknowledgements

Opinions expressed, and conclusions arrived at, are those of the author and are not necessarily to be attributed to the NRF or the MRC.


Disclosure statement

The authors declare no conflict of interests.

Funding

This work was supported by the National Research Foundation (NRF) of South Africa under Grant number [91460]; the South African Medical Research Council (MRC) under Self-Initiated Research grant and DrugMode A/S and MC2 Biotek (Denmark). We would also like to acknowledge the support of the COST actions CM1407 (Challenging organic synthesis inspired by nature - from natural products chemistry to drug discovery) and CA16119 (*In vitro* 3-D total cell guidance and fitness).

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CHAPTER 4**MANUSCRIPT SUBMITTED TO INTERNATIONAL JOURNAL OF
MOLECULAR SCIENCES**

Chapter 4 is presented in the form of a research manuscript submitted to the journal *Toxicology Mechanisms and Methods* in 2018. The complete guide for authors for this journal is provided in Appendix B, which states that the manuscript should be written in Times New Roman font. This chapter differs from the rest of the thesis as it is written in U.S. English and not U.K. English. Furthermore, the data shown in the manuscript and the certificate of analysis of the Uzara commercial product used, are presented in Appendix C and H, respectively.

Toxicity and anti-proliferative properties of *Xysmalobium undulatum* water extract during short term exposure to two-dimensional and three-dimensional spheroid cell cultures

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Abstract

Xysmalobium undulatum (Uzara) is one of the most widely used indigenous traditional herbal remedies in Southern Africa. Traditionally, the roots containing the cardenolide cardiac glycoside, uzarin, is utilized in the treatment of various maladies. Although a toxic digitalis-like action on the heart has been reported for extremely high doses, there is no hepatotoxicity or cytotoxic reports for Uzara as yet. Commercially available Uzara plant material was used to prepare a crude aqueous extract, of which the toxicity potential was investigated in the hepatic HepG2/C3A cell line in both traditional two-dimensional (2D) and rotating three-dimensional (3D) spheroid cell cultures. These cultures were treated over a period of 4 days at concentrations of 200, 350, 500, and 750 mg/kg. Basic physiological parameters of the cell cultures were measured during exposure, including cell proliferation, glucose uptake, intracellular adenosine triphosphate (ATP) levels and adenylate kinase (AK) release. The results indicated that all physiological parameters monitored were affected in a dose dependent manner, with the highest concentration of Uzara crude water extract (750 mg/kg) resulting in toxicity. Anti-proliferating effects of Uzara crude water extract were observed in both the 2D and 3D cell cultures, with the most pronounced effects at concentrations of 350, 500 and 750 mg/kg. Discrepancies between results obtained from the 2D and 3D cell culture models may be attributed to the type of repair system that is initiated upon exposure, depending on where cells are within the cell

cycle. DNA repair systems differ in cells within the G1 phase and non-dividing cells, thus cells found predominantly in 3D and the *in vivo* situation.

Keywords: C3A, Cell culturing, Hepatotoxicity, Herbal medicine, Spheroid, Three-dimensional cell culture, Uzara, Uzarin.

1. Introduction

Traditional herbal medicine remains a popular means of treating various ailments in developing and developed countries (Rivera *et al.*, 2013; Hosseinzadeh *et al.*, 2015). An estimated 75% of Africa's population still consult traditional healers and rely on traditional herbal medicine to meet their health care needs (Taylor *et al.*, 2001; Holmes, 2015; Marais *et al.*, 2015). The popularity of traditional herbal medicines can be attributed to the patient's ideologies, cultural beliefs and religion. In certain instances, traditional herbal medicine is more accessible and affordable than available western medicine. Furthermore, an unsupported perception exists that herbal medicine offers safer and more effective treatment (Taylor *et al.*, 2001; Vermani & Garg, 2002; Fennell *et al.*, 2004; Cordier & Steenkamp, 2011). It is true that certain traditional herbal medicines have been proven to be pharmacologically active, contributing to the establishment of new lead compounds. However, due to poor or no regulation and lack of chemical characterization of these traditional herbal medicines, toxicity and herb-drug interactions remain a critical problem and is rarely documented (Vermani & Garg, 2002; Cordier & Steenkamp, 2011; Singh *et al.*, 2011; Teschke & Eickhoff, 2015).

One of the most widely used traditional herbal medicines in Southern Africa is *Xysmalobium undulatum* (L.) W.T. Aiton (Apocynaceae) known as Uzara, or alternatively milk bush, milkwort (Eng.); melkbos, bitterwortel (Afr.); iyezaelimphe, ishongwane (Xhosa); iShinga (Zulu). Uzara is a traditional herbal medicine indigenous to sub-Saharan Africa (Bester, 2009; Schmelzer & Gurib-Fakim, 2013). This almost stalkless robust geophyte grows 0.5 - 2.0 m in height, has large, hairy and heart shaped leaves, and produces cream-green to yellowish flowers growing in small clusters around the stem (Bester, 2009; Schmelzer & Gurib-Fakim, 2013; Vermaak *et al.*, 2014). The roots are fleshy, having an almost carrot-like appearance with a nauseating smell (Schmelzer & Gurib-Fakim, 2013). Although the stems are poisonous, Uzara roots are used in traditional remedies while the leaves are ingested as spinach supplement (Reid *et al.*, 2006). Traditional uses of either a water decoction or poultice, depending on application, include the treatment of indigestion and stomach aches, diarrhoea, dysentery, malaria, colic, headaches, sores, wounds and abscesses, afterbirth cramps, hysteria as well as food poisoning (Steenkamp *et al.*, 2004; Reid *et al.*, 2006; Van Wyk, 2011; Vermaak *et al.*, 2014). The chief pharmacologically active constituents from Uzara root include the cardenolide cardiac glycosides uzarin (5.6%), xysmalorin (1.5%) and the isomers allouzarin (0.4%) and alloxysmalorin

(0.1%). Minor constituents are the cardenolideaglycones uzarigenin and xysmalogenin, as well as allouzarigenin, alloxysmalogenin, ascleposide, coroglaucigenin, corogluaucigenin, alloxysmalogenin, ascleposide, coroglaucigenin, corogluacigenin-3-*O*-glucoside, pachygenol, pachygenol-3 β -*O*-glucoside, desglucouzarin, smalogenin, desglucoxysmalorin, uzaroside, pregnenolone and β -sitosterol (Vermaak *et al.*, 2014). No hepatotoxicity has been reported for Uzara in literature, but a toxic digitalis-like action on the heart has been reported when administered in extremely high concentrations (Vermaak *et al.*, 2014). Since the liver is the major site for xenobiotic bio-transformation and detoxification, hepatotoxicity is specifically of concern.

The current gold standard in *in vitro* pre-clinical drug screening and hepatotoxicity testing remain primary hepatocytes or hepatocyte cell lines, grown on static flat surfaces formally known as traditional two-dimensional culture (2D) (Davila *et al.*, 2007; Wrzesinski *et al.*, 2014; Bell *et al.*, 2016). However, when considering drug discovery and development to discern possible hepatotoxicity, one wishes to implement an experimental model that best mimics the *in vivo* environment of man (Edmondson *et al.*, 2014; Antoni *et al.*, 2015; Breslin & O'Driscoll, 2016; Fang & Eglen, 2017; Lelievre *et al.*, 2017). Organs such as the liver boast a unique three-dimensional cellular architecture where various cells interact with each other as well as the extracellular matrix, creating a complex communication network through biochemical and mechanical signals. More recently, many publications have shown proof of concept that three-dimensional cell culturing (3D) is revolutionizing the evaluation of herb-drug interactions and toxicity owing to the models' improved correlation to this *in vivo* environment (Fey & Wrzesinski, 2012; Breslin & O'Driscoll, 2013, 2016; Calitz *et al.*, 2018; Wrzesinski & Fey, 2018).

There are a few important and distinct differences between 2D, 3D cell culturing and the *in vivo* situation that should be mentioned. Due to the unnatural two-dimensional environment of cells in 2D, the metabolism and biochemical features are altered, possibly influencing pharmacological drug responses (Baker & Chen, 2012; Werner *et al.*, 2017; Langhans, 2018). Also, differences in cell morphology and geometry affects the cell cytoskeleton responsible for the regulation of gene and protein expression, prompting cellular and physiological response and functionality (Lee *et al.*, 2008; Baker & Chen, 2012; Wrzesinski *et al.*, 2014; Langhans, 2018; Wrzesinski & Fey, 2018). Finally, definite differences exist in the cell cycle kinetics and proliferation rates and event events like apoptosis of cells grown in 2D versus 3D. Cell aggregates grown in 3D, most often spheroids, are at various growth stages and found within different cellular layers. The latter supports native physiological shape and morphology, which in turn enhances cell-cell regulatory mechanisms and biochemical and mechanical signalling systems (Wrzesinski *et al.*, 2014; Yono *et al.*, 2015; Brasja *et al.*, 2016; Calitz *et al.*, 2018; Wrzesinski & Fey, 2018). These critical differences between 2D and 3D models culminate in discrepancies towards drug responses within these systems, suggesting that 3D

models may be able to provide a more accurate representation of how a specific organ could react. (Lin & Chang, 2008; Baker & Chen, 2012; Fey & Wrzesinski, 2012; Page *et al.*, 2013; Brasja *et al.*, 2016; Breslin & O'Driscoll, 2016; Duval *et al.*, 2017; Calitz *et al.*, 2018; Wrzesinski & Fey, 2018).

Various types of 3D cell culture models are currently available and being explored, including forced-floating devices, hanging drop cultures, agitation-based spheroid cultures, matrices, scaffolds and microfluidic cell culture platforms (Breslin & O'Driscoll, 2013; Calitz *et al.*, 2018). The choice of system depends on the hypothesis stated, as not one system is superior to the other and each offers various advantages and disadvantages (Calitz *et al.*, 2018). The dynamic micro-gravity spheroid 3D cell culture model developed by Wrzesinski and colleagues, exhibited the ability to overcome many of the shortcomings of traditional 2D cell cultures. These spheroids are produced using microwell plates, capable of producing large numbers of size-controlled spheroids (Fey & Wrzesinski, 2012a; Razian *et al.*, 2013; Fey & Wrzesinski, 2013; Wrzesinski *et al.*, 2013; Wrzesinski *et al.*, 2014; Aucamp *et al.*, 2017; Wrzesinski & Fey, 2018). Spheroids produced from these microwells can then be grown in micro-gravity rotating bioreactors for a period of 42 days, while still exhibiting stable physiological functionality comparable to values within the liver (Wrzesinski & Fey, 2013; Wrzesinski *et al.*, 2013; Wrzesinski *et al.*, 2014; Wrzesinski & Fey, 2018). Due to the low and equal shear forces found within the micro-gravity bioreactors, spheroids of uniform size are maintained (standard deviation of 21%) while increasing nutrient and gas exchange (Wrzesinski & Fey, 2013; Wrzesinski *et al.*, 2013; Wrzesinski *et al.*, 2014; Wrzesinski & Fey, 2018).

This model has also proven to be able to predict drug toxicity of well-known hepatotoxic compounds, including acetaminophen, diclofenac, metformin and valproic acid, more comparable to published *in vivo* data than the 2D cultures (Fey & Wrzesinski, 2012a; Fey & Wrzesinski, 2013; Wrzesinski & Fey, 2018). Furthermore, Wrzesinski and colleagues (2014) determined that spheroids grown in a 3D environment differ in terms of structure as well as metabolic capabilities when compared to their 2D counterparts. The latter correlates well to previous observations stating that these spheroids are able to mimic physiological responses similar to those found within the intact human liver. Similar observations have been made by others employing 3D cultures in toxicological evaluation of drugs (Calitz *et al.*, 2018). Recently, it was established that HepG2/C3A spheroids grown in these micro-gravity bioreactors had accumulation of cells in the G1/G0 phase of the cell cycle, similar to *in vivo* tumour cells and healthy counterparts (Wrzesinski & Fey, 2018). The latter results in an increase of intracellular ATP within these spheroids, influencing the spheroids' ability to "cope" with xenobiotic onslaughts.

This study aimed to investigate the potential toxic and anti-proliferic effects of a crude aqueous extract prepared from commercially available *Xysmalobium undulatum* (Uzara) plant material. To determine possible hepatotoxic events in the 2D HepG2/C3A model, as well as the 3D spheroid model, we evaluated cell growth, intracellular ATP and extracellular AK levels. The results obtained from these 2 models was then compared to determine their suitability and comparability in predicting possible herbal hepatotoxicity.

2. Materials and Methods

2.1 Preparation of the Uzara crude aqueous extract

Milled *Xysmalobium undulatum* (Uzara) plant material was purchased from Afrinatural holdings (Prestige laboratory supplies, Mt Edgecombe, Johannesburg). A crude aqueous extract was prepared by forming a suspension of Uzara plant material powder in water, in a 1:10 product to water ratio. The suspension was sonicated in a Eumax ultrasonic bath at 45°C for 45 min, and subsequently centrifuged for 10 min at 5000 xg. The supernatant was collected while the pellet was re-suspended in 100 mL water, sonicated and centrifuged to collect the supernatant again. The collected extract was filtered and frozen overnight at -80°C and then lyophilised on a Virtis freeze dryer (SP Scientific, Gardiner, New York).

2.2 Liquid Chromatography-Mass Spectrometry (LC-MS) characterisation of Uzara crude aqueous extract

Ultrahigh-pressure liquid chromatography (UPLC) analysis of the Uzara crude aqueous extract was performed on a Waters Acquity Ultra Performance Liquid Chromatographic system with PDA detector (Waters, Milford, MA, USA). UPLC separation was achieved on an Acquity UPLC BEH C18 column (150 mm × 2.1 mm, i.d., 1.7 µm particle size, Waters) maintained at 40°C. The mobile phase consisted of 0.1% formic acid in water (solvent A) and acetonitrile (solvent B) at a flow rate of 0.3 ml/min; a gradient elution was as follow: 85% A: 15% B to 65% A: 35% B in 10 min, changed to 50% A: 50% B in 0.5 min, keeping for 1 min and back to initial ratio in 0.5 min. The running time was 12 min. The samples were injected in the mobile phase with an injection volume of 1.0 µl (full-loop injection). Mass spectrometry was operated in positive ion electrospray mode. N₂ was used as the desolvation gas. The desolvation temperature was set to 250°C at a flow rate of 600 l/hr and the source temperature was 100°C. The capillary and cone voltages were set to 3000 and 40 V, respectively. Data were collected between 100 and 1000 m/z, and processed by chromatographic software Masslynx 4.1. The quantitative aspect of the method was validated by determining the linearity, recovery, and limit of detection (LOD) and the limit of quantification (LOQ).

2.3 Two-dimensional cell culturing

A clonal derivative of the hepatocellular carcinoma cell line HepG2, namely HepG2/C3A (American Type Culture Collection [ATCC] cat. no. CRL-10741, sixth passage after receipt from ATCC, Manassas, VA), was cultured using standard tissue culture conditions in Dulbecco's Modified Eagle's Medium (DMEM) (1g glucose/l) (Gibco, Carlsbad, CA; Cat. no. 11880-028) with 1% non-essential amino acids (Gibco; Cat. no. 11140-035); 10% fetal calf serum (FCS) (Sigma, St Louis, MO; Cat. no. F 7524); 0.5% penicillin/streptomycin (Gibco; Cat. no. 15140-122) and 1% GlutaMAX (Gibco; Cat. no. 35050-038). Cultures were incubated in a humidified atmosphere at 37°C, 5% CO₂ and 95% air.

2.3.1 Two-dimensional culture experimental setup

Five experimental groups were setup in 96-well plates. Cells were seeded at a density of 3000 cells per well to measure physiological parameters, namely intracellular ATP, extracellular AK and protein content. Experimental groups were as follows: crude Uzara aqueous extract at 200 mg/kg, 350 mg/kg, 500 mg/kg and 750 mg/kg, and an untreated control group with normal growth medium. Drug treatment was initiated 6 h after seeding, allowing cells to adhere; all experimental groups were treated for 96 h.

2.4 Protein determination in 2D cell culture model

Protein content was determined using the colorimetric Bradford assay in three replicates of each experimental group in clear flat bottom 96-well plates. Cells were treated with Uzara water extract for 96 h, with medium exchanges in 24 h intervals. Bovine serum albumin (BSA) (BioRad, Sandton, South Africa, Cat No 500-0206) was diluted to 1 µg/µl in PBS for the reference standard. Growth medium was removed from wells and replaced with 158 µl water. Lysis buffer (2 µl) and 40 µl colour reagent (BioRad, Sandton, South Africa, Cat No 500-0006) were added to all wells containing standard and samples. The plate was incubated at room temperature for 5 min. Absorption was measured with a SpectraMax[®] Paradigm[®] plate reader (Molecular Devices Inc, Separations, Randburg, South Africa) at 595 nm. The data was normalized with reference to a protein standard curve and the untreated control.

2.5 Intracellular ATP determination in 2D cell culture model

Cell viability was determined based on ATP production, using the CelTiter Glo Luminescent cell viability assay. Intracellular ATP concentration was determined in six replicates of each experimental group, in black clear bottom 96-well plates. Cells were treated with the Uzara water extract for 96 h,

and medium was exchanged in 24 h intervals. ATP was measured at time points 0, 4, 12 and 24 h after each exchange. Growth medium was removed from each well and the volume adjusted to 100 μ l with PBS. The cells were then lysed with 100 μ l lysis buffer (CellTiter-Glo luminescent cell viability assay, Promega, Fitchburg, WI; Cat. no. G7571) and shaken in the dark for 40 min before the luminescence was measured in a SpectraMax[®] Paradigm[®] plate reader (Molecular Devices Inc, Separations, Randburg, South Africa). The data was normalized with reference to a standard curve for ATP and the untreated control.

2.6 Adenylate kinase determination in 2D cell culture model

Adenylate kinase was measured in the growth medium (140 μ l) of each experimental group in six replicates to determine cytotoxicity, at time points 0, 4, 8, 12 and 24 h following each medium exchange for the duration of the study, using the Lonza Toxilight assay kit (Cat No LT07-117). This assay measures release of adenylate kinase from the mitochondria, indicating loss of membrane integrity. Growth medium was placed in black clear bottom 96-well plates in triplicate, and diluted with five volumes of adenylate kinase detection reagent. The plate was then incubated in the dark for 20 min before measuring luminescence using a SpectraMax[®] Paradigm[®] plate reader (Molecular Devices Inc, Separations, Randburg, South Africa). A standard curve was prepared in the same way for each assay plate using a dead cell standard (4.29 million C3A cells/ml lysed in lysis buffer).

2.7 Three-dimensional cell culturing

2.7.1 Spheroid preparation using AggreWell[™]400 plates

HepG2/C3A cell spheroids were prepared using an AggreWell[™]400 plate (Stemcell Technologies, Grenoble, France; Cat. no. 27845) according to manufactures specifications and as previously described (Fey & Wrzesinski, 2012a; Razian *et al.*, 2013; Fey & Wrzesinski, 2013; Wrzesinski *et al.*, 2013; Wrzesinski *et al.*, 2014; Aucamp *et al.*, 2017; Wrzesinski & Fey, 2018). Wells were rinsed twice with 1 ml Aggrewell[™] Rinsing solution (Stemcell Technologies, Grenoble, France; Cat. no. 07010). Air bubbles were removed from the well surface through centrifugation for 3 min at 3000 xg. HepG2/C3A cells were seeded into each of the wells of the AggreWell[™] plate at a seeding density of 1.2×10^3 , and centrifuged for 3 min at 120 xg. Following seeding, the cells were left in the AggreWell[™] plate overnight to ensure aggregation and spheroid formation.

2.7.2. Spheroid culture in rotating bioreactors

Spheroids were detached from the AggreWell™ by gently washing the wells with pre-warmed growth medium. Detached spheroids were collected and the spheroid quality was evaluated using a light microscope. Spheroids were selected based on visual quality, similar areas of compactness, as well as equal size and roundness. Selected spheroids were placed into the rotating bioreactors system (BAM system 4.6 CelVivo, Denmark and MC2 Biotek, Hørsholm, Denmark; Cat. no. 010), approximately 300 per bioreactor, and cultivated at 37°C, 5% CO₂, 95% air in a humidified incubator for 21 days with continuous rotation of the bioreactors.

The day of spheroid removal from the AggreWell™ is referred to as day 0, and an estimated 90% of growth medium is removed and replaced on day 3, and thereafter three times a week for a period of 21 days. The rotation speed of the bioreactors is initially set between 9 and 11 rotations per minute (rpm) and monitored closely within the first week to compensate for cell growth. The spheroid population density was reduced, and experimental group setup was performed on days 8 and 17, respectively (Fey & Wrzesinski, 2012a; Razian *et al.*, 2013; Fey & Wrzesinski, 2013; Wrzesinski *et al.*, 2013; Wrzesinski *et al.*, 2014; Aucamp *et al.*, 2017; Wrzesinski & Fey, 2018).

2.8 Experimental design

2.8.1. Three-dimensional culture experimental setup

Five experimental group bioreactors were initiated, each containing exactly 239 of the 21 day-old spheroids that were gently pipetted into each 10 ml bioreactor before treatment, to ensure sufficient material for analysis. Experimental groups consisted of the following: crude Uzara aqueous extract at various concentrations (200 mg/kg, 350 mg/kg, 500 mg/kg and 750 mg/kg) as well as an untreated control group. Spent media was removed at 24 hour intervals and exchanged with 10 ml medium containing the crude Uzara aqueous extract at the various concentrations. Physiological parameters, namely glucose consumption, surface area of spheroids, intracellular ATP and extracellular AK was measured in all experimental groups for a duration of 96 h.

2.9 Glucose determination in 3D cell culture spheroid model

Growth medium samples (50 µl) for glucose determination were collected from each experimental group at time points 0, 2, 4, 8, 12 and 24 h after medium exchange for the duration of the study. Samples were measured using a Onetouch Vita glucose meter (MediqDanmark, Cat No 6407078) and test strips (MediqDanmark, Cat No 6407079). The instrument was calibrated daily using a 1 mg/ml D-

glucose standard solution (Sigma-Aldrich, Cat No G3285-5ML) and Onetouch Vita control solution (MediqDanmark, Cat No 6407081). Measurements entailed spotting of 3 μl of each sample onto the test strip (6 replicates for each experimental group). The glucose concentration (mmol/l) was read immediately and noted.

2.10 Planimetry in 3D cell culture spheroid model

Photomicrographs of spheroids were taken using an Olympus IX81 motorized microscope and an Olympus DP71 camera. Images were transferred to the Olympus AnalySiSDocu program (Soft Imaging System) and the „shadow“ area of spheroids measured using the „Fitted Polygon Area“ function which calculates the planar surface of the spheroids in μm^2 .

2.11 Intracellular ATP determination in 3D cell culture spheroid model

Spheroids from each experimental group (six replicates; 2 spheroids per assay point) were collected at time points 0, 4, 12 and 24h after each exchange of medium, for a duration of 96 h, and transferred to white opaque microtitre plates (Nunc, Roskilde, Denmark; Cat No 165306). Growth medium was removed, and the volume adjusted to 100 μl with Hanks buffered saline solution without Ca^{2+} and Mg^{2+} (Gibco, Cat No 14175-129). The cells were then lysed with 100 μl lysis buffer (CellTiter-Glo luminescent cell viability assay, Promega, Fitchburg, WI; Cat No G7571) and shaken in the dark for 40 min before the luminescence was measured in a FLUOstar OPTIMA (BMG Labtech, Ortenberg, Germany), using the following parameters: one kinetic window, 10 measurement cycles with 0.3 s of measurements interval time, 2 s delay per measurement, additional 0.5 s delay per position change (repeated twice for each measured plate). The data was normalized with reference to a standard curve for ATP and the untreated control.

2.12 Adenylate kinase determination in 3D cell culture spheroid model

Adenylate kinase was measured in the growth medium (140 μl) of each experimental group to determine cytotoxicity, at time points 0, 4, 8, 12 and 24 h following each medium exchange, for a duration of 96 h, using the Lonza Toxilight assay kit (Cat No LT07-117). Growth medium was placed in microtitre plates in triplicate, and diluted with five volumes of adenylate kinase detection reagent. The plate was then incubated in the dark for 20 min before measuring luminescence using a FluoStar Omega[®] (BMG Labtech, Ortenberg, Germany). A standard curve was prepared in the same way for each assay plate, using a dead cell standard (4.29 million C3A cells/ml lysed in lysis buffer). Gain was adjusted based on the highest standard curve values.

2.13 Statistical analyses

Repeated measures ANOVA was performed on the three-dimensional cell culture data using Statistica software version 13.2.

3. Results

3.1 Characterization and yield of the crude Uzara aqueous extract

Figure 1 shows the liquid chromatography mass spectrometry (LC-MS) chromatogram of the prepared Uzara aqueous extract. The presence of the major active constituent, uzarin, was confirmed and quantified (226.4 $\mu\text{g}/\text{mg}$ uzarin ($n = 2$)). The chemical composition of the Uzara aqueous extract as shown in the chromatogram correlates well with previously published data (Kanama *et al.*, 2016). The uzarin concentration in each of the experimental treatment solutions are presented in Table 1. A yield of 30% Uzara aqueous extract was achieved.

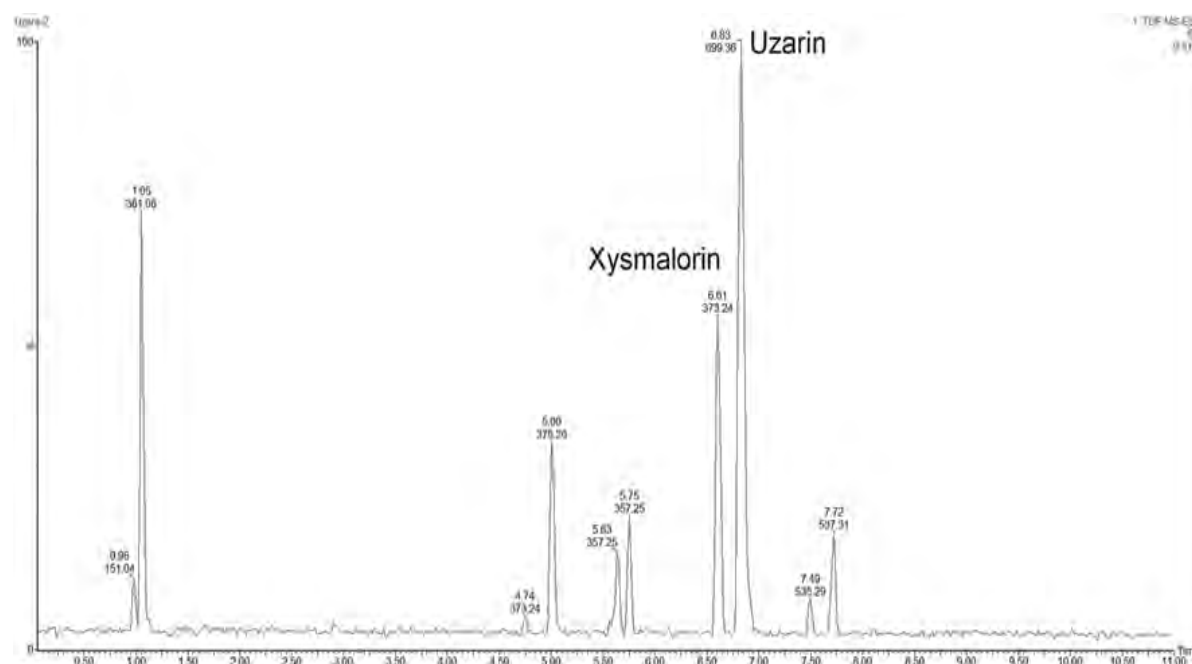


Figure 1. LC-MS chromatogram of Uzara crude aqueous extract, indicating the presence of uzarin and xysmalorin.

Table 1. Concentration of uzarin (mg/kg) applied to the cell culture models in each of the experimental treatment groups

Experimental group	Uzarin concentration (mg/kg) applied to the cell culture models
Uzara crude aqueous extract: 200 mg/kg	45.28
Uzara crude aqueous extract: 350 mg/kg	79.24
Uzara crude aqueous extract: 500 mg/kg	113.20
Uzara crude aqueous extract: 750 mg/kg	169.80

3.2 Two-dimensional culture model

3.2.1. Protein content in the 2D cell culture model

The protein content of the cells in each experimental group was quantified at various time points as a means to determine cell growth as a function of time, and enabled expression of intracellular ATP and AK levels in relation to the protein content. Figure 2 depicts the average protein content per experimental group, following treatment with Uzara crude aqueous extract. Growth inhibition was observed for all the experimental groups treated with Uzara crude aqueous extract when the protein content was compared to that of the untreated control group. The experimental groups treated with 200 and 500 mg/kg extract showed more sustained growth, although slower proliferation appears to have occurred in comparison to the untreated control group. The 350 and 750 mg/kg extract treated groups showed a greater measure of growth inhibition from time point 48 h when compared to the untreated control group, as well as the other experimental groups. At time point 72 h, the 750 mg/kg extract treatment induced significant growth inhibition when compared to the other groups. It is probable that treatment with 750 mg/kg crude Uzara aqueous extract might even be significantly lower than the control group at time point 96h, when the standard deviation of the control group is taken into consideration, however this cannot be confirmed statistically. The untreated control group data correlates well with previously published data, suggesting the doubling time of HepG2/C3A cells in exponential growth to be 3 days and the optimal time for determining cellular effects is within this time frame (Wrzesinski *et al.*, 2014). This also suggests that experimental conditions in 2D culture differ from day to day, and its usefulness is limited to the first 72 hours.

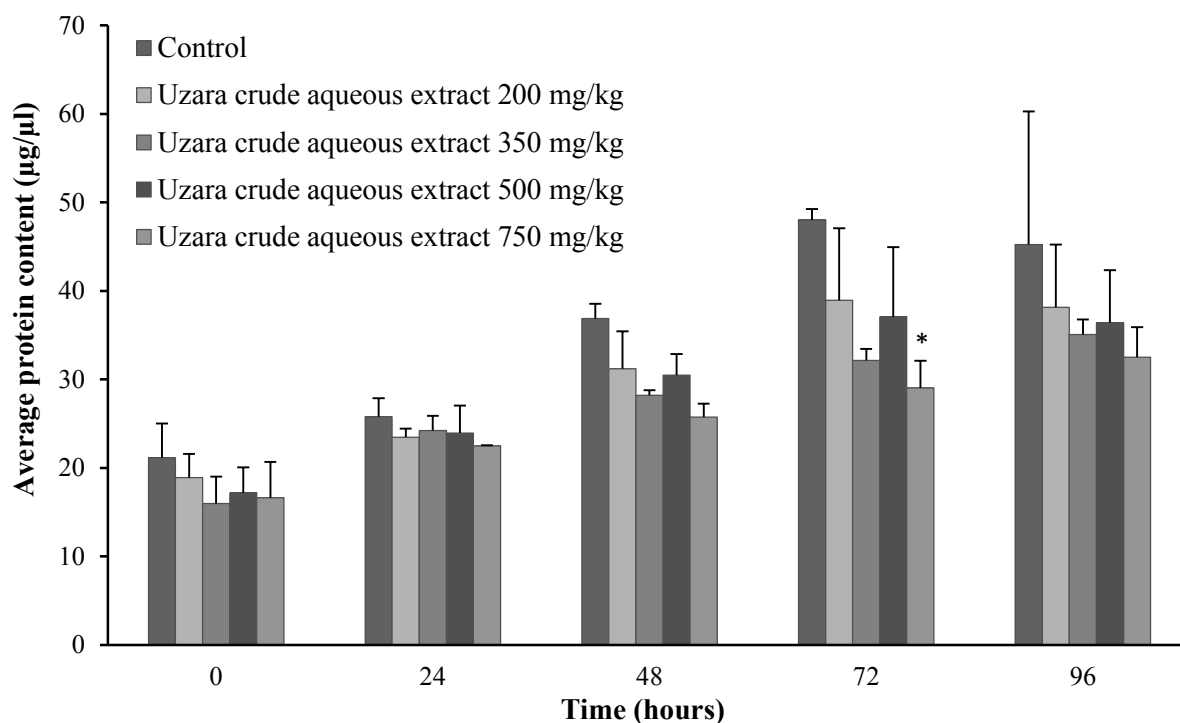


Figure 2. Average protein content ($\mu\text{g}/\mu\text{l}$) of 2D cultured HepG2/C3A cells treated with 200, 350, 500 and 750 mg/kg Uzara crude extract in 24 h intervals, measured prior to each new dosage application ($n = 3$, error bars = SD).

3.2.2. Intracellular ATP levels and AK release within 2D cultures

Data from the 2D cultures are graphically depicted in Figure 3, showing the average intracellular ATP levels in terms of average protein content, normalized to the untreated control group. Within the first 24 h post-treatment, the intracellular ATP levels increased noticeably for three of the experimental groups treated with the Uzara crude aqueous extract, relative to the control group. Between time point 24 and 48 h, all the experimental groups showed intracellular ATP levels similar to that of the untreated control group. However, at time point 48 h there was an increase in the amount of intracellular ATP for the 750 mg/kg treated group when compared to the other experimental groups and the control group values. After 48 h, the ATP levels increased for three of the experimental groups (i.e. 200, 350 and 750 mg/kg) with 750 mg/kg being significantly increased in relation to the untreated control group between 72 and 96 h. The 500 mg/kg experimental group showed a slight increase in ATP at 52 h, decreasing to values below the control at 60 h, while reaching values similar to the untreated control from time points 72 to 96 hours.

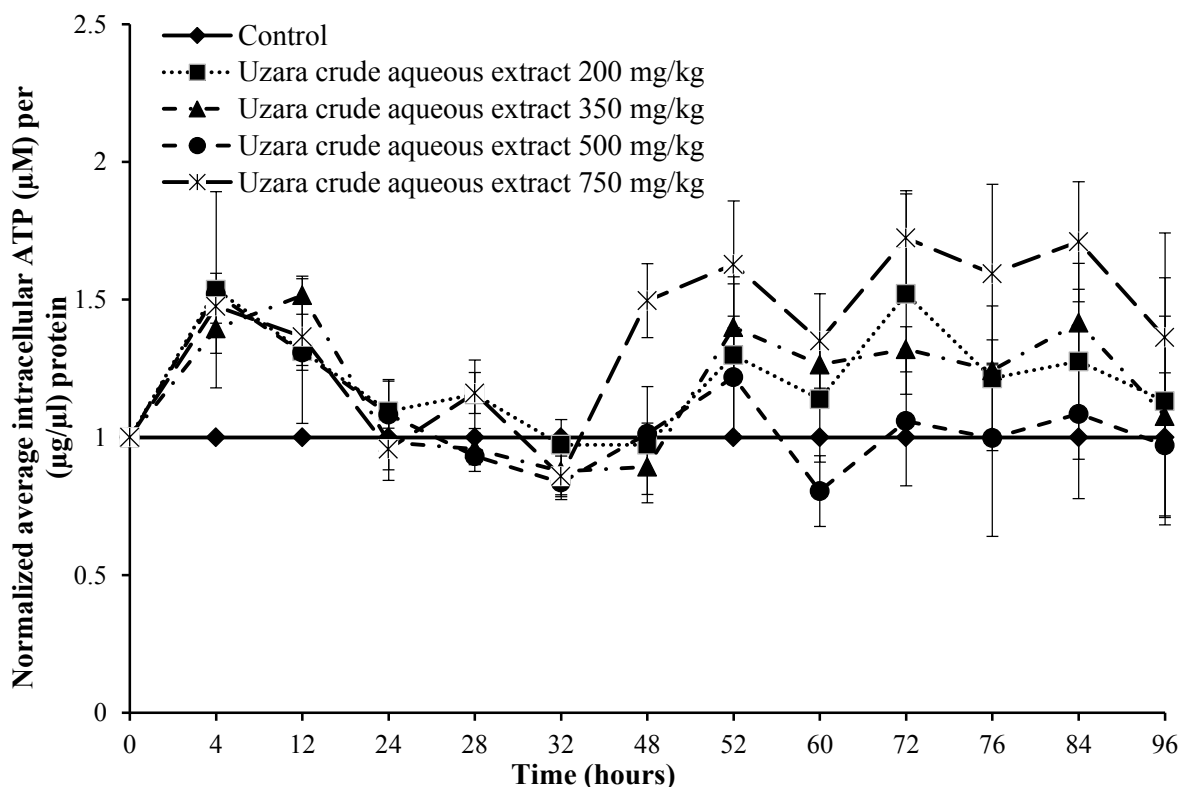


Figure 3. Average intracellular ATP concentration (μM) per protein content ($\mu\text{g}/\mu\text{l}$) for 2D cultured HepG2/C3A cells treated with 200, 350, 500 and 750 mg/kg Uzara crude extract, normalised to the untreated control group ($n = 3$, error bars = SD)

AK released from the 2D model groups are depicted in Figure 4. A slight decrease in AK released within the first 24 h is observed for all experimental groups in comparison to the untreated control group. At time points 28 and 32 h, all experimental groups showed AK release similar to that of the untreated control group. After 48 h, three of the experimental groups (i.e. 350, 500 and 750 mg/kg) again showed a slight increase in AK release, returning to levels similar to the control groups at time points 52 and 60 h. Between 72 and 48 h there was an overall increase in AK release for all the experimental groups, with 350, 500 and 750 mg/kg reaching statistically significant increases at time point 96 h, when compared to the untreated control.

When considering the interplay between intracellular ATP levels and active AK release, it appears that cells grown under 2D conditions are initially able to cope efficiently with the onslaught of the crude Uzara aqueous extract at all the concentrations. However, towards the end of the study the statistically significant increased AK and ATP levels indicate severe cytotoxicity, possibly due to mitochondrial dysfunction leading to reactive oxygen species (ROS) production and loss of cell viability within the 2D model. In the 2D culture the effects of the Uzara treatment appear to be attenuated towards the end

of the experiment, probably due to cells in 2D reaching their growth decline phase considering the growth characteristics presented in Figure 4.

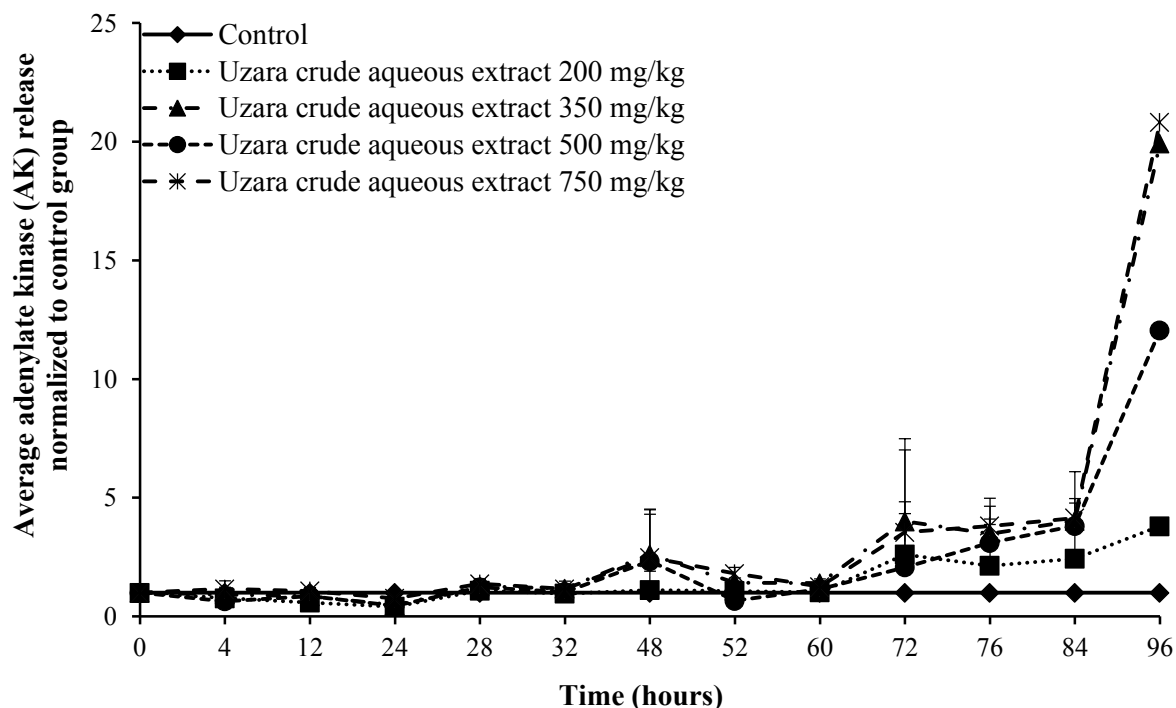


Figure 4. Average adenylate kinase (AK) released in terms of protein content ($\mu\text{g}/\mu\text{l}$) for 2D cultured HepG2/C3A cells treated with 200, 350, 500 and 750 mg/kg Uzara crude extract, normalized to the control group ($n = 3$, error bars = SD).

3.3 Three-dimensional culture model

3.3.1. Planimetry of the 3D cell culture model

The average spheroid surface area of the experimental and control groups, measured at pre-determined time points, are shown in Figure 5. The planimetric spheroid surface area gives an indication of the growth of spheroids, since it is relatable to both the protein content of each spheroid and the total protein content of the spheroid population within each experimental group (Wrzesinski & Fey; 2012 Wrzesinski & Fey 2018). The latter enables normalization of the data in terms of the protein content, corresponding to *in vivo* toxicology studies where treatment quantity (i.e. dose) with a compound is determined in terms of subject weight (mg/kg). The average spheroid surface area for all the experimental groups at time point 0 h was approximately $8.3 \times 10^5 \pm 0.1 \mu\text{m}^2$. From the planimetric data shown in Figure 4, the spheroids experienced significant growth inhibition at time points 48, 72 and 96 h following treatment with Uzara crude aqueous extract at 350, 500 and 750 mg/kg, when compared to the control group. The

spheroids receiving 200 mg/kg extract showed moderate growth, with significant growth inhibition after 96 h. This accumulative and concentration dependant growth inhibition seen over the range of concentrations for both the 2D and 3D data, indicates cytotoxic properties of the Uzara crude aqueous extract, possibly due to the cardenolide uzarin. Similar anti-proliferating effects have been observed in various other cancer cell lines, where cardenolides selectively induced anti-proliferative effects and cell death (Khrisna *et al.*, 2015).

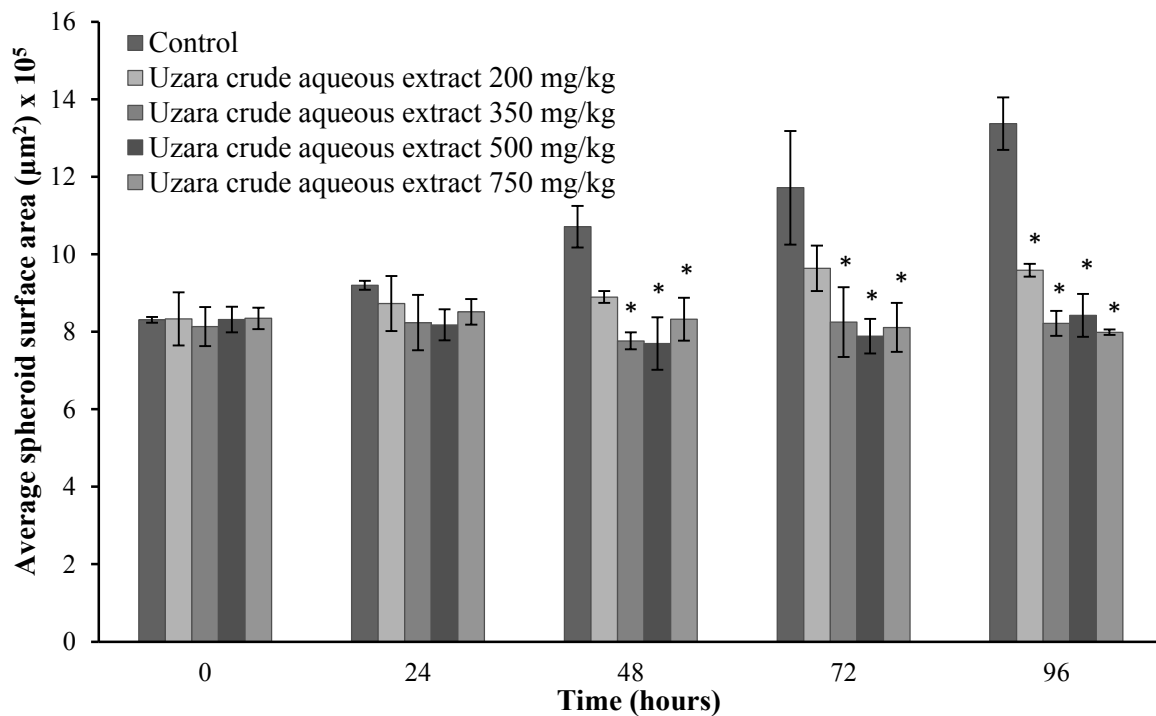


Figure 5. Average spheroid surface area (μm^2) for 3D cultured HepG2/C3A spheroids treated with 200, 350, 500 and 750 mg/kg Uzara crude extract in 24 h intervals, measured prior to each new dosage application ($n = 3$, error bars = SD).

3.3.2. Glucose consumption in the 3D cell culture model

Figure 6 depicts the average concentration glucose consumed per spheroid surface area, in 24 h intervals. At 24 h post treatment, it can be observed that all the treatment groups imported significantly higher quantities glucose per spheroid surface area in comparison to the control group. The 200 mg/kg experimental group consumed considerably more glucose than the control group, with all the other experimental groups achieving significantly higher values throughout the experiment. The level of glucose consumed did, however, decrease over time for the experimental groups treated with 350, 500 and 750mg/kg extract. This effect appeared to be concentration dependent, with 350 mg/kg reaching levels similar to the untreated control towards the end of the experiment. Higher

Uzara extract concentrations clearly affected the ability of the spheroid cells to import and use glucose from the growth medium. At time points 72 and 96 h, the ability of spheroids treated with 750 mg/kg crude Uzara aqueous extract to import glucose proved to be impaired significantly.

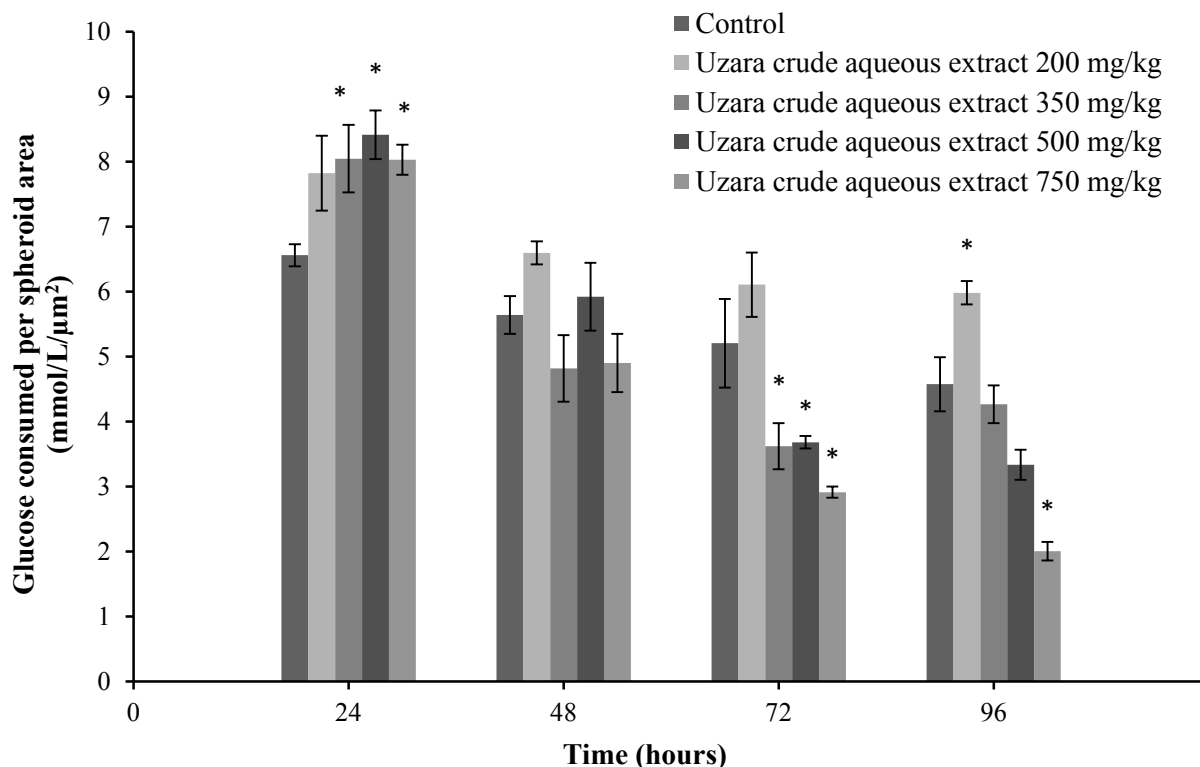


Figure 6. The average glucose consumption (mmol/l per spheroid surface area (μm^2)) for 3D cultured HepG2/C3A spheroids treated with 200, 350, 500 and 750 mg/kg Uzara crude extract in 24 h intervals, measured immediately prior to replacement of growth medium ($n = 3$, error bars = SD).

3.3.3. Intracellular ATP levels and AK release within 3D cultures

Figure 7 gives a graphical depiction of the intracellular ATP levels per spheroid area, normalized with reference to the control group. Within the first 4 h post-treatment, intracellular ATP levels decreased noticeably for all experimental groups relative to the control group, steadily recovering back to the untreated control levels over 24 h. From 24 to 48 h there was a dose-dependent increase in ATP levels for groups treated with 200, 350 and 500 mg/kg extract, reaching values higher than those of the control group. However, the 750 mg/kg experimental group remained below the control, only reaching levels similar to the control group at 48 h. 52 h post-treatment, the ATP levels again decreases in a dose-dependent manner. The experimental groups treated with Uzara extract at dosages of 200, 350 and 500 mg/kg resulted in a decrease in ATP with each successive dosage. ATP levels increased, however, to baseline levels or above within 12 h. This decrease and subsequent increase of

ATP levels noticed throughout the study may potentially be the result of an attempt by the cells to recover from the onslaught of treatment with the crude Uzara aqueous extract. It is important to note that ATP levels for the 750 mg/kg treatment group did not recover to the levels of the control group beyond 48 h. This suggests impaired mitochondrial function brought on by the crude Uzara aqueous extract at 750 mg/kg.

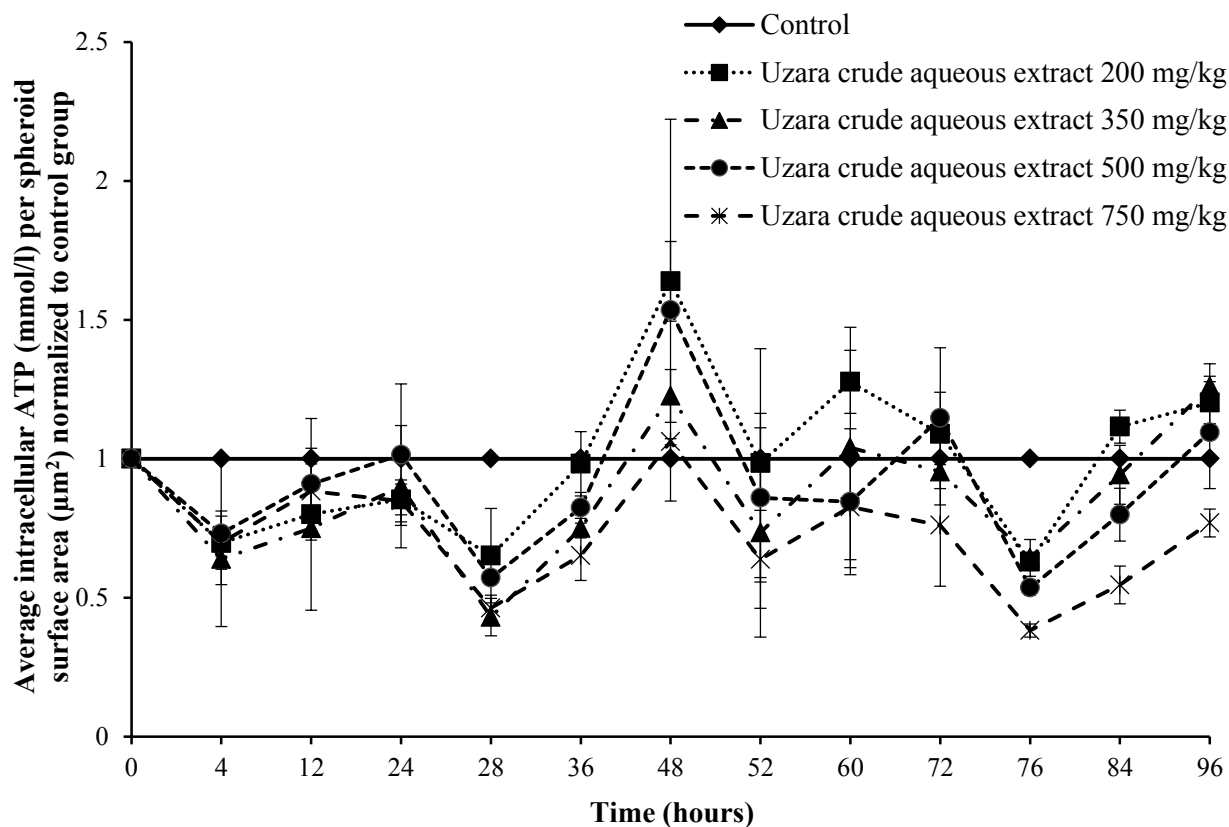


Figure 7. Average intracellular ATP concentration (μM) per average spheroid surface area (μm^2) for 3D cultured HepG2/C3A spheroids treated with 200, 350, 500 and 750 mg/kg Uzara crude extract, normalised relative to the untreated control group ($n = 3$, error bars = SD)

The AK release data depicted in Figure 8 shows an inverted trend compared to the intracellular ATP levels, as results indicated an initial increase in AK within the first 4 h post-treatment for all experimental groups, and a subsequent decrease over 24 h when compared to the untreated control. At 28 h post treatment, AK again increased whilst steadily decreasing over the next 48 h and throughout the study to levels below the untreated control for all experimental groups. However, the 750 mg/kg extract treatment resulted in a steady increase in AK release at 96 h indicating cytotoxicity, and this corresponded well to the diminished ATP levels and mitochondrial dysfunction seen in Figure 7.

This interplay between intracellular ATP levels and AK release from the spheroids indicates the cytotoxic effect and the loss of cell viability as a result of treatment with crude Uzara aqueous extract on the spheroids for all the experimental groups, but in particular the 750 mg/kg treatment. There is also an attempt of the spheroids treated with 200, 350 and 500 mg/kg extract to recover from each successive dosage administered, indicated.

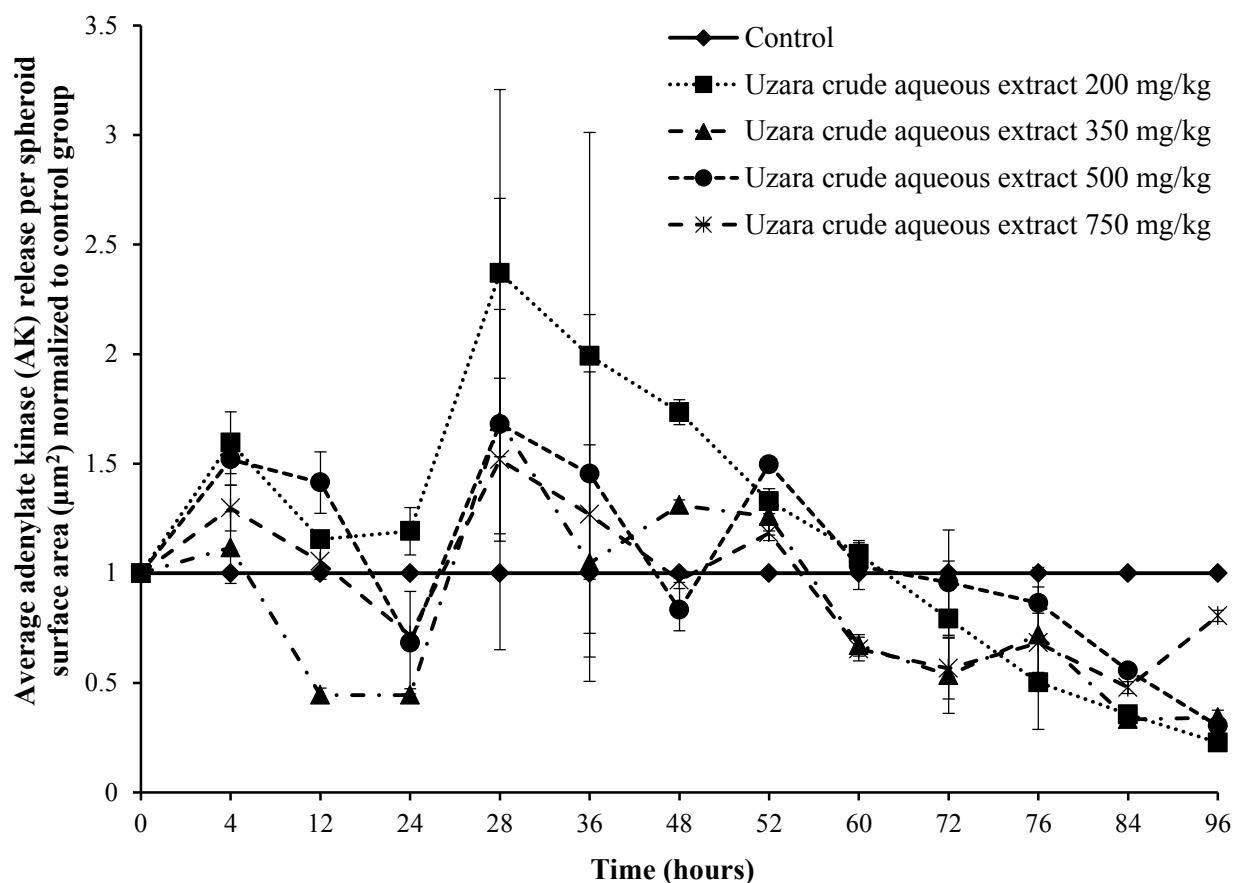


Figure 8. Average adenylate kinase (AK) released per spheroid surface area (μm^2) for 3D cultured HepG2/C3A spheroids treated with 200, 350, 500 and 750 mg/kg Uzara crude extract, normalised to the control group ($n = 3$, error bars = SD).

4. Discussion

This study aimed to investigate the potential toxic effects of short term exposure of the HepG2/C3A liver cell line, to a crude aqueous extract prepared from *Xysmalobium undulatum* (Uzara) plant material in both traditional flat cultures (2D) and a dynamic micro-tissue spheroid model (3D). The protein data obtained from the 2D model showed growth inhibition and anti-proliferating effects due to treatment, with significant anti-proliferating effects for the 750 mg/kg experimental group after 72 hours. Treatment with the crude Uzara aqueous extract in 3D spheroids also showed significant growth inhibition and anti-proliferating abilities in a dosage dependent manner, especially when the inability to utilize glucose and the inhibition in spheroid surface area/biomass is taken into consideration.

Toxicity on the organelle level is in many instances associated with damage to the mitochondria, which is attributed to its proximity to reactive oxygen species (ROS) production during oxidative phosphorylation (Anderson & Borlak, 2007). Upon being bombarded with stressful stimulus, the mitochondria will undergo mitochondrial dysfunction that will determine the commitment of the cell to survival, or apoptosis/necrosis by releasing proteins that acquire key apoptotic functions (Rasda & Bernardi, 2007). One means of determining mitochondrial dysfunction, and ultimately toxicity and cell viability on the molecular level, is through evaluation of intracellular ATP levels and the release of the intermembrane proteins Adenylate kinase (AK) and cytochrome *c* (Cyt *c*). AK is a key mitochondrial intermembrane space protein, responsible for maintenance of metabolic equilibrium within all eukaryotic cells. During stressful states, AK up- or down-regulates ATP production, influencing cell viability and toxicity. Furthermore, during the active release of AK and Cyt *c* the execution caspase cascade is activated, resulting in cell death (Single *et al.*, 1998; Leist & Singel, 1999; Hardie, 2003; Anderson & Borlak, 2007; Rasda & Bernardi, 2007).

The physiological parameters such as intracellular ATP levels and actively released AK levels were therefore used in this study as indicators of cell viability and cytotoxicity, respectively. Intracellular ATP levels in the 2D model were increased for all of the treatments, and only decreased towards the end of the experiment coinciding with a sharp increase in AK released. These results indicated significant cytotoxicity and impaired mitochondrial functionality due to the Uzara water extract treatment for all the experimental groups, when compared to the untreated control group. However, intracellular ATP and AK results from the 3D cell culture model showed potential recovery from toxicity damage at the three lower concentrations tested (i.e. 200, 350 and 500 mg/kg). To this end, the 750 mg/kg Uzara crude aqueous extract treatment group showed mitochondrial dysfunction and cytotoxicity when taking into consideration both the intracellular ATP levels lower than the levels seen in the untreated control group, and the increase of actively released AK levels towards the end of treatment.

This correlates well with data regarding plant extracts containing cardenolides, such as the major active constituent uzarin that is present in the prepared crude Uzara aqueous extract. Krishna and associates (2015) indicated that cardenolides found in plant material are ligands for Na^+/K^+ -ATPase responsible for signalling various cascades that affects numerous targets, including the mitochondria, and subsequent activation of the caspase cascade leading to apoptosis. It also results in a blockage of the NF- κ B signal, resulting in anti-proliferating capabilities as suggested by the 2D protein concentrations and spheroid growth inhibition in the 3D model. However, cardenolides have been shown to have different modes of action in normal cells, in that it stimulates proliferation and inhibits cell death (Khrisna *et al.*, 2015).

Results obtained from the 3D spheroid model showed extensive growth inhibition by the Uzara crude aqueous extract compared to a lesser extent of growth inhibition in the 2D model. This could be a result of cells in the *in vivo* environment, such as the hepatic cells within the liver, are in various stages of the cell growth cycle. Similarly, cells within 3D structures such as the spheroids also find themselves within various stages of the growth cycle, with the outermost layer of the spheroids being predominantly in the active growth phase (S and M phase), while cells in the core of the spheroids find themselves within the G_0 and G_1 phase (Wrzesinski *et al.*, 2014; Yono *et al.*, 2015; Brasja *et al.*, 2016; Calitz *et al.*, 2018; Wrzesinski & Fey, 2018). Cells grown in 2D are at a disadvantage to both the *in vivo* and 3D situations, since they find are in a continuous exponential growth phase due to continuous trypsinization and sub-culturing. Trypsinization at regular intervals results in the disruption of advanced cellular functions, signal transduction, gene expression, extra cellular matrix (EMC), repair processes and cell structure (Wrzesinski & Fey, 2018). Any effect of the applied crude Uzara aqueous extract on cells in the 2D model may therefore be repaired more rapidly, due to DNA repair systems involved in cells within the G_1 phase. The latter is attributed to the type of repair system that is initiated depending on where the cell is within the cell growth cycle. Cells within the G_1 phase, such as cells found in normal 2D cultures, actively repair damage from endogenous ROS species prior to replication, whereas most DNA-repair pathways are attenuated in non-dividing cells, thus a large portion of the cells found in the spheroids. These non-dividing cells have dedicated significant and abundant repair mechanisms to maintain DNA integrity (Branzei *et al.*, 2008). These differences may explain the discrepancies in results obtained from the 2D and 3D models.

These discrepancies can also be observed in the results from the ATP levels and AK release. The cells in the spheroids seem to “cope” more effortlessly with the Uzara water extract, while cells in the 2D model initially appears less affected than the 3D model. However, after 72 h when cells in the 2D model have reached the end of their exponential growth phase, the crude Uzara aqueous extract induces considerable toxicity over the whole concentration range, compared that level of toxicity only being observed for the 750 mg/kg treatment in the 3D model.

5. Conclusions

Taking all the physiological parameters measured, namely the inability to import glucose, lack of proliferation and ATP levels below normal physiological levels with increasing adenylate kinase release into consideration, it can be deduced that the crude Uzara aqueous extract showed an acute toxic effect between 500 and 750 mg/kg, as indicated by the decrease in intracellular ATP levels and increase in actively released AK, indicating mitochondrial dysfunction. The crude Uzara aqueous extract at concentrations of 350, 500 and 750 mg/kg also presented with significant anti-proliferating effects similar to previously reported data on the anti-proliferating effects of cardenolides. To determine if the anti-proliferating effects of the crude Uzara aqueous extract seen in this investigation is limited only to cancer cells, further investigations must be initiated. When considering the behaviour of the three-dimensional and two-dimensional screening models, the results suggest that 3D models may be a superior model in the elucidation of possible herbal toxicity compared to conventional 2D models, due to the lack of physiological relevance of the 2D models. Discrepancies in the data obtained from the 2D and 3D models indicate that the 2D model may result in false positives, as the data suggests that the 2D model initially showed limited toxicity, possibly due to different modes of DNA repair systems in their exponential growth phase. However, as the cells enter the decline phase of the growth curve they seemed to also be severely affected by the crude Uzara aqueous extract at all concentrations. In the 3D spheroid model, the majority of the cells within the spheroid structure are predominantly non-dividing and only a few cells are believed to be in the G1 phase of the cell cycle. The latter correlates well to the *in vivo* situation and intact organs, where cells are also at various stages of the cell cycle or non-dividing, suggesting that a 3D spheroid model will give a more accurate representation on how an organ, in this instance the liver, will respond to a herbal xenobiotic such as Uzara.

Acknowledgments: This work was supported by the National Research Foundation (NRF), South Africa. Funding for work undertaken at the Tshwane University of Technology was made available through the NRF/DST SARChI program as well as the South African Medical Research Council. The financial assistance of the NRF is hereby acknowledged. Opinions expressed, and conclusions arrived at, are those of the authors and are not to be attributed to the NRF.

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

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CHAPTER 5**MANUSCRIPT PREPARED FOR SUBMISSION TO BIOCHEMICAL
PHARMACOLOGY**

Chapter 5 consists of a research manuscript which has been prepared for submission to the journal *Biochemical Pharmacology*. The complete guide for authors for this journal is provided in Appendix D. This chapter differs from the rest of the thesis as it is written in U.S. English and not U.K. English. Furthermore, all data not included in the manuscript is presented in Appendix E, and a diagram of the complete *in vivo* study design is provided in Appendix F. The *in vivo* study has been approved by the AnimCare committee (ethical approval for the *in vivo* studies using animal subjects) of the North-West University, and proof is provided in Appendix G. The certificates of analysis of Uzara commercial product and Valproic acid used are presented in Appendix H.

HepG2/C3A spheroid cultures as a screening tool for sub-chronic hepatotoxicity studies compared to an *in vivo* model

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2017

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Abstract:

Various *in vitro* and *in vivo* models are available for the pre-clinical assessment of drug and herbal hepatotoxicity. However, more reliable and readily available *in vitro* models are needed, which are capable of bridging the gap between existing models and real life. Three-dimensional (3D) spheroid cultures offer higher physiological relevance, overcoming many of the shortcomings of traditional two-dimensional (2D) cell cultures. This study investigated the potential of the 3D HepG2/C3A spheroid model to determine hepatotoxic and anti-proliferative effects of the crude aqueous *Xysmalobium undulatum* (Uzara) extract during a sub-chronic study (21 days). This was done by evaluating cell spheroid growth, intracellular adenosyl triphosphate (ATP) levels and extracellular adenylate kinase (AK). To determine the applicability of the results from this model, they were compared to *in vivo* toxicity effects (i.e. the serum chemistry of Sprague Dawley rats) treated with the same compounds over 21 days. The results from the *in vitro* study clearly indicated hepatotoxic effects and possible liver damage following treatment with valproic acid, as indicated by the growth inhibition, the loss of cell viability and the increased cytotoxicity as indicated by the reduced intracellular ATP levels and increased AK levels. These results were supported by the increased *in vivo* levels of AST, ALT and LDH and the slight increase in triglycerides following treatment of the Sprague Dawley rats with valproic acid, indicative of hepatic cellular damage that may result in hepatotoxicity. The data from both the 3D spheroid model and the Sprague Dawley model was also able to predict the potential concentration dependent hepatotoxicity of the crude Uzara aqueous extract. The results obtained confirm the ability of 3D spheroid models to effectively and reliably predict the long-term outcomes of possible hepatotoxicity.

Key words: *Three-dimensional cell culturing, in vivo model, Uzara, Valproic acid, hepatotoxicity, microgravity, cell spheroids.*

1. Introduction

Various *in vitro* and *in vivo* models are available for the pre-clinical assessment of drug biotransformation, hepatotoxicity and herb-induced liver injury (Tingle & Helsby, 2006; Wrzesinski & Fey 2015). Two-dimensional (2D) cell cultures, with cells cultured in suspension or on solid flat surfaces have been used in drug discovery and screening for decades, but due to poor inter-cellular communication, compromised signalling pathways, the short experimental duration time available and several tissue specific properties lacking, its usefulness as a pre-clinical safety screening model is limited (Lin & Chang, 2008; Breslin & O'Driscoll, 2012; Antoni *et al.*, 2015; Wrzesinski & Fey 2015; Fang & Eglen, 2017).

For obvious reasons, the gold standard for pre-clinical assessment of drug hepatotoxicity remains *in vivo* animal studies. Over the last few years, the use of animal models has become the subject of increasing criticism and from an ethical point of view, a strong need exists for the development of novel *in vitro* screening models to effectively reduce the number of animals used in research projects (Baumans, 2004; Liedtke *et al.*, 2013; Soldatow *et al.*, 2013; Freires *et al.*, 2017). Expression of liver-specific enzymes relevant to drug metabolism and toxicity differ between animals and humans, influencing pre-clinical outcomes (Martignoni *et al.*, 2006; Sabolic *et al.*, 2011; Dalgaard, 2015). Furthermore, *in vivo* animal studies are costly and time-consuming (Soldatow *et al.*, 2013; Sabolic *et al.*, 2011).

Clearly, more reliable and readily available *in vitro* models are needed, which are capable of bridging the gap between the existing models and the *in vivo* environment. Development of novel three-dimensional (3D) cell culturing techniques attempted to overcome these obstacles by providing a cellular environment more closely related to the *in vivo* state, which are able to increase predictability of drug efficacy and toxicity prior to the initiation of clinical trials (Antoni *et al.*, 2015; Wrzesinski & Fey, 2015; Fang & Eglen, 2017). This is important since it has been estimated that almost 39% of new drug candidates are withdrawn from development programmes because of pharmacokinetic deficiencies (i.e. inadequacies in absorption, distribution, biotransformation and excretion), while toxicity contributed to withdrawal of a further 21% of drug candidates during the developmental phase (Tingle & Helsby, 2006).

Various advances have been made with 3D cell culturing, making *in vitro* models more reliable systems that offer longer term cell culturing and testing periods and more accurate determination of drug biotransformation and toxicity (Antoni *et al.*, 2015; Brasjsa *et al.*, 2016;

Fang & Eglén, 2017). Of the various 3D models, spheroids have been extensively characterized and offers reproducibility, simplicity and show resemblances of native *in vivo* organ systems (Tung et al., 2011; Hirschhaeuser *et al.*, 2010). Natural processes occurring during embryogenesis, morphogenesis and organogenesis are mimicked during spheroid assembly with dominating cell-cell interactions and the formation of molecular gradients (Tung *et al.*, 2011; Hirschhaeuser *et al.*, 2010; Achilli *et al.*, 2012; Fang & Eglén, 2017). Spheroids can be cultured by employing various systems, but Wrzesinski and colleagues have developed a dynamic micro-tissue spheroid cell model capable of overcoming many of the shortcomings of traditional 2D cell cultures. These micro-tissue spheroids present with multi-cellular arrangement, mimicking *in vivo* organ architecture and behaviour (Fey and Wrzesinski, 2012a, 2012b; Wojdyla *et al.*, 2016; Wrzesinski and Fey, 2013; Wrzesinski *et al.*, 2013, 2014; Aucamp *et al.*, 2017).

Xysmalobium undulatum (L.) W.T. Aiton (Apocynaceae), also known as Uzara, is a widely used traditional herbal medicine in Southern Africa to treat various maladies (Vermaak *et al.*, 2014: 137,138; Van Wyk, 2011:825). The predominant pharmacologically active constituents extracted from Uzara root include the cardenolide cardiac glycosides uzarin (5.6%), xysmalorin (1.5%) and the isomers allouzarin (0.4%) and alloxysmalorin (0.1%). No hepatotoxicity has been described for Uzara, but a toxic digitalis-like action on the heart has been reported when administered in extremely high concentrations (Vermaak *et al.*, 2014). Herbal medicines and supplementation are generally believed to be safe due to their natural origin with fewer adverse effects, however, this is not only misleading but also untrue (Ekor, 2014; Calitz *et al.*, 2015; Boadu & Asase, 2017). And although many of these herbal medicines do offer the potential of becoming valuable candidates in the treatment of various disease states, the majority remain untested and their usage unmonitored while they are introduced as commercial products, without any prior safety or toxicological evaluation (Ekor, 2014). Liver injury caused by herbal supplements range from asymptomatic elevation of liver enzymes, to cirrhosis and in certain instances even acute liver failure (Calitz *et al.*, 2015). This study investigated the use of the *in vitro* 3D dynamic micro-tissue HepG2/C3A spheroid culture model as a pre-clinical screening tool to evaluate hepatotoxicity and anti-proliferic effects, which were compared to the results of an *in vivo* study.

2. Materials and Methods

2.1. Preparing a crude Uzara aqueous extract

Commercially available dried and milled Uzara material was purchased from Afrinatural holdings (Prestige laboratory supplies, Mt Edgecombe, Johannesburg). A water extract was prepared by creating an Uzara-water suspension in a 1:10 product to water ratio. The

suspension was sonicated in a Eumax ultrasonic bath at the following parameters, 45°C for 45 min, followed by centrifugation for 10 min at 5000 x *g*. The pellet was re-suspended in 100 ml water, and the suspension was sonicated and centrifuged again to collect the supernatant. The collected supernatant was filtered and frozen at -80°C. The frozen filtrate was lyophilised on a Virtis freeze dryer (SP Scientific, Gardiner, New York).

The chemical composition of the Uzara aqueous extract was determined on a Waters Acquity Ultra Performance Liquid Chromatographic system with PDA detector (Waters, Milford, MA, USA), as previously published (Calitz et al., 2017). The presence of the major active constituent, uzarin, was confirmed and quantified as 226.4 µg/mg Uzara (n=2).

2.2. Two-dimensional cell culture

The clonal derivative, HepG2/C3A (American Type Culture Collection [ATCC] cat. no. CRL-10741, sixth passage after receipt from ATCC, Manassas, VA), of the hepatocellular carcinoma cell line HepG2, was cultured using standard tissue culture conditions in Dulbecco's Modified Eagle's Medium (DMEM) (1g glucose/l) (Gibco, Carlsbad, CA), with 1% non-essential amino acids (Gibco); 10% fetal calf serum (FCS) (Sigma, St Louis, MO); 0.5% penicillin/streptomycin (Gibco) and 1% GlutaMAX (Gibco). Cultures were incubated in a humidified atmosphere at 37°C, 5% CO₂ and 95% air.

2.3. Three-dimensional cell culture

2.3.1. Spheroid preparation using AggreWell™400 plates

HepG2/C3A cell spheroids were prepared using an AggreWell™400 plate (Stemcell Technologies, Grenoble, France) according to the manufacturer's specifications and as previously published (Wrzesinski & Fey, 2012; Aucamp et al., 2017). Wells were rinsed twice with 1 ml Aggrewell™ Rinsing solution (Stemcell Technologies, Grenoble, France), and HepG2/C3A cells were seeded into each of the wells of the AggreWell™ plate at a seeding density of 1.2×10^4 . Following centrifugation for 3 min at 120 x *g*, the plate was incubated overnight to allow spheroid formation.

2.3.2. Spheroid culture in bioreactors

Spheroids were detached from the AggreWell™ wells by gently washing the wells with pre-warmed growth medium. Detached spheroids were then collected, and the spheroid quality was determined using a light microscope. Spheroids were selected based on visual quality, by comparing compactness, size and roundness. Selected spheroids were placed into bioreactors (MC2 Biotek, Hørsholm, Denmark), approximately 300 per bioreactor, and cultivated at 37 °C, 5% CO₂, 95% air in a humidified incubator for 17 days with continuous

rotation of the bioreactors and replacement of medium three times a week as described below.

The day of spheroid removal from the AggreWell™ wells is referred to as day 0 and an estimated 90% of growth medium was removed and replaced on day 3. Thereafter, the medium was removed and replaced three times a week. The rotation speed of the bioreactors was initially set between 9 and 11 rotations per minute (rpm) and was adjusted as necessary to compensate for spheroid size due to cell growth. The spheroid population was split on day 8 and when experimental groups were initiated on day 17 and introduced to new bioreactors.

2.3.3. *In vitro* experimental design

The design of the *in vitro* experiments on the 3D HepG2/C3A spheroids is shown in Figure 1. Four experiment groups were prepared, and each group consisted of four biological replicates in individual bioreactors. Each bioreactor on which the experiments were conducted contained 207 spheroids (17 days old) as illustrated in Figure 1. The experimental groups consisted of spheroids treated with crude Uzara aqueous extract at various concentrations (200 mg/kg and 250 mg/kg), spheroids treated with Sodium valproate (Sigma, Johannesburg, South Africa) at 10 g/kg served as a positive control group for hepatotoxicity, and spheroids in normal growth medium as the untreated control group. Treatment concentrations for Uzara were based on available literature and previously conducted acute and sub-chronic *in vitro* studies (Vermaak *et al.*, 2014; Calitz *et al.*, 2017), while the valproic acid concentration (10 mg/mg protein translating to 10g/kg) was based on a value below the acute lethal threshold of 20 mg/mg protein in spheroids as determined by Fey and Wrzesinski (2013). The valproic acid concentration was chosen to be very high to ensure potential hepatotoxic effects would be observable in the model if only limited effects were instigated. The uzarin concentration in the 200 mg/kg and 250 mg/kg experimental treatment solutions were 45.28 mg and 56.60 mg, respectively.

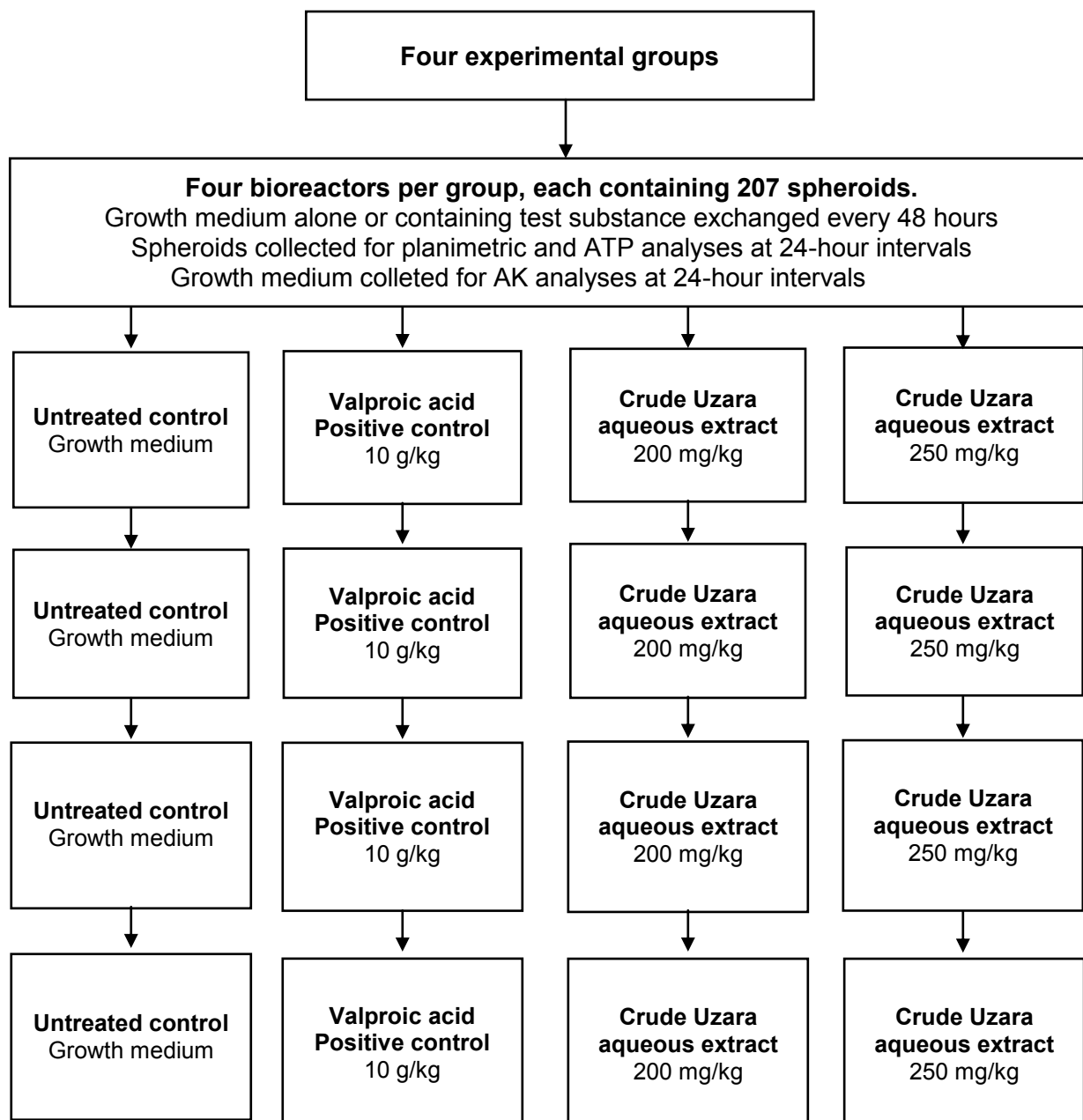


Figure 1: Diagram depicting *in vitro* experimental design

To initiate drug treatment, the bioreactor rotation was stopped, and spheroids were allowed to settle. Growth medium was removed and exchanged with medium containing the various treatments. Spheroids and medium samples were collected for planimetry, adenosine triphosphate (ATP) and adenylate kinase (AK) analyses. Growth medium containing treatment was exchanged at 48-hour intervals, for a duration of 21 days.

2.3.4. Spheroid microscopy and planimetric analyses

Photomicrographs were taken of three spheroids per time point, in 24h intervals for the duration of the 21-day study, using an Olympus IX81 motorized microscope and an Olympus DP71 camera. Images were transferred to the Olympus AnalySiS Docu program (Soft Imaging System) and the “shadow” areas of spheroids were measured using the “Fitted Polygon Area” function. This indicated the planar surface area of the spheroids in μm^2 , and allowed for the ATP and AK to be related to spheroid surface area. In a previous study by Wrzesinski and Fey (2012), a correlation was found between the spheroid surface area and the protein content of the spheroids, allowing the normalization of data in terms of protein content. Furthermore, this allowed dosages of Uzara extract to be administered to the spheroids per mass (mg/kg), which corresponded with dosages administered to animal subjects during the *in vivo* studies.

2.3.5. In vitro adenosyl triphosphate (ATP) quantification

Cell viability was based on the ability of the spheroids to produce ATP. Spheroids from each experimental group (three replicates consisting of two spheroids per 24h time point) were collected for the duration of the 21 days, and transferred to white opaque microtitre plates (Nunc, Roskilde, Denmark). Growth medium was removed, and the volume adjusted to 100 μl with Hanks buffered saline solution without Ca^{2+} and Mg^{2+} (Gibco, Carlsbad, CA). The cells were then lysed with 100 μl lysis buffer (CellTiter-Glo luminescent cell viability assay, Promega, Fitchburg, WI) and shaken in the dark for 40 min, before the luminescence was measured in a FLUOstar OPTIMA (BMG Labtech, Ortenberg, Germany) using the following parameters: one kinetic window, 10 measurement cycles with 0.3 s of measurements interval time, 2 s delay per measurement, additional 0.5 s delay per position change (repeated twice for each measured plate). The data was normalized with reference to a standard curve for ATP and the untreated control.

2.3.6. In vitro adenylate kinase (AK) quantification

AK content was measured in the growth medium (140 μl) of each experimental group to determine cell death in 24h intervals for the duration of the 21-day study, using the Lonza Toxilight assay kit. Growth medium was placed in microtitre plates in triplicate and diluted with five volumes of adenylate kinase detection reagent. The plate was then incubated in the dark for 20 min before measuring luminescence, using a FluoStar Omega[®] (BMG Labtech). A standard curve was prepared in the same way for each assay plate using a dead-cell standard (4.29 million HepG2/C3A cells per ml, which were lysed in lysis buffer). Gain was adjusted based on the highest standard curve values.

2.4. *In vivo* study

2.4.1. *In vivo* experimental design

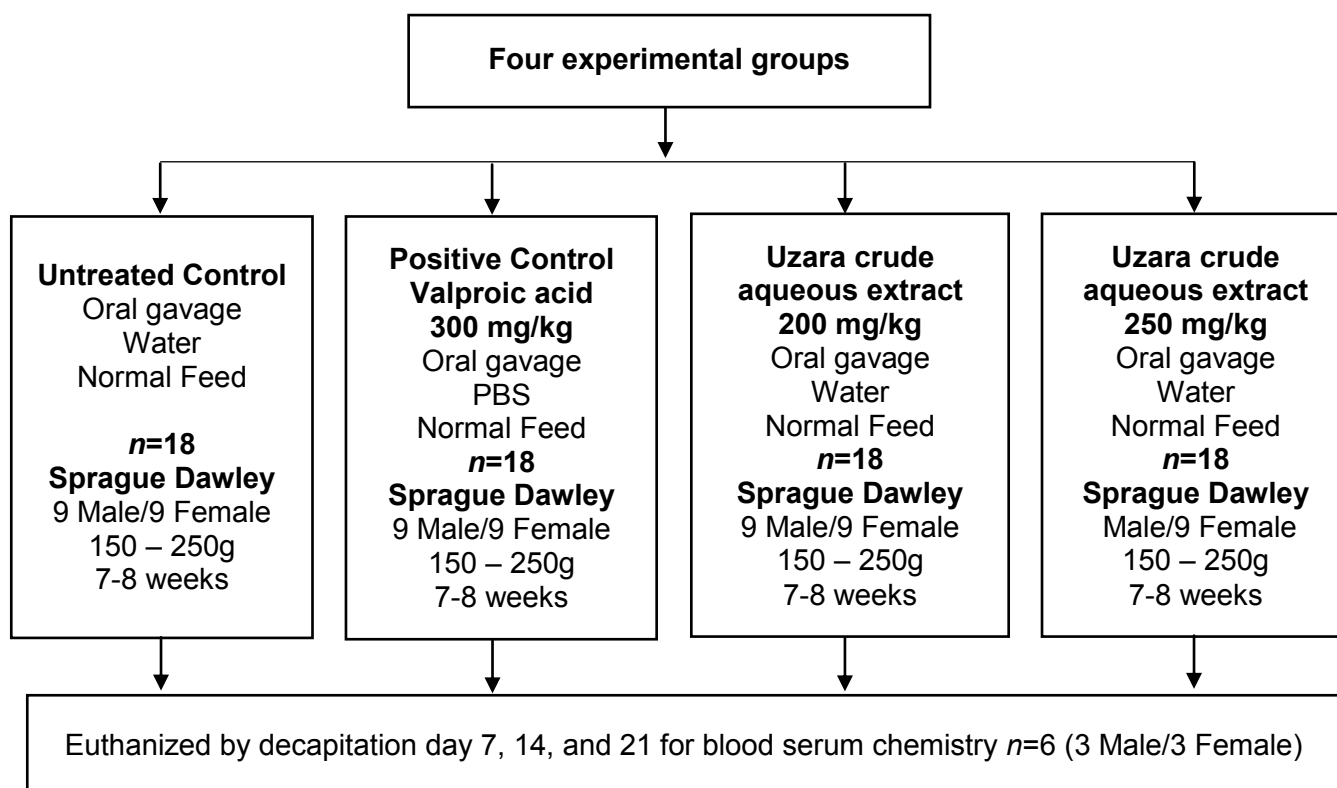


Figure 2: Diagram depicting sub-chronic 21-day *in vivo* experimental design.

The *in vivo* study was approved by the AnimCare ethics committee of the North-West University (NWU-00276-17-S5), prior to initiation of the study. Specific pathogen free male and female Sprague Dawley rats were obtained from the North-West University vivarium (Potchefstroom, South Africa; South African Veterinary Council registered). Figure 2 gives a graphical depiction of the experimental design. Test subjects weighing between 150-250g and aged 7-8 weeks were allowed seven days of acclimatisation to laboratory conditions prior to the initiation of the experiment. Housing was limited to groups of three per cage according to sex, with corncob bedding and an artificial 12-hour light cycle in a room with a temperature of $22 \pm 2^{\circ}\text{C}$ and relative humidity of $55 \pm 10\%$. Test subjects were fed a conventional laboratory diet with drinking water supplied *ad libitum*. The weight of each test subject was recorded prior to the initiation of the experiment, and bi-weekly thereafter. General clinical observations were made daily, and all test subjects was checked for morbidity and mortality twice daily. All procedures conformed to the North-West University vivarium's laboratory animal care standard operating procedures to ensure that good

laboratory practice was adhered to in all instances according to national and international accepted principles and standards for humane handling of animals.

The test substances consisted of 200 and 250 mg/kg crude Uzara aqueous extract in water and 300 mg/kg valproic acid in phosphate buffered saline (PBS) as positive control, with the control group receiving only PBS. Treatment concentrations for Uzara were based on available literature and previously conducted acute and sub-chronic *in vitro* studies (Vermaak *et al.*, 2014; Calitz *et al.*, 2017), while the valproic acid concentration was based on its EC₅₀ (485 mg/kg) and LD₅₀ (600 mg/kg) values in rodents (Löscher, 2007; Lee *et al.*, 2008; Drugbank, 2017). Fresh stock solutions of the test substances were prepared daily, and diluted according to the weight of each test subject prior to administration thereof. The test solutions were administered by means of oral gavage as 21 single dosages at 24 h intervals. Each treatment group consisted of 18 rats (9 male, 9 female). On day 7, 14 and 21, a sample population of 6 rats (3 male, 3 female) from each group were euthanatized by means of decapitation, and terminal blood samples were collected for serum chemistry analyses.

2.4.2. Adverse events

The dosage selected for the valproic acid treatment was initially 600 mg/kg, based on similar studies in literature (Lee *et al.*, 2008; Zhang *et al.*, 2014). However, during the first 24 h post-administration of the first dosage, severe morbidity could be observed, and some mortalities occurred. Subjects presented with difficulty breathing and lethargy approximately 7 h post-treatment, and would succumb in their sleep 9 to 10 h post-treatment. Some participants were not severely affected, but did show a loss of weight of approximately 5% of the initial body weight. Several test subjects presented with rails. An adverse event was immediately reported to the ethical committee of the North-West University, and the study was halted to allow amendment of the study design. Following careful consideration, and taking into account the EC₅₀ value (485 mg/kg) and the LD₅₀ value (600 mg/kg) of valproic acid, the treatment was changed to 300 mg/kg valproic acid.

2.4.3. In vivo serum chemistry

Terminal blood samples ($n = 6$), were collected at time points 0, 7, 14, and 21 days to analyse markers of liver damage. The following parameters were monitored: triglycerides, total albumin, total bilirubin, alkaline phosphatase (ALP), alanine transaminase (ALT), aspartate aminotransferase (AST), and lactate dehydrogenase (LDH). Serum chemistry was analysed by PathCare Pathologists (Potchefstroom, South Africa). Briefly, samples were centrifuged for 15 min at 4000 rpm in a Biofuge™ Primo™ centrifuge (Heraeus Instruments, Thermo Fisher Scientific, Johannesburg, South Africa). Collected serum was then analysed

using a Beckman Coulter AU480 Biochemistry analyser (Beckman Coulter, South Africa, Johannesburg).

2.5. Statistical analyses

Repeated measures ANOVA was performed on the three-dimensional cell culture data using Statistica software version 13.2.

3. Results and discussion

3.1. *In vitro* study

3.1.1. Planimetry and spheroid growth

The planimetric data shown in Figure 3, clearly indicate significant concentration-dependent growth inhibition following treatment with the crude Uzara aqueous extract at both 200 mg/kg and 250 mg/kg when compared to the untreated control group. The experimental group treated with 10 g/kg valproic acid showed even more extensive growth inhibition. The average spheroid surface area at time point 0 for all the experimental groups was $5.38 \pm 0.11 \mu\text{m}^2$, and at day 21 this increased to $17.73 \pm 0.62 \mu\text{m}^2$ for the untreated control, $6.36 \pm 0.36 \mu\text{m}^2$ for valproic acid and $11.23 \pm 0.79 \mu\text{m}^2$ and $11.51 \pm 0.28 \mu\text{m}^2$ for the crude Uzara aqueous extract at 200 mg/kg and 250 mg/kg, respectively. This growth inhibition corresponds well to previous studies with valproic acid and Uzara crude aqueous extract at various concentrations (Wrzesinski & Fey, 2012; Calitz *et al.*, 2018). Uzara contains the cardenolide uzarin which has been shown to possess cytotoxic and anti-proliferic effects (Krishna *et al.*, 2015; Calitz *et al.*, 2018).

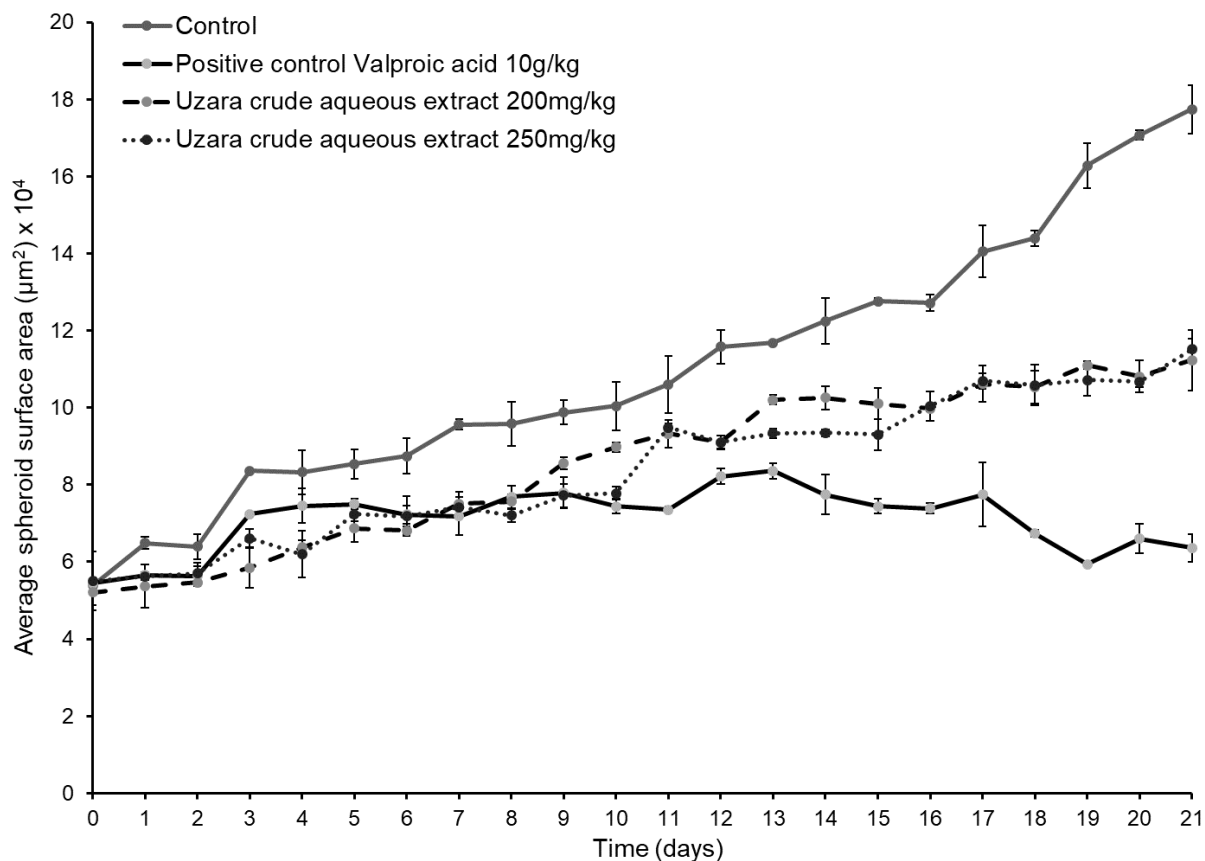


Figure 3: Average spheroid surface area (μm^2) for the spheroids of each experimental group as a function of time over a 21-day period ($n = 3$) (Error bars = standard deviation)

3.1.2. Intracellular ATP and AK release

The intracellular ATP levels expressed relative to the spheroid surface area are depicted in Figure 4. There was a sharp increase in ATP levels for all the experimental groups within the first 24 h, but they decreased to levels below that of the untreated control group after 48 h. ATP levels for the valproic acid positive control (10 g/kg) group remained below the untreated control group, decreasing daily from day 5 to day 14. By day 14, the intracellular ATP levels for the valproic acid group was significantly lower than that of the untreated control group, as well as the crude Uzara aqueous extract experimental groups. Following treatment with the various concentrations crude Uzara aqueous extract, the intracellular ATP levels increased post-treatment, but decreased prior to each successive treatment. By day 19 and 21, ATP levels increased significantly when compared to the untreated control, and this may indicate an attempt of the spheroids to recover from the continuous onslaught of treatment with the crude Uzara aqueous extract. This corresponds well with previous data on crude Uzara aqueous extract (Calitz *et al.*, 2017), albeit in this study it was shown after a longer duration of exposure.

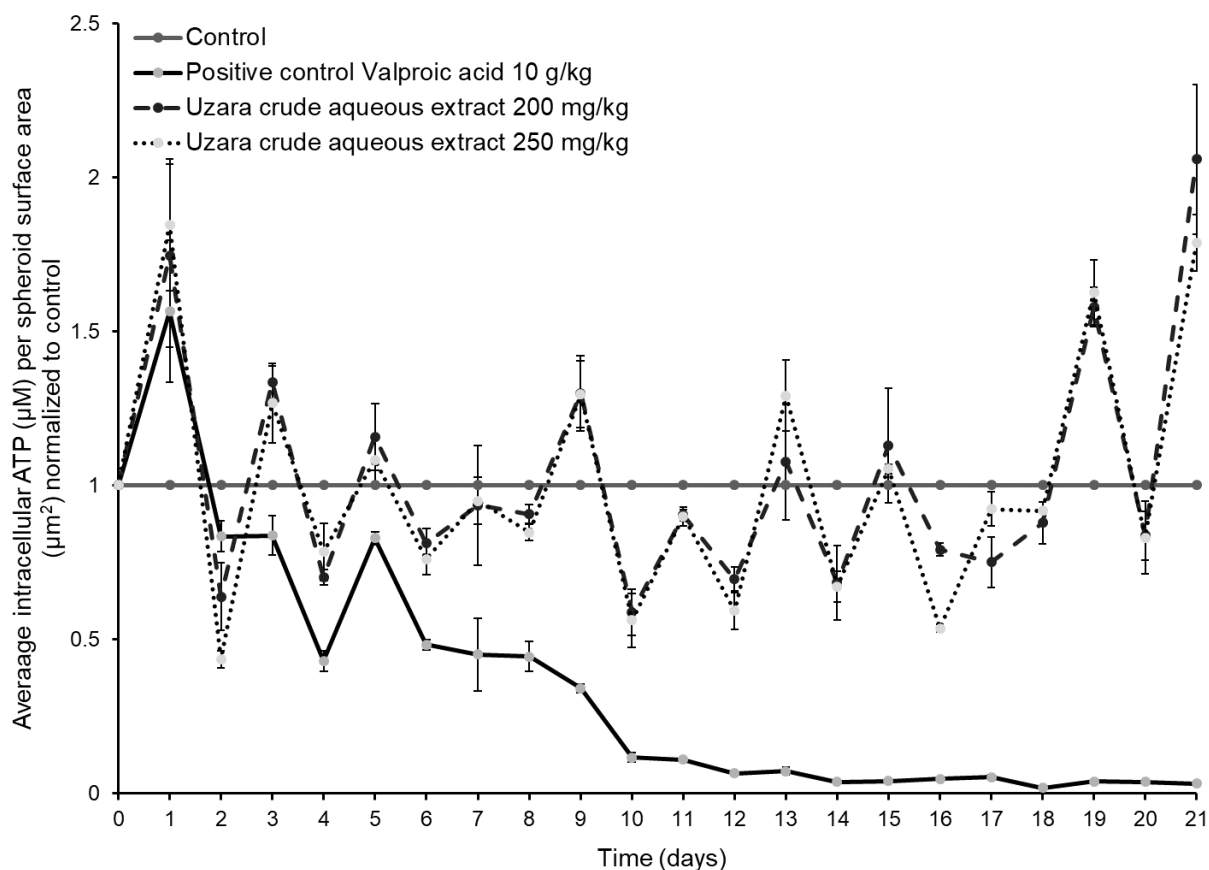


Figure 4: Average intracellular ATP levels (μM) per spheroid surface area (μm^2) normalized to the untreated control as a function of time ($n = 3$) (Error bars = standard deviation).

Figure 5 shows the active AK release per spheroid surface area. An inverted trend can be observed between the AK released and the intracellular ATP levels depicted in Figure 4. AK released appears to have decreased for all experimental groups relative to the untreated control within the first 24 h post-treatment. AK increased to levels above the untreated control for the valproic acid treated group, while remaining below the untreated control for the crude Uzara aqueous extract-treated groups. On day 6, there was a significant increase in AK for all the experimental groups, but by day 7 the AK levels for all the experimental groups decreased to levels low than that of the untreated control again. AK levels remained below the untreated control group from day 7 to day 14 for the valproic acid treated group, but on days 15, 17, 19 and 21, the AK levels once again increased above that of the untreated control. The AK release for the crude Uzara aqueous extract treated groups remained lower than that of the untreated control for the most part of the remaining treatment period.

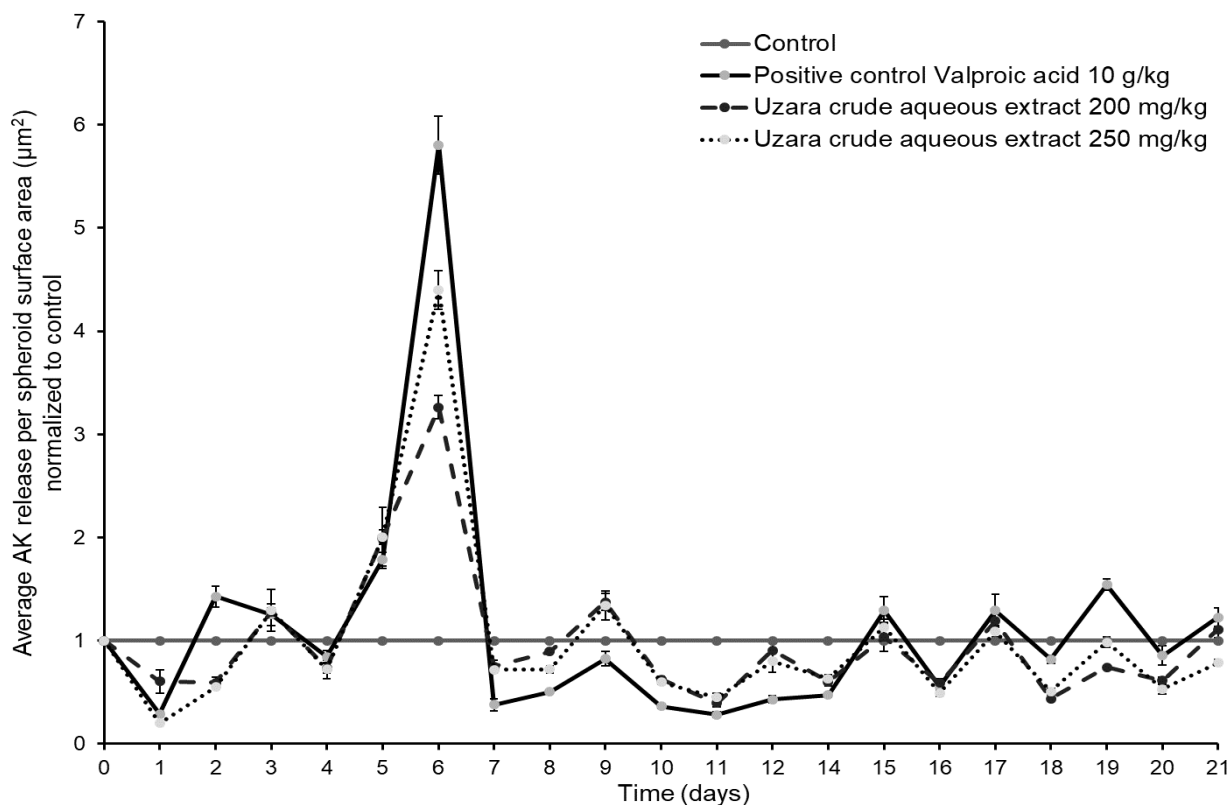


Figure 5: Average AK release per spheroid surface area (μm^2), normalized to the untreated control as a function of time ($n = 3$) (Error bars = standard deviation).

An interplay between intracellular ATP levels and the levels of AK released exists, and can clearly be seen for both the valproic acid and crude Uzara aqueous extract treated groups. The increase and subsequent decrease in intracellular ATP levels, and the increase in AK release by the spheroids treated with the valproic acid, is indicative of the toxic effect of this known hepatotoxin and the loss in cell viability with continuous administration thereof. Results also indicated an initial loss of cell viability and increased toxicity for both concentrations of crude Uzara aqueous extract.

In many instances, hepatotoxicity is associated with the production of reactive oxygen species (ROS) and ultimately with damage to the mitochondria on the organelle level. The latter results in the dysfunction of the mitochondria, determining the fate of the cell, i.e. survival and/or apoptosis/necrosis (Rasda & Bernardi., 2007; Singh *et al.*, 2011). The interplay between intracellular ATP levels and the release of the intermembrane proteins AK and cytochrome *c* (Cyt *c*), can be used to determine mitochondrial dysfunction on the molecular level and ultimately as indicators of cell viability and toxicity. AK release during stressful cellular states is responsible for the up and/or down regulation of intracellular ATP levels, and additionally, the release of AK and Cyt *c* activates the execution caspase

cascade, resulting in apoptosis/necrosis (Single *et al.*, 1998; Leist *et al.*, 1999; Anderson & Borlak, 2007; Rasda & Bernardi, 2007; Hardie, 2003; Singh *et al.*, 2011).

3.2. *In vivo* study

Biochemical markers are clinically employed in the diagnosis of liver injuries caused by hepatotoxins like drugs and/or herbal constituents. These liver injury-related biochemical markers include, but are not limited to, albumin, total bilirubin, alkaline phosphatase (ALP), alanine transaminase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH) and triglycerides (Singh *et al.*, 2011). These markers were therefore quantified in serum samples obtained following treatment of male and female Sprague Dawley rats with valproic acid (300 mg/kg) and crude Uzara aqueous extract (200 and 250 mg/kg), and are shown in Table 1.

Albumin and total bilirubin levels (data not shown) did not change as a result of the various treatments. Similarly, the ALP levels for all the various experimental groups remained unchanged. Following treatment with the crude Uzara aqueous extract at 250 mg/kg, AST levels increased relative to the untreated control group for the male participants on day 7 and on day 21 for the female participants, while ALT levels were increased at all time points compared to the untreated control group. The valproic acid group also had increased AST and ALT levels after 14 and 21 days' treatment for both male and female test subjects. Increased AST and ALT levels can be indicative of hepatitis, associated with the leakage of these enzymes from damaged mitochondria and cytoplasm (Singh *et al.*, 2011; Featherstone, 2007).

Increased LDH levels are also frequently associated with hepatotoxicity, indicating the disruption of the mitochondria and the sarcoplasmic reticulum, and leakage of the enzyme from damaged tissue. Furthermore, it is also indicative of possible hepatocellular necrosis (Singh *et al.*, 2011). Following treatment with the crude Uzara aqueous extract at 250 mg/kg, the LDH levels increased for both male and female participants on day 7, and for the male participants on day 21. The increased LDH levels were also seen for the male participants of the 200 mg/kg crude Uzara aqueous extract treated group on day 21 when compared to the control group. The valproic acid treated test subjects also showed a cumulative increase in LDH levels from day 14 to 21, for both the male and female participants. Furthermore, an increase in TG levels were observed at all time points following treatment with 250 mg/kg crude Uzara aqueous extract, and also on day 14 for the individuals treated with 200 mg/kg crude Uzara aqueous extract. The valproic acid treated group showed only a slight increase in TG levels on days 14 and 21. Since the liver is responsible for lipid metabolism, liver dysfunction due to hepatotoxin exposure could also influence lipid metabolism. An increase

in TG levels has been shown to be indicative of dysfunctional liver metabolism, potentially resulting in steatosis if left untreated (Featherstone, 2007; Merrel & Cherrington, 2010).

Table 1: Serum chemistry following sub-chronic treatment of male and female Sprague Dawley rats with valproic acid and crude Uzara aqueous extract (Alkaline phosphatase (ALP), alanine transaminase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), triglycerides (TG), male (M; $n = 3$), female (F; $n = 3$))

Time point (day)	Parameter	Male/ Female	Control (Valproic acid 300 mg/kg (Uzara crude aqueous extract 200 mg/kg (Uzara crude aqueous extract 250 mg/kg
7	ALP (IU/L)	M	757.33 ± 21.29	427.66 ± 34.56	665.33 ± 77.04	569.33 ± 37.57
		F	597 ± 76.08	386.33 ± 49.38	420.66 ± 41.33	538.66 ± 25.22
	AST (IU/L)	M	247.00 ± 45.10	203.66 ± 27.18	222.00 ± 8.16	305.33 ± 58.78
		F	299.66 ± 63.98	213.00 ± 4.96	271.00 ± 19.30	290.33 ± 47.04
	ALT (IU/L)	M	82.66 ± 7.84	76.66 ± 3.77	76.00 ± 4.32	85.33 ± 7.03
		F	80.00 ± 4.32	68.00 ± 4.32	79.66 ± 5.24	94.00 ± 3.74
	LDH (IU/L)	M	861.66 ± 396.00	695.66 ± 201.55	797.33 ± 78.10	1235.33 ± 349.77
		F	1080.00 ± 267.49	863.66 ± 63.54	910.00 ± 65.67	1086.00 ± 187.51
TG (mg/dL)	M	1.15 ± 0.15	0.93 ± 0.13	0.87 ± 0.10	1.29 ± 0.14	
	F	0.76 ± 0.01	1.17 ± 0.11	0.70 ± 0.19	0.87 ± 0.14	
14	ALP (IU/L)	M	646.66 ± 54.16	454.66 ± 23.45	555.66 ± 4.10	546.33 ± 15.19
		F	439 ± 16.87	307 ± 22.64	494.66 ± 52.00	449.33 ± 33.63
	AST (IU/L)	M	196.66 ± 31.35	280.00 ± 19.61	199.00 ± 28.39	163.66 ± 4.92
		F	207.66 ± 30.72	276.66 ± 26.7	193.00 ± 33.17	215.66 ± 17.55
	ALT (IU/L)	M	71.66 ± 5.24	93.66 ± 8.21	76.00 ± 4.08	68.00 ± 1.63
		F	63.00 ± 0.81	77.00 ± 2.44	72.00 ± 3.55	76.66 ± 6.64
	LDH (IU/L)	M	654.66 ± 159.91	975.66 ± 109.36	653.66 ± 199.06	542.66 ± 28.52
		F	588.33 ± 96.23	989.66 ± 50.28	645.00 ± 183.58	705.66 ± 67.60
TG (mg/dL)	M	1.01 ± 0.11	1.14 ± 0.46	1.14 ± 0.24	0.96 ± 0.05	
	F	0.64 ± 0.04	0.79 ± 0.01	0.78 ± 0.08	1.53 ± 1.03	
21	ALP (IU/L)	M	584.66 ± 40.54	568.00 ± 45.74	542.33 ± 22.29	491.00 ± 24.91
		F	444.66 ± 58.02	379.00 ± 4.00	456.33 ± 52.68	379.00 ± 21.77
	AST (IU/L)	M	213.33 ± 23.15	288.00 ± 11.34	212.33 ± 28.94	185.66 ± 16.35
		F	197.66 ± 27.82	376.00 ± 40.00	187.66 ± 25.31	214.33 ± 23.80
	ALT (IU/L)	M	68.00 ± 4.24	93.00 ± 4.08	71.66 ± 1.24	70.66 ± 7.36
		F	65.00 ± 7.07	98.00 ± 4.00	61.33 ± 3.09	78.00 ± 10.03
	LDH (IU/L)	M	615.33 ± 114.42	1252.00 ± 116.01	640.66 ± 67.59	662.66 ± 56.38
		F	647.66 ± 110.97	1708.50 ± 244.50	594.33 ± 78.01	514.00 ± 73.33
TG (mg/dL)	M	1.51 ± 0.20	1.14 ± 0.29	1.42 ± 0.31	1.52 ± 0.54	
	F	0.96 ± 0.16	1.08 ± 0.05	0.81 ± 0.30	1.31 ± 0.36	

4. Conclusion

The use of animal models has become the subject of increasing criticism, urging the scientific community to provide more reliable, relevant and predictive *in vitro* models. The results from the *in vitro* study clearly indicated hepatotoxic effects and possible liver damage following treatment with valproic acid, a compound known to cause hepatotoxic effects in certain individuals, as indicated by the growth inhibition, the loss of cell viability and the increased cytotoxicity as indicated by the reduced intracellular ATP levels and increased AK levels. These results also correlated well with previously published data in both 3D spheroids and *in vivo* systems, and is supported by the increased *in vivo* levels of AST, ALT and LDH and the slight increase in triglycerides following treatment of the Sprague Dawley rats with valproic acid, indicative of hepatic cellular damage that may result in hepatotoxicity.

The *in vitro* 3D spheroid model was also able to predict the potential concentration dependant hepatotoxicity of the crude Uzara aqueous extract. Similarly, the results obtained from the *in vivo* Sprague Dawley model indicated moderate hepatotoxic potential.

The results confirmed the usefulness of the HepG2/C3A spheroid culture model to effectively and reliably predict potential hepatotoxicity following long-term exposure to compounds. This model therefore offers a readily available *in vitro* model, capable of bridging the gap between current two-dimensional *in vitro* screening models and the pre-clinical *in vivo* animal models. Implementing this screening model in the pre-clinical evaluation process of new drug entities could therefore reduce costs and time as the use of animal models could be reduced as compounds with potential hepatotoxic properties can be eliminated prior to *in vivo* testing.

Through comparison of the *in vitro* results to those obtained following treatment of an *in vivo* animal model and available literature, the validity and potential of the 3D HepG2/C3A model as a screening tool could be confirmed.

5. Declaration of interest

The authors declare no conflict of interest.

6. Funding

This work was supported by the National Research Foundation (NRF), South Africa. Opinions expressed, and conclusions arrived at, are those of the authors and are not to be attributed to the NRF.

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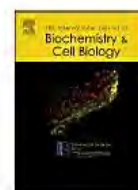
CHAPTER 6**ARTICLE PUBLISHED IN INTERNATIONAL JOURNAL OF
BIOCHEMISTRY AND CELL BIOLOGY**

A research manuscript published in the journal *International Journal of Biochemistry and Cell Biology* in 2017 (doi: 10.1016/j.biocel.2017.06.014) is presented in Chapter 6. The complete guide for authors for this journal is provided in Appendix I. This chapter differs from the rest of the thesis as it is written in U.S. English and not U.K. English. Furthermore, the research for this publication was a collaboration with the department of Biochemistry at the North-west University, and it should be noted that both J. Aucamp and C. Calitz contributed equally to this manuscript, sharing first authorship.



Contents lists available at ScienceDirect

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Cell-free DNA in a three-dimensional spheroid cell culture model: A preliminary study



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ARTICLE INFO

Keywords:

Cell-free DNA
3D cell culture
Spheroid
Capillary electrophoresis
Rotating bioreactors

ABSTRACT

Background: Investigating the biological functions of cell-free DNA (cfDNA) is limited by the interference of vast numbers of putative sources and causes of DNA release into circulation. Utilization of three-dimensional (3D) spheroid cell cultures, models with characteristics closer to the *in vivo* state, may be of significant benefit for cfDNA research.

Methods: cfDNA was isolated from the growth medium of C3A spheroid cultures in rotating bioreactors during both normal growth and treatment with acetaminophen. Spheroid growth was monitored via planimetry, lactate dehydrogenase activity and glucose consumption and was related to isolated cfDNA characteristics.

Results: Changes in spheroid growth and stability were effectively mirrored by cfDNA characteristics. cfDNA characteristics correlated with that of previous two-dimensional (2D) cell culture and human plasma research.

Conclusions: 3D spheroid cultures can serve as effective, simplified *in vivo*-simulating “closed-circuit” models since putative sources of cfDNA are limited to only the targeted cells. In addition, cfDNA can also serve as an alternative or auxiliary marker for tracking spheroid growth, development and culture stability.

Biological significance: 3D cell cultures can be used to translate “closed-circuit” *in vitro* model research into data that is relevant for *in vivo* studies and clinical applications. In turn, the utilization of cfDNA during 3D culture research can optimize sample collection without affecting the stability of the growth environment. Combining 3D culture and cfDNA research could, therefore, optimize both research fields.

1. Introduction

Since the discovery of cell-free DNA (cfDNA) in human plasma and other biological fluids (Fleischhacker and Schmidt, 2007; Peters and Pretorius, 2011), investigation of these peculiar cfDNA fragments has become a fast-growing research field due to its immense potential as a non-invasive diagnostic and prognostic marker for both disease and normal physiological conditions. Intercellular and inter-organ messaging functions have also been proposed for cfDNA in the form of newly synthesized, spontaneously released DNA/RNA-lipoprotein complexes (Aucamp et al., 2016; Gahan, 2006; Gahan and Stroun, 2010). Apart from the active release of cfDNA (Gahan and Stroun, 2010; Van der Vaart and Pretorius, 2007), practically any physiological source or process that can result in the release of DNA into biological fluids can contribute to cfDNA content (refer to Thierry et al. (2016) as an

example of the multiple putative cfDNA sources in cancer). From an *in vivo* perspective this multitude of putative cfDNA sources results in background noise and false results. This interference, in concurrence with a lack of knowledge regarding the biological functions of cfDNA, are factors that prevent the effective translation of cfDNA research into clinical practice.

The study of the biological characteristics and functions of cfDNA through the utilization of *in vitro* cell cultures has been proposed. Our previous research showed that an osteosarcoma cell culture model has a distinct pattern of apoptotic and actively released DNA levels (Bronkhorst et al., 2016). However, two-dimensional (2D) cell cultures have their own set of difficulties regarding cfDNA research, one being whether the obtained information sufficiently reflects that of *in vivo* conditions. To date it has been well acknowledged that 2D cell cultures are not representative of the cellular environment found in organisms

Abbreviations: cfDNA, cell-free DNA; 2D, two-dimensional; 3D, three-dimensional; LDH, lactate dehydrogenase; CE, capillary electrophoresis; APAP, acetaminophen

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<http://dx.doi.org/10.1016/j.biocel.2017.06.014>

Received 16 January 2017; Received in revised form 7 June 2017; Accepted 22 June 2017

Available online 24 June 2017

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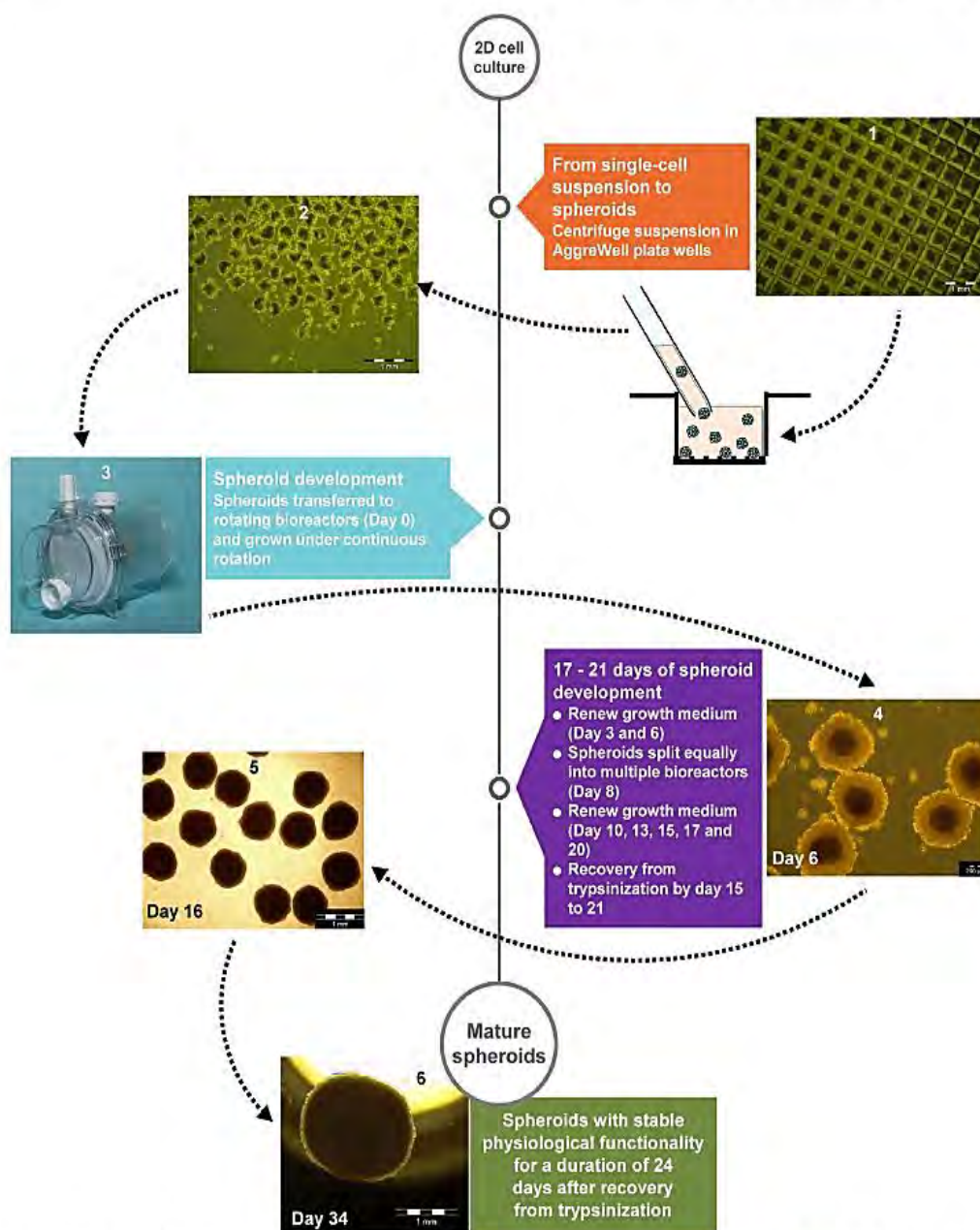


Fig 1. The development of HepG2/C3A spheroids via the rotating bioreactor technique (Wrzesinski and Fey, 2013; Wrzesinski et al., 2014). (1) AggreWell™400 with cells after centrifugation of the single cell suspension and 24 h of incubation; (2) Spheroids collected from the AggreWell™400; (3) Rotating bioreactor (MC2 Biotek) (Fey and Wrzesinski, 2012a); (4) Light microscopy of 6 day old HepG2/C3A spheroids; (5) Light microscopy of 16 day old HepG2/C3A spheroids; (6) Light microscopy of a HepG2/C3A spheroid 14 days after development (34 days old).

as they cannot effectively simulate tissue-specific architecture, mechanical and biochemical cues and cell-cell communication (Edmondson et al., 2014; Pampaloni et al., 2007). The use of living organisms, on the other hand, has its own unique scientific, ethical and social challenges (Edmondson et al., 2014). 2D cultures serve as “closed-circuit” models that promote the restriction of putative DNA sources to only that of the cell type in question. One will no longer have a “closed-circuit” model when using living models and, therefore, no control over the targeted physiological environment, resulting in background noise due to unforeseen biological influences. Moreover, the use of living organisms over *in vitro* methods also contravenes the 3Rs principle (Flecknell, 2002).

Comparison of the size profile of cfDNA from 2D cell cultures

(Bronkhorst et al., 2016) to that of plasma samples (Applied-Biosystems, 2015) showed striking similarities, as both samples contained nucleosomal fragment patterns, as well as DNA fragments with a size of 2 000 bp, which was determined to not be of apoptotic or necrotic origin (Bronkhorst et al., 2016). This observation bridges the gap between the utilization of cell cultures and biological fluids in researching the biological functions of cfDNA. To further benefit from the simple, highly flexible, morally acceptable and less invasive approach of cell cultures, while maintaining the physiologically relevant cellular behavior of living organism models, the utilization of three-dimensional (3D) cell cultures has been investigated.

1.1. Three-dimensional cell culturing

Traditional 2D cell culture models often lack tissue –specific properties found within *in vivo* systems. Cells within whole organisms (*in vivo*) form part of an intricate system having interactions with both neighbouring cells as well as the extracellular matrix (ECM). These interactions between cells and the ECM result in a complex communication network made possible by both biochemical and mechanical signals (Fey and Wrzesinski, 2012b; Lin and Chang, 2008; Page et al., 2013). The overall 3D architecture of a tissue is essential to its function. Due to the fact that 2D cell models are lacking several advanced physiological functions necessary to correlate *in vitro* conditions with those present *in vivo*, cells grown in 2D cannot be seen as equivalent to those present in intact organs (Fey and Wrzesinski, 2012b; Page et al., 2013; Wrzesinski and Fey, 2013; Wrzesinski et al., 2014). 3D cell culture models attempt to bridge the gap between cell-based experimental approaches, *in vivo* animal models and humans (Antoni et al., 2015; Fey and Wrzesinski, 2012b; Lin and Chang, 2008; Wojdyla et al., 2016; Wrzesinski and Fey, 2015).

A variety of 3D cell culture techniques are currently being explored, each offering various advantages and disadvantages. Multi-cellular 3D spheroid cell culture systems, in particular, have the possibility of overcoming the difficulties presented by both *in vivo* animal models and 2D cell culture models (Antoni et al., 2015; Breslin and O'Deiscoll, 2013; Lin and Chang, 2008). These spheroids have multi-cellular arrangements that mimic the 3D architecture of tissues, with sizable cell–cell interactions like that of tight junctions, and diffusion limits mimicking *in vivo* physiological barriers found during drug transport (Fey and Wrzesinski, 2012a, 2012b; Metha et al., 2012; Tvardovskiy et al., 2015; Wojdyla et al., 2016; Wrzesinski and Fey, 2013, 2015; Wrzesinski et al., 2013, 2014). Different multi-cellular spheroid systems are currently under investigation, including hanging drop cultures, non-adhesive surfaces, spinner flasks, NASA rotary system, micro-molding, 3D scaffolds, PNIPAAm cell sheets, primaria dishes, galactosylated substrates, pellet cultures, monoclonal growth and external force enhancement (Breslin and O'Deiscoll, 2013; Lin and Chang, 2008).

1.2. Dynamic micro-tissue spheroid cultures

The dynamic micro-tissue spheroid culturing technique utilizes rotating bioreactors that is revolutionizing mainstream *in vitro* cell culture work, providing better *in vivo* correlation than traditional 2D cell culturing models, and its application in drug toxicity studies has great potential (Fey and Wrzesinski, 2012a; Fey and Wrzesinski, 2012b; Wojdyla et al., 2016; Wrzesinski and Fey, 2015). Wrzesinski and colleagues developed, characterized and established this rotating bioreactor spheroid cell culturing system using the immortal hepatic HepG2/C3A cell line (Fey and Wrzesinski, 2012a, 2012b; Wojdyla et al., 2016; Wrzesinski and Fey, 2013, 2015; Wrzesinski et al., 2013, 2014). Cell suspensions are centrifuged in AggreWell® 400 plates (Fey and Wrzesinski, 2012b; Razian et al., 2013) to create spheroids, which are transferred to resealable bioreactors (Fey and Wrzesinski, 2012a) and continuously rotated in order to prevent the spheroids from adhering to one another and the bioreactor surface (Fig. 1). Whereas other spheroid culturing methods quickly produce spheroids via self-aggregation or scaffolding support systems, this rotating bioreactor technique involves three weeks of development within the rotating reactors, resulting in:

(1) An *in vitro* cell culture that provides physiologically relevant toxicity data

Fey and Wrzesinski (2012b) investigated the median lethal dose (LD_{50}) of six commonly used drugs (acetaminophen (APAP), amiodarone, diclofenac, metformin and valproic acid) in toxicity studies, by means of rotating bioreactors and the HepG2/C3A cell line, as well as comparing that to 2D cell culturing conditions and available *in vivo* observations. To circumvent uncertainty experienced regarding cell

numbers and population size, the spheroid data was normalized to amount of protein (μg) present within the spheroids. The latter allowed for dosages administered to spheroids to correlate with dosages administered to animal models during *in vivo* toxicity studies (mg/kg). Microscopy, planimetry and protein content were measured and it was found that the comparison of planimetric area and protein content of the spheroids demonstrated a clear correlation with a relative standard deviation of 21%. Intracellular ATP content, measured to determine cell viability for each of the drug treatments, correlated well with published *in vivo* data, with noted differences in LD_{50} values obtained in 2D cultures compared to 3D spheroid cultures, indicating the usefulness of this *in vitro* technique for determination of LD_{50} values.

(2) The recovery of cultured cells from trypsinization

Trypsinization of cells during sub-culturing is a process wherein the protease trypsin proteolytically degrades ECM proteins resulting in a single cell suspension. Trypsinization at regular intervals such as is needed for continuous 2D cell culturing will result in a disruption of advanced cellular functions, signal transduction, gene expression as well as influencing ECM repair processes and natural cell structure (Page et al., 2013; Wrzesinski and Fey, 2013). Wrzesinski and Fey (2013) found that cells recover after trypsinization in both 2D and 3D cultures. In 2D cultures this typically takes place after 5 days, however cells then again have to be trypsinized, placing 2D cells in a continuous “wound healing” cycle. For the 3D spheroids, on the other hand, this recovery of physiological functions and ultra-structural traits continues up until 15–18 days, as suggested by changes in adenylate kinase, ATP, urea, and cholesterol production. This corresponded well with published literature as several other cell lines namely: Caco-2, HT 29, MDCK, MCF-10A and HepG2, also reported similar changes between 15 and 21 days. Wrzesinski and Fey thus proposed that this is a pervasive recovery process rather than differentiation, which may explain the physiological capabilities that more closely resemble *in vivo* conditions within 3D spheroid culturing.

(3) The development of structures with tissue-like qualities and stable physiological functionality

Cells grown in 3D constructs such as spheroids at dynamic equilibrium are focused on functionality effectively mimicking the *in vivo* condition. It was determined that the HepG2/C3A 3D spheroids exhibited stable physiological functionality for a duration of 24 days after recovery from trypsinization (Wrzesinski et al., 2013). The 3D spheroid cultures provided a metabolically competent homeostatic cell model that remains within equilibrium with the culturing environment for a period of 24 days. Such a stable system permits it to be used as a means to a determine drug toxicity and mode of drug action, evaluation of biomarkers as well as the study of system biology all of which requires metabolic functions to be stable over a long term period (Antoni et al., 2015; Justice et al., 2009; Wrzesinski et al., 2013). Furthermore, it was concluded that the spheroids developed significant changes within every aspect of cellular metabolism, oxidation, transport, growth and morphology that serves as the foundation of architectural, functional and physiological differences within cells (Wrzesinski et al., 2014) (Fig. 2). The spheroids develop cholesterol, urea and ATP levels close to the physiological levels of liver tissue. A significant increase in haemoglobin levels compensates for oxygen transport throughout the spheroid and results in a reduction in protein oxidation. DNA content decreases with a concomitant increase in DNA repair enzymes, indicating increased DNA function and repair. The bioreactor culturing technique also results in a “pressurized” system that promotes the penetration of growth medium, nutrients and oxygen into spheroids (Wrzesinski and Fey, 2017), preventing the formation of the so-called necrotic core observed in other spheroid culture techniques due to a lack of access to nutrients and clearance of waste products (Alvarez-Pérez et al., 2005; Gong et al., 2015; Lin and Chang, 2008). Moreover, electron microscopy has revealed that spheroids develop microscopic structures similar to that of *in vivo* liver tissues, including bile canaliculi, sinusoid channels with microvilli and glycogen granules

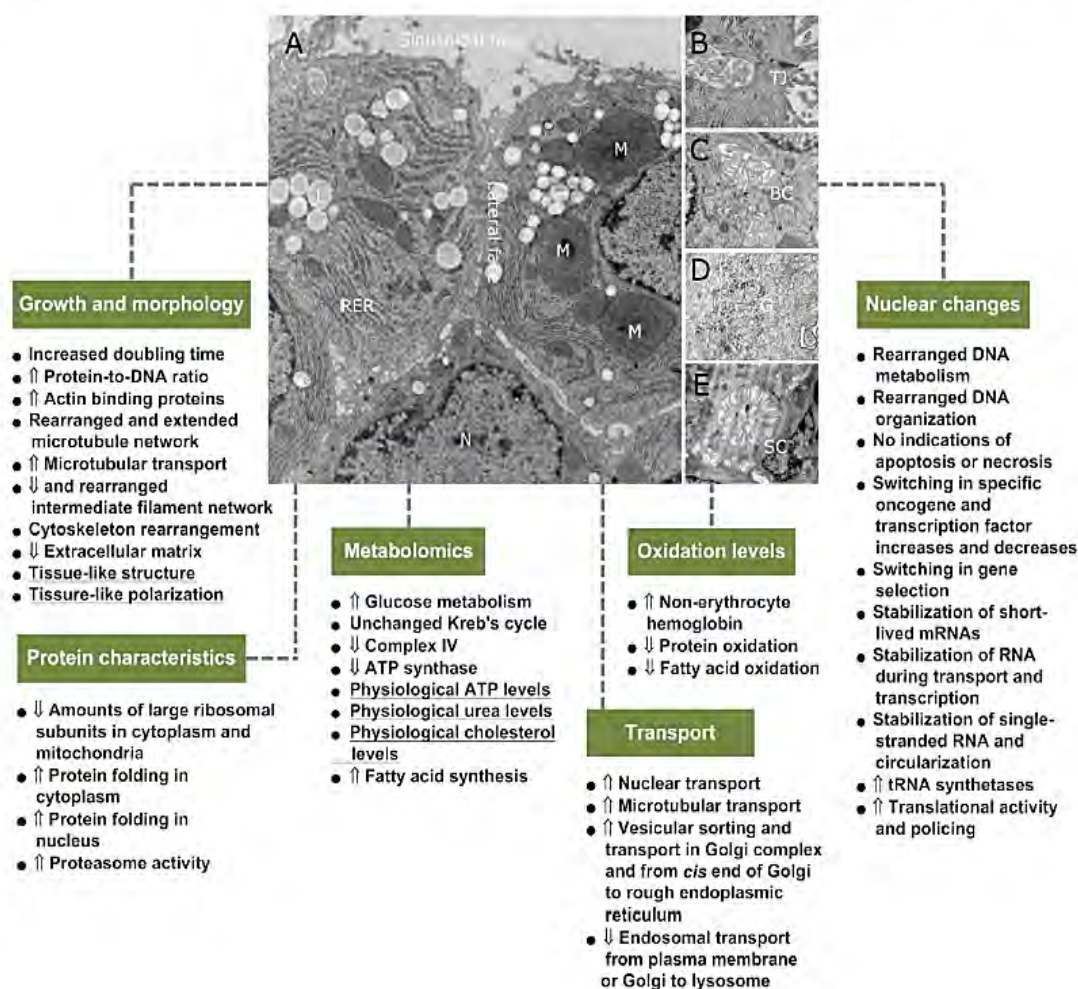


Fig. 2. Summary of the morphological and biochemical characteristics of mature HepG2/C3A spheroids (Wrzesinski and Fey, 2013; Wrzesinski et al., 2014). Electron microscopy of five 21 day old HepG2/C3A spheroids (A, B, C, D and E) with lipid droplets (L), mitochondria (M), rough endoplasmic reticulum (RER), nucleus (N), tight junctions (TJ), bile canaliculi (BC), glycogen granules (G) and sinusoidal channels (SC) (Wrzesinski and Fey, 2013).

(Wrzesinski and Fey, 2013) (Fig. 2).

2. Materials and methods

2.1. Two-dimensional cell culture conditions

Hepatocellular carcinoma derivative cells of the HepG2 cell line, HepG2/C3A (ATCC[®] CRL-10741™), were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 1 g glucose/l (Gibco) fortified with 1% non-essential amino acids (Lonza), 10% fetal bovine serum (Gibco), 1% penicillin streptomycin (Lonza), 1% L-glutamine (Lonza) and 1% amphotericin B (Biochrom). Cells were grown in 75 cm² flasks (Corning) at 37 °C and 5% CO₂ in humidified atmosphere.

2.2. Three-dimensional cell culture preparation

2.2.1. Spheroid preparation using AggreWell™400 plates

Cell spheroids were prepared using an AggreWell™400 plate (Stemcell Technologies) according to manufacturer's specifications and previously published data (Fey and Wrzesinski, 2012b; Razian et al., 2013; Wrzesinski and Fey, 2013; Wrzesinski et al., 2013; Wrzesinski et al., 2014). HepG2/C3A cells were seeded into each of the wells of the AggreWell™ plate at a seeding density of 1.2×10^6 , and centrifuged in a Sigma 3-16KL centrifuge (Sigma, Germany) for 3 min at 120 x g. Following seeding, the cells were incubated in the AggreWell™ plate

overnight to ensure aggregation.

2.2.2. Spheroid culture in bioreactors and growth medium collection

Spheroids were detached from the AggreWell™ by gently washing the wells with pre-warmed growth medium (Razian et al., 2013). Detached spheroids were then collected into Petri dishes and the spheroid quality was determined using a light microscope. Spheroids were selected based on visual quality, showing similarities in areas of compactness, as well as equal size and roundness. Selected spheroids were then placed into bioreactors (MC2 Biotek, Hørsholm) and cultivated at 37 °C, 5% CO₂, 95% air in a humidified Galaxy 170R incubator (Eppendorf Company, Stevenage, United Kingdom) for 21 days (Fey and Wrzesinski, 2012a; Fey and Wrzesinski, 2012b; Wrzesinski and Fey, 2013; Wrzesinski et al., 2013; Wrzesinski et al., 2014). The growth medium of each reactor was regularly renewed (Fig. 1) and the spent growth medium was collected for cDNA extraction at each round of medium change for 21 days during the development of the spheroids (prior to the initiation of the experiment) and thereafter from mature spheroids before each dosage of APAP. The collected medium was centrifuged (EBA 21 Hettich Zentrifugen, Tuttlingen, Germany) at 5000 × g for 5 min and the supernatant frozen at –20 °C.

2.2.3. Drug treatment and experimental group setup

APAP was purchased from Sri Krishna Pharmaceuticals. Two experimental groups were prepared in new bioreactors on day 20 by

placing 115 spheroids into each, with two biological replicates (bioreactors) prepared for each experimental group. Spheroids were treated with APAP (100 mg/kg) and growth medium (negative control) every 2 days for a period of 15 days. Dosages were based on the protein mass, spheroid size and the amount of spheroids at each specific day of treatment (Fey and Wrzesinski, 2012b). For all experimental groups, microscopy and planimetry was performed in six-fold with three technical replicates each, resulting in an average of 18 measurements. Growth medium (200 μ L) was collected every 2 days and centrifuged for 5 min at $140 \times g$. The supernatant was centrifuged at $15\,000 \times g$ for 15 min, 130 μ L supernatant was collected, snap frozen and stored at -150°C for lactate dehydrogenase (LDH) analysis. All other spent medium was collected and processed for cDNA analysis as described in Section 2.2.2.

2.2.4. Glucose quantification

Glucose consumption was measured every 2 days in spent medium using the One Touch Select™ blood glucose monitoring system (Life Scan, Sug, Switzerland) and One Touch Select™ glucose measurement strips (Life Scan, Sug, Switzerland) ($n = 6$).

2.2.5. Microscopy and planimetry

Photomicrographs of the spheroids were taken using a Nikon Eclipse TS100 light microscope (Nikon Instruments, Tokyo, Japan) and a DFK 72AUC02 USB 2.0 color industrial camera (The Imaging Source, Bremen, Germany). Images were analysed by measuring the “shadow area” of the spheroids using the ImageJ software (open-source, Java-based imaging program) to calculate the planar surface in μm^2 .

2.2.6. Lactate dehydrogenase assay

An LDH activity assay kit was purchased from Sigma-Aldrich (Sigma, Germany) and prepared according to the manufacturer's specifications in a 96 well plate (TPP Company).

2.3. Cell-free DNA extraction and quantification

cDNA was extracted with the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's PCR clean-up instructions. Samples were thawed at 37°C , vortexed and centrifuged briefly. For each biological replicate the cDNA was extracted in triplicate. Extraction samples were prepared by mixing growth medium 1:2 with binding buffer NTI. The samples were vortexed, centrifuged briefly, the entire volume added to spin columns in three 0.6 mL regiments, and centrifuged at $11\,000 \times g$ for 1 min. Wash buffer was used to clean the spin columns and the cDNA eluted into 20 μ L of elution buffer. The cDNA was quantified using the Qubit® dsDNA High Sensitivity Assay kit and Qubit® 2.0 Fluorometer (Invitrogen, Life Technologies) according to the manufacturer's instructions. The concentrations were then normalized with reference to the amount of protein present (calculated from data of established spheroid growth rate and protein concentrations during the 21 day development period), or to the planimetry data where established protein data was not available (see Method as described in Fey and Wrzesinski 2012b).

2.4. Capillary gel electrophoresis

Capillary electrophoresis (CE) was performed to analyse the size distribution of cDNA extracted at the different time intervals. The High Sensitivity DNA kit (Agilent Technologies) was used according to the manufacturer's instructions and the analysis performed with the Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA) equipped with Expert 2100 software. cDNA (0.8 ng) was separated analogously to CE, normalized to a ladder and two DNA markers, the band sizes calculated automatically by the software and the final results were represented as electropherograms. To better observe the ratios of the

nucleosomal to larger fragments of APAP-treated spheroids, the amount of DNA used during CE was doubled for the day 8–14 samples.

2.5. Statistics

Statistical analysis of the results was performed with the Tukey multiple comparisons test via GraphPad software. The results of the APAP-treated samples were statistically compared with that of the negative control.

3. Results and discussion

Tissue cells interact with one another and with the ECM through biochemical and mechanical cues and these interactions establish communication networks that maintain tissue specificity and homeostasis. Experimental 3D cell culture systems that re-establish such physiological interactions can mimic the specificity of real tissues (Pampaloni et al., 2007). 3D cell cultures can serve as simplified and flexible models in the form of cellular aggregates called spheroids, which can be produced from single and/or co-cultures via scaffolding/matrix or scaffold-free culturing methods (Wrzesinski and Fey, 2015). Spheroid cultures consist of cells in various stages (proliferating outer layers and quiescent, apoptotic, hypoxic and necrotic core cells) relating them more to *in vivo* tissues compared to 2D cultures (Edmondson et al., 2014). Their growth conditions affect cellular growth rate, spatial organization of cell surface receptors, signal transduction from the outside to the inside of cells, gene and protein expression, cell proliferation, differentiation and cellular behaviour.

For this study, a scaffold- and matrix-free 3D spheroid culture method that requires development over 21 days in rotating bioreactors is of particular interest (Wrzesinski and Fey, 2013). Wrzesinski and Fey (2013) showed that the trypsinization process required to dislodge cells from culture flasks immediately suppresses several important physiological functions. Normal 2D cell cultures could not successfully recover these functions due to required trypsinization when the cells reach confluence, while the 3D spheroids re-establish these physiological conditions after ~ 18 days. Most morphological, physiological and metabolic functions were shown to reach physiological levels and remain stable for a further 24 days. Wrzesinski and Fey (2013)'s method for 3D spheroid development, therefore, efficiently overcomes the physiological restrictions of *in vitro* cell culture methods and does not contain synthetic matrices and scaffolds to form the spheroids. The release patterns and characteristics of cDNA from these 3D cultures were elucidated during their development (first 21 days) and treatment (after 21 days) with a medicinal compound, APAP, in order to determine whether (i) 3D spheroids produce similar patterns of cDNA contents as 2D cultures and plasma samples, (ii) the cDNA yield can be improved through the use of 3D cultures and (iii) cDNA release and fragment patterns can provide useful information regarding the stability of the cultures and therapeutic and/or toxicological effects of treatments. APAP was chosen as model compound as this study formed part of a pilot hepatotoxicity study, and APAP is a well-known and characterized hepatotoxic drug.

3.1. Developing spheroids

Fig. 3A depicts the amount of cells and protein in the bioreactors during 3D spheroid development, which, after 20 days, is a third more than that which can be maintained in a standard 75 cm^2 flask. From these cultures almost microgram amounts of cDNA could be isolated from the growth medium by the end of spheroid development, compared to nanogram amounts from the growth medium of 2D cell cultures (Aucamp et al., 2017), which is of great benefit for subsequent cDNA-related research, e.g. mutation screening or sequencing. Quantification of cDNA over time (Fig. 3B) revealed patterns that correlate well with the growth conditions in the bioreactors. Initially each

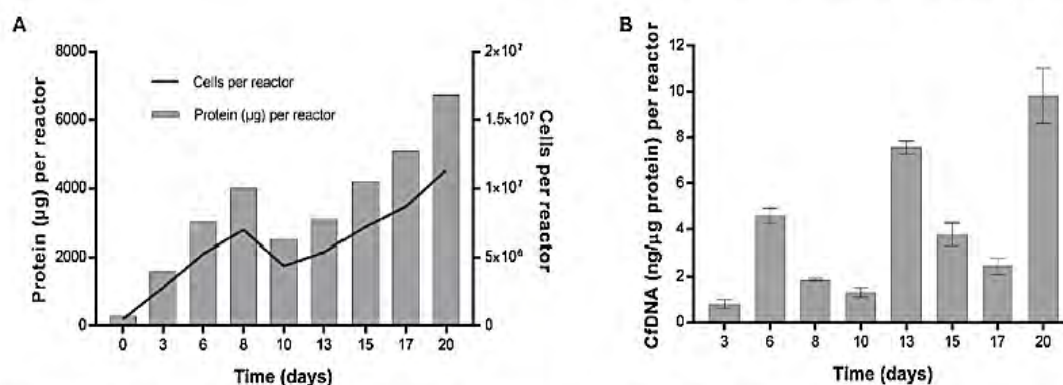


Fig. 3. 3D spheroid development over three weeks. (A) The calculated bioreactor protein and cell content of 3D bioreactors over time. (B) cDNA release per protein content per bioreactor over time \pm SD, $n = 6$ (three replicates from two bioreactors). Growth medium was exchanged every 2–3 days and the amount of spheroids per bioreactor reduced on day 8 and 20 after sample collection.

bioreactor contained 300 HepG2/C3A cell aggregates produced from a quarter of a 75 cm² flask, correlating to the low cDNA yield for the first three days. The growth medium was replaced with fresh medium every 2 days with the exception of day 6, 13 and 20, which were replaced after 3 days when an increase in cDNA yield was observed at these time intervals. On day 8 the spheroids were divided into more bioreactors with each bioreactor containing 150 spheroids, resulting in the slight reduction of cDNA levels in day 10 compared to day 8.

Electropherograms of the cDNA collected during spheroid development (Table 1) revealed fragment patterns similar to that of 2D cultures (Bronkhorst et al., 2016) and plasma samples (Applied-Biosystems, 2015). The presence of small DNA fragments (in small amounts at 42–60 bp in length), nucleosomal DNA fragment patterns (associated with apoptosis (Bronkhorst et al., 2016)) and a fragment peak at ~2000 bp (associated with actively released DNA (Bronkhorst et al., 2016)) were detected during each time interval. The ratio (percentage) between the amount of 2000 bp DNA fragments and the amount of DNA fragments from 45 to 950 bp, including nucleosomal fragments, in the cDNA samples indicates that the four nucleosomal fragment peaks are predominant in the bioreactors during spheroid development. The 2 000 bp fragment peak levels (in fluorescent units) were found to increase by day 3 with a concomitant decrease in the nucleosomal fragments. The 2000 bp fragments continued to increase up to day 8 with a concomitant increase in nucleosomal fragments, causing the nucleosomal fragment-to-actively released fragment ratio to increase. As this ratio increases, the fourth nucleosomal peak combines with the 2000 bp peak, causing the peak value to shift to 1000–2000 bp rather than 2000–4000 bp. After sample collection on day 8 in order to ensure balanced culture conditions the number of spheroids per bioreactor was reduced from 300 to 150, resulting in the slight increase of the nucleosomal fragment-to-actively released fragment ratio by day 10 due to induced stress on the spheroids during the spheroid removal process (which requires that bioreactor rotation must be suspended and the spheroids be allowed to sink to the bottom of the bioreactor to remove growth medium without removing any spheroids).

After 3 days of incubation, both the nucleosomal and larger DNA fragments slightly increased on day 13, but the nucleosomal fragment-to-actively released fragment ratio decreased. By day 15, 17 and 20 the nucleosomal fragment-to-actively released fragment ratio significantly increased as the four nucleosomal fragment peak levels increased with a concomitant decrease in the 2 000 bp peak levels. This occurrence could be due to: (1) the spheroids reaching maturity, thereby switching from proliferative cells to a functional cellular form, implying that the actively released cDNA is connected to cell proliferation and decreases due to the switch in cellular functionality; (2) Increased stress on cells due to the overcrowding of the larger spheroids in the bioreactors. For the subsequent treatment experiments spheroids were transferred to the

necessary amount of bioreactors. In this case the electropherogram data may indicate that the amount of spheroids should be reduced earlier to prevent unnecessary stress on spheroids during the last days of development. The cDNA in bioreactor growth medium may, therefore, assist in the monitoring of spheroid growth and development.

3.2. Viability assays

3.2.1. Spheroid growth and glucose consumption

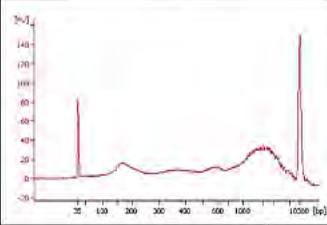
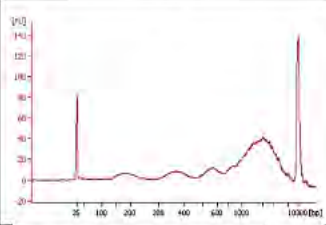
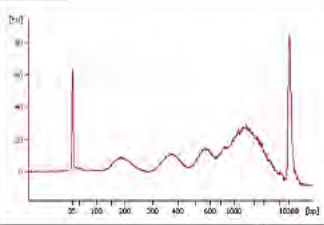
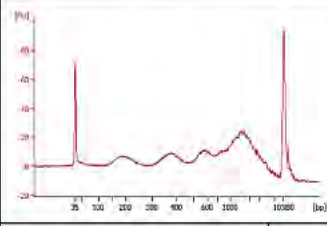
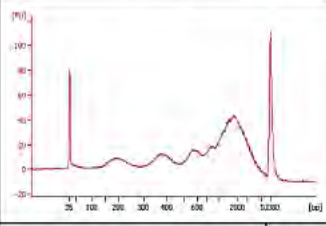
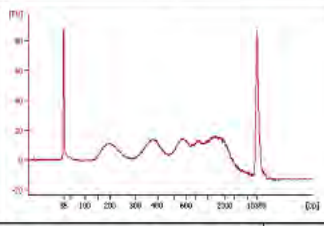
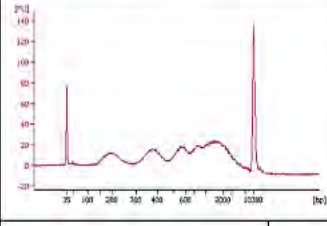
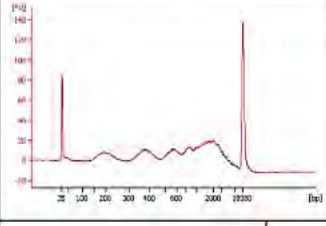
Planimetry was used to determine the average size of the spheroids (Fig. 4A) and the LDH activity used to monitor the viability of the spheroids (Fig. 4B) in each bioreactor. The glucose consumption was also used to determine cell viability or activity (Fig. 4C). The untreated (negative control) and APAP-treated spheroids showed similar high levels of growth with a slight, statistically significant decrease in spheroid growth for APAP at day 10, correlating well with decreased viability (LDH activity) and glucose consumption. The reduction in APAP-treated spheroid growth at 10–12 days resulted in a decrease in LDH activity and a concomitant increase in glucose consumption per spheroid area. Though not statistically significant, the results show a tendency towards a positive correlation between spheroid size and LDH activity (decreased viability). Bigger or older, spheroids develop a significantly slower proliferation rate, resulting in a decrease in glucose consumption in relation to total spheroid mass, thereby explaining the possible inverse correlation observed between spheroid size and glucose consumption at 10–12 days.

3.2.2. Cell-free DNA release per spheroid area

cDNA quantification in the case of the control spheroids revealed an initial increase in cDNA release at day 2, followed by a decrease and eventual stable level of further cDNA release by day 6 (Fig. 5). The increase is likely from stressed cells due to the transfer of the spheroids to multiple bioreactors. The total amount of cDNA per bioreactor increased from 2.79 to 3.89 µg during day 0–6 and decreased steadily to 3.17 µg by day 14. Electropherogram data (Table 2) showed initially high levels of both nucleosomal fragments and the longer DNA fragments with more nucleosomal fragment DNA than actively released DNA, likely due to the transfer of the spheroids to respective bioreactors. Increasing levels of these peaks from day 2–12 correlate with the amount of cDNA released per bioreactor, with the nucleosomal fragment-to-actively released fragment ratio gradually decreasing over time possibly indicating stabilization of the spheroids. By day 14 it appears that both nucleosomal fragment and actively released fragment peak levels increased with a slight nucleosomal fragment-to-actively released fragment ratio increase indicating that the increased nucleosomal fragment levels are more than that of the larger DNA fragments. This could be due to the beginning of a proliferative response to the

Table 1

Capillary electropherograms of the cfDNA released during 3D spheroid development and the percentage of the ~2000 bp actively released fragments and the other DNA fractions, including nucleosomal DNA fragments and a smaller fragment, present in 800 pg of cfDNA sample.

DAY 3		DAY 6		DAY 8	
					
42–60 bp (small fragments)	1 %	42–60 bp (small fragments)	1 %	42–60 bp (small fragments)	2 %
42–950 bp (small + nucleosomal DNA fragments)	62 %	42–883 bp (small + nucleosomal DNA fragments)	48 %	42–883 bp (small + nucleosomal DNA fragments)	60 %
950–8 900 bp (actively released DNA)	35 %	883–8 900 bp (actively released DNA)	49 %	883–8 900 bp (actively released DNA)	37 %
DAY 10		DAY 13		DAY 15	
					
42–60 bp (small fragments)	2 %	42–60 bp (small fragments)	2 %	42–60 bp (small fragments)	2 %
42–900 bp (small + nucleosomal DNA fragments)	63 %	42–910 bp (small + nucleosomal DNA fragments)	57 %	42–900 bp (small + nucleosomal DNA fragments)	73 %
900–8 900 bp (actively released DNA)	33 %	910–8 900 bp (actively released DNA)	40 %	900–8 900 bp (actively released DNA)	23 %
DAY 17		DAY 20			
					
42–60 bp (small fragments)	1 %	42–60 bp (small fragments)	2 %		
42–900 bp (small + nucleosomal DNA fragments)	67 %	42–910 bp (small + nucleosomal DNA fragments)	64 %		
900–8 900 bp (actively released DNA)	29 %	910–8 900 bp (actively released DNA)	33 %		

increased availability of nutrients due to the removal of spheroids during previous sampling.

Spheroids treated with 100 mg/kg APAP released significantly less cfDNA per bioreactor and per spheroid area compared to that of the negative control (Fig. 5) at day 0, likely due to less stressed cells due to

the transfer of the spheroids to multiple bioreactors compared to that of the negative control. However, by day 2 the amount of cfDNA per bioreactor and per spheroid area became comparable to that of the negative control. At day 10 the amount of cfDNA released increased significantly, followed by a decrease at day 12–14, to levels closer to

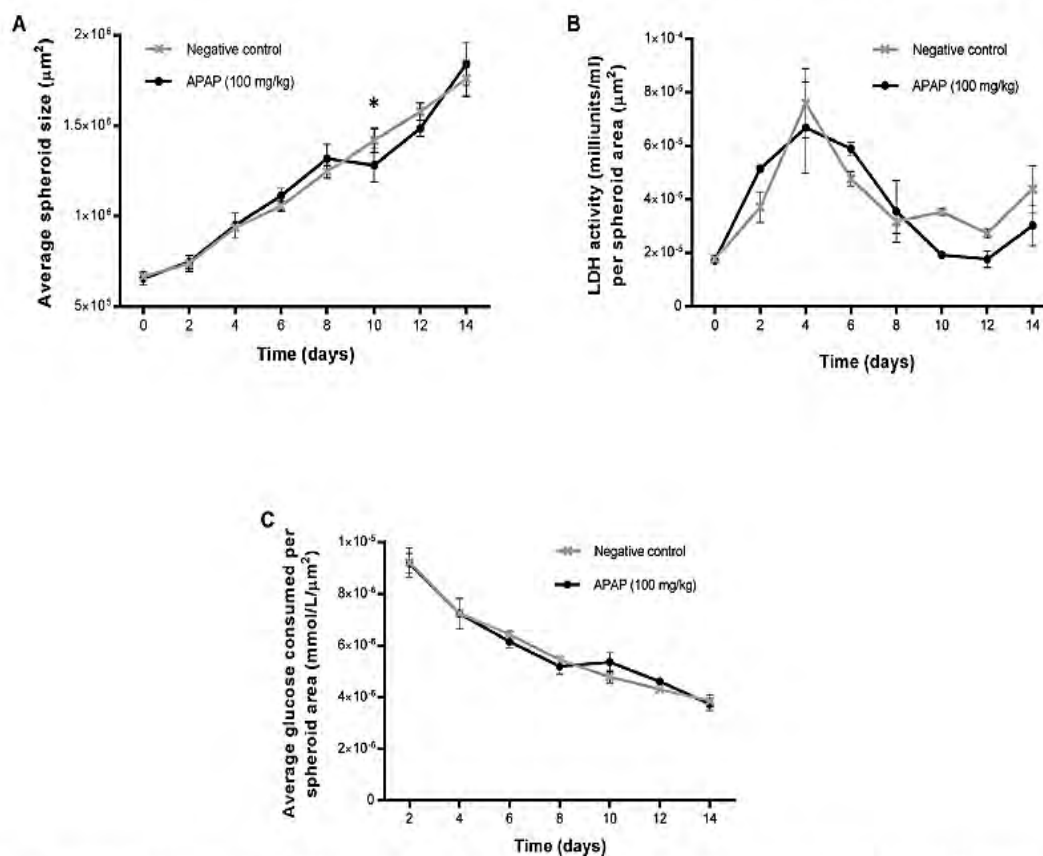


Fig. 4. Average growth rate and glucose consumption of 3D spheroids. (A) Average spheroid size \pm SD, $n = 6$ (six replicates from one bioreactor), * $p < 0.05$ versus negative control), (B) LDH activity \pm SD, $n = 2$ (two replicates from one bioreactor) and (C) glucose consumption per spheroid area \pm SD, $n = 6$ (six replicates from one bioreactor) over time. Growth medium was exchanged and 6 spheroids removed from each reactor every 2 days.

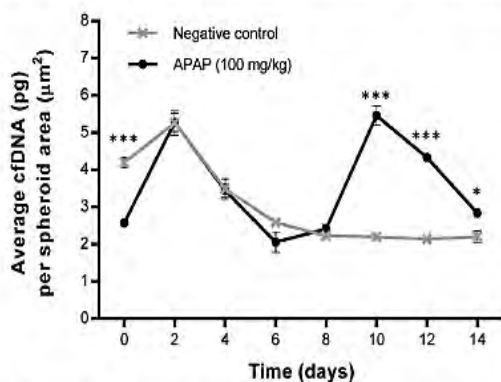


Fig. 5. Average cfDNA release per spheroid area in the presence of APAP over time (Error bars represent SD, $n = 3$) (three replicates from one bioreactors and repeated twice). Growth medium was exchanged and 6 spheroids removed from each reactor every 2 days. *** $p < 0.001$ and * $p < 0.05$ versus negative control.

that of the negative control spheroids. These changes correlate with the time at which changes were observed in spheroid growth, glucose consumption and LDH activity compared to that of the negative control (Fig. 4). Therefore, the significant changes in the amount of cfDNA from day 10 indicates that the changes observed in Fig. 4, though not statistically significant, does indeed indicate that a biological reaction occurred in response to the APAP treatment. Electropherograms (Table 3) showed an increase in nucleosomal fragment-to-actively released fragment ratio from day 0–2, followed by a gradual decrease from day 2–8 as the 2000 bp fragment peak levels gradually increased. As observed in section 3.1, an increase in this ratio results in a shift of

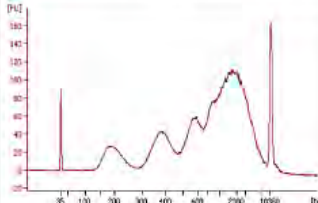
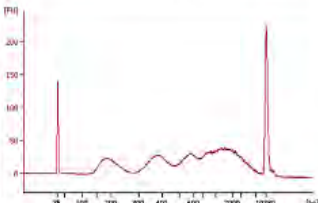
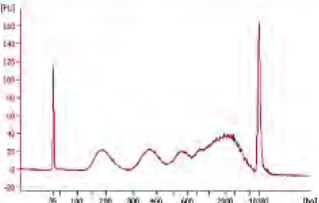
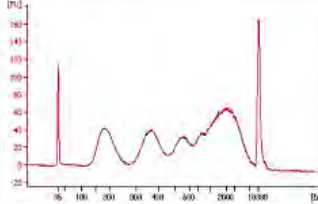
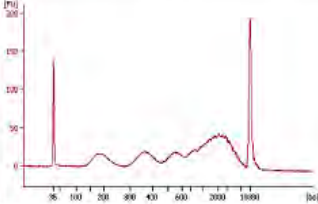
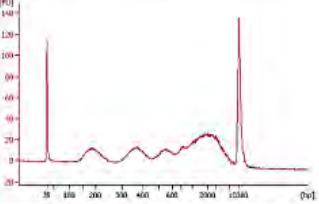
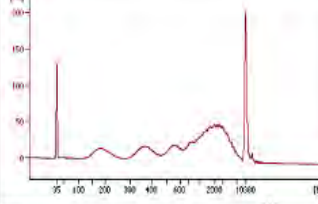
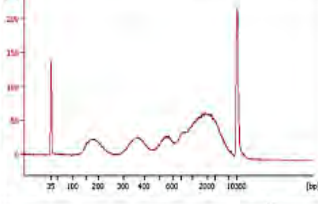
the 2000 bp peak to 1000–2 000 bp. To better observe the nucleosomal fragment-to-actively released fragment ratio, the amount of DNA used for CE was doubled for the day 8–14 samples. A significant decrease in nucleosomal fragment-to-actively released fragment ratio followed by day 10 as the 2 000 bp fragment peak levels reached a significantly high level, which exceeded the amount of nucleosomal fragment fraction in the sample by 31%. A subsequent significant increase in nucleosomal fragment-to-actively released fragment ratio from day 10–14 may indicate that the last optimal therapeutic level for 100 mg/kg APAP could have been reached at day 10 and that subsequent dosages started to induce hepatotoxicity commonly associated with APAP overdose (Kon et al., 2007; McGill et al., 2012). This may be corroborated by the initial decreased growth and increased energy consumption observed at day 10 in Fig. 4. Investigation regarding further subsequent dosages of APAP is encouraged to substantiate this.

3.2.3. Amount of nucleosomal DNA fragments in cell-free DNA samples

The fragment patterns of the spheroid cfDNA correlates with that of both 2D cell cultures (Bronkhorst et al., 2016) and human plasma samples (Applied-Biosystems, 2015), with the exception that there is a continuous presence of nucleosomal fragment DNA, proposed to represent apoptotic cfDNA (Bronkhorst et al., 2016), throughout spheroid development and in both the untreated control and treated bioreactors during the toxicological studies. The promotion of growth medium, nutrient and oxygen penetration into spheroids due to the “pressurized” system of the bioreactors (Section 1.2) negates the possibility of increased cell death due to increased spheroid size, decreased access to nutrition, respiration and waste clearance. The continuous presence of nucleosomal DNA fragments, particularly in the samples of the spheroids that completed 21 day development and were used for the

Table 2

Capillary electropherograms of cDNA from the negative control bioreactors over time and the percentage of the ~2000 bp actively released fragments and nucleosomal DNA fragments present in 800 pg of cDNA sample.

DAY 0		DAY 2		DAY 4	
					
42 – 900 bp (small + nucleosomal DNA fragments)	55 %	42 – 910 bp (small + nucleosomal DNA fragments)	68 %	42 – 900 bp (small + nucleosomal DNA fragments)	62 %
900 – 8 900 bp (actively released DNA)	44%	910 – 8 900 bp (actively released DNA)	31 %	900 – 8 900 bp (actively released DNA)	37 %
DAY 6		DAY 8		DAY 10	
					
42 – 900 bp (small + nucleosomal DNA fragments)	64 %	42 – 900 bp (small + nucleosomal DNA fragments)	58 %	42 – 900 bp (small + nucleosomal DNA fragments)	59 %
900 – 8 900 bp (actively released DNA)	35 %	900 – 8 900 bp (actively released DNA)	41 %	900 – 8 900 bp (actively released DNA)	40 %
DAY 12		DAY 14			
					
42 – 883 bp (small + nucleosomal DNA fragments)	52 %	42 – 883 bp (small + nucleosomal DNA fragments)	56 %		
883 – 8 900 bp (actively released DNA)	46 %	883 – 8 900 bp (actively released DNA)	43 %		

toxicological studies, may be a likely indication of equilibrium between cell proliferation and cell death, a homeostatic trait of *in vivo* tissue (Fulda et al., 2010). This 3D culture method can, however, still serve as an effective “closed-circuit” model despite the nucleosomal DNA fragments as the model still restricts the putative sources and causes of cDNA to that of the target cell type.

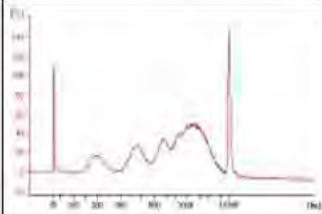
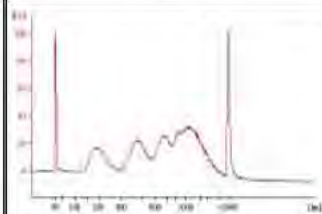
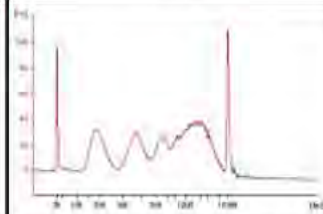
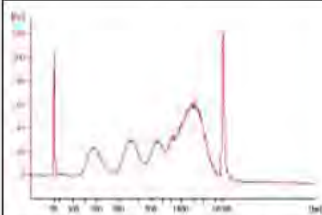
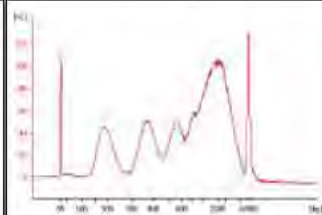
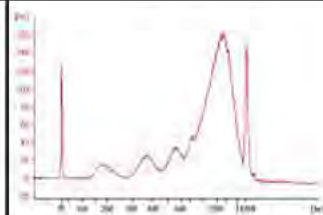
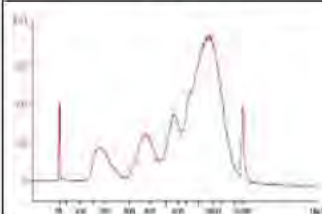
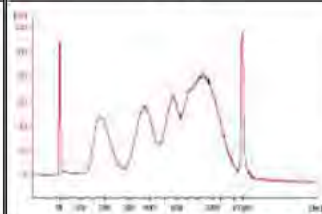
3.2.4. Utilizing cell-free DNA in three-dimensional cell culture monitoring

Two significant specificities of this 3D spheroid development method are (i) the duration of spheroid development, depending on the cell line's ability to stabilize growth under rotating conditions (in the

case of C3A's, 18 days are required), and (ii) the sensitivity of the spheroid cultures to sampling. The average 2D culture flask of 75 cm² can contain up to 1 × 10⁵ cells/cm². The 3D spheroids of a single bioreactor, on the other hand, is developed from 3 × 10⁵ individual cells with an initial doubling time similar to that of 2D cell cultures that decreases slightly as time progresses (Wrzesinski and Fey, 2013), resulting in ~1.1 × 10⁷ cells per reactor by day 20 (1.5 times higher than the yield of a 75 cm² flask). This is only the development phase of the spheroids and can, therefore, result in even larger cell numbers per bioreactor when grown for experimental purposes since spheroid cultures can remain stable for up to a further 24 days after completion of

Table 3

Capillary electropherograms of cfDNA from bioreactors treated with 100 mg/kg acetaminophen over time and the percentage of the ~2000 bp actively released fragments and nucleosomal DNA fragments present in 800 pg (day 0–6) and 1.6 ng (day 8–14) of cfDNA sample.

DAY 0		DAY 2		DAY 4	
					
42 – 850 bp (small + nucleosomal DNA fragments)	61 %	42 – 883 bp (small + nucleosomal DNA fragments)	70 %	42 – 883 bp (small + nucleosomal DNA fragments)	67 %
850 – 8 900 bp (actively released DNA)	40 %	883 – 8 900 bp (actively released DNA)	29 %	883 – 8 900 bp (actively released DNA)	32 %
DAY 6		DAY 8		DAY 10	
					
42 – 883 bp (small + nucleosomal DNA fragments)	59 %	42 – 833 bp (small + nucleosomal DNA fragments)	57 %	42 – 833 bp (small + nucleosomal DNA fragments)	34 %
883 – 8 900 bp (actively released DNA)	40 %	833 – 8 900 bp (actively released DNA)	42 %	833 – 8 900 bp (actively released DNA)	65 %
DAY 12		DAY 14			
					
42 – 833 bp (small + nucleosomal DNA fragments)	51 %	42 – 833 bp (small + nucleosomal DNA fragments)	67 %		
833 – 8 900 bp (actively released DNA)	48 %	833 – 8 900 bp (actively released DNA)	31 %		

their 21 day development. A 3D culture bioreactor can, therefore, easily surpass the amount of cells that a 2D culture flask can maintain and as the size of the spheroids become larger, the availability of nutrients among the spheroids reduces. In effect, removing an amount of spheroids from bioreactors for analytic purposes can have a large impact on the growth environment of the remaining spheroids, resulting in altered growth rate and size due to sudden availability of more nutrients. The maintenance of stable growth environments becomes difficult when spheroids have to be removed from reactors for cell growth and development monitoring and/or long term experiment sampling. Methods that can monitor cell growth, development and other processes using

growth medium instead of the cells themselves can, therefore, be a great advantage during spheroid culture experiments.

The cfDNA characteristics observed during both spheroid development and treatment effectively mirrored minor changes in growth rate and glucose consumption and correlated well with previous observations of spheroidal growth and development (Wrzesinski and Fey, 2013, 2017; Wrzesinski et al., 2013), indicating that cfDNA can serve as an effective marker to monitor spheroid development, growth rate and bioreactor environment stability. For the quantification of cfDNA in bioreactors only 1.8 mL of bioreactor growth medium was used. Due to the significantly larger amount of cells in bioreactors and longer

incubation periods compared to standard 2D cell cultures the bioreactors provide significantly larger yields of cfDNA (in micrograms), indicating that the amount of growth medium used for quantification can be significantly reduced. The replacement of spheroid-requiring monitoring methods with low volume, growth medium-requiring cfDNA quantifying methods and CE can, therefore, serve as a new and efficient approach to monitoring spheroid cultures without affecting the growth environment of the spheroids.

The cfDNA results appear to reveal a positive correlation between cfDNA release and spheroid glucose consumption, and a negative correlation between cfDNA release and spheroid growth. Further investigation is required to confirm whether these correlations are common occurrences. Furthermore, the data provided more insight regarding the effect of the length of time between doses of compounds for toxicology testing, as there was little evidence of increased apoptosis and necrosis indicative of increased toxicity. This implies that (i) the samples were collected after the spheroids recovered from the toxic effects of the compounds and (ii) that the dosage interval may, therefore, be too long to be considered as a therapeutically representative dosage regimen. This regimen, however, is normally effective for 2D cell cultures and, therefore, illustrates the adaptation of these 3D cultures to more physiologically relevant conditions.

4. Conclusions

In this study it was determined that cfDNA release and fragment patterns mirror the brief and/or minor changes in the growth and glucose consumption of spheroids developed in rotating bioreactors during both the development phase and toxicological studies. The fragment patterns of the spheroid cfDNA correlates with that of both 2D cell cultures (Bronkhorst et al., 2016) and human plasma samples (Applied-Biosystems, 2015). The microgram yields of cfDNA compared to that of 2D cell cultures is highly beneficial for subsequent studies of the origin, purpose and biological functions of the cfDNA. Moreover, cfDNA data obtained from 3D cultures will likely be more relateable to *in vivo* conditions due to their re-establishment of tissue cell communication networks that maintain tissue specificity and homeostasis. This study, therefore proposes (i) the utilization of cfDNA in 3D spheroid-utilizing studies as a marker for 3D spheroid growth and development to spare precious spheroids and maintain a stable physiological environment and (ii) the utilization of Wrzesinski and Fey (2013)'s method of spheroid development in rotating bioreactors as a "closed-circuit" model for cfDNA research to study the biological functions and origins of cfDNA as replacement for or for use in conjunction with *in vivo* models in order to aid the discovery of novel biomarkers for clinical non-invasive diagnostic, prognostic and theranostic applications.

Conflict of interest

Authors declare no conflict of interest.

Acknowledgements

This work was supported by the National Research Foundation (NRF), South Africa [SFH14061869958, RCA13091339132]. The financial assistance of the NRF is hereby acknowledged. Opinions expressed and conclusions arrived at, are those of the authors and are not to be attributed to the NRF.

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CHAPTER 7**MANUSCRIPT SUBMITTED TO THE JOURNAL OF CELL BIOLOGY**

Chapter 7 is presented in the form of a methodology manuscript submitted to the journal *Journal of Cell Biology* in 2017. The complete guide for authors for this journal is provided in Appendix J. This chapter differs from the rest of the thesis as it is written in U.S. English and not U.K. English.

Novel sodium alginate encapsulated LS180 cell spheroids cultured in rotating bioreactors

Abbreviated title: Novel sodium alginate encapsulated LS180 cell spheroids

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Abstract

Current models to assess drug biotransformation cannot always accurately predict *in vivo* biotransformation and elimination. Three-dimensional cell culturing techniques attempt to overcome these obstacles by providing a cellular environment closer to the *in vivo* state, which increases predictability of drug efficacy and toxicity prior to the initiation of clinical trials. Spheroid cultures are seen as one of the most recognized three-dimensional culturing techniques have been investigated since the early 1980's for tissue culturing. Some cell lines need biomaterials that mimic the extracellular matrix in order to efficiently form spheroids. This manuscript presents a novel application of sodium alginate micro-encapsulation to create a three-dimensional cell culture model based on LS180 cells, cultured in dynamic rotating micro-gravity bioreactors. The parameters established to be most ideal for creating sodium alginate encapsulated LS180 cell spheroids, were a seeding density of 1,500 cells per 1 µl Sodium Alginate (2% w/v), exposed to 0,5 µl cross-linker for 5 min. These encapsulated LS180 spheroids have the potential to be employed as a novel long-term culture method.

Key words: Hydrogels, LS180, rotating bioreactors, spheroids, sodium alginate, three-dimensional cell culture.

1. Introduction

Withdrawal of candidates from drug development programs have been estimated at 39% due to deficiencies in pharmacokinetics (i.e. absorption, distribution, biotransformation and excretion), with a further 21% due to toxicity indirectly attributed to disparities in biotransformation (Tingle & Helsby, 2006). Current pre-clinical models used in the assessment of drug biotransformation cannot always accurately predict *in vivo* biotransformation and elimination (Brandon *et al.*, 2006; Fang & Eglen, 2017). Since the early 1950's, cells cultured either in suspension or on solid flat surfaces as two-dimensional monolayers (2D) have been used in drug discovery. Although 2D cell culture models are convenient, efficient and have contributed to the establishment of a myriad of drug candidates, these systems have certain disadvantages due to deficient cellular communication as compared to cells *in vivo*, as well as limitations in terms of the time period over which they can be grown (Antoni *et al.*, 2015; Fang & Eglen, 2017). Novel three-dimensional (3D) cell culturing techniques attempt to overcome these obstacles by providing a cellular environment more closely related to the *in vivo* state, which will be able to increase predictability of drug efficacy and toxicity prior to the initiation of clinical trials (Antoni *et al.*, 2015; Wrzesinski & Fey, 2015; Fang & Eglen, 2017).

Chemical entities entering the body undergo biotransformation to produce more polar water-soluble molecules. This is accomplished by phase I and II enzymatic systems mainly within the liver and epithelial cells of the gastrointestinal tract (Liska, 1998; Paine *et al.*, 2006; Tingle & Helsby, 2006). Phase I reactions involve oxidation, reduction and hydrolysis, converting most non-polar drugs to reactive oxygen intermediates that are further biotransformed by phase II conjugation reactions to more polar compounds with increased water solubility (Liska, 1998; Shargel *et al.*, 2005). The phase I system is governed by the mixed-function oxidase (MFO) enzyme system with the cytochrome P450 iso-enzyme family (CYP) central to this system (Sheweita, 2000; Shargel *et al.*, 2005), which accounts for 30% of hepatic and 70% of intestinal bio-transformation activities (Pal & Mitra., 2006; Hellum & Nilsen 2007). CYP3A is the most abundant CYP subfamily found within the intestinal epithelium enterocytes, comprising of CYP3A3, 3A4, 3A5, 3A7 and 3A43 (Paine *et al.*, 2006; Thelen & Dressman, 2009). The majority of orally administered drugs undergoes biotransformation by CYP3A (50%), CYP2D6 (25%) and CYP2C (20%) (Brandon *et al.*, 2006).

The human colon carcinoma cell line, LS180, is a micro-villus expressing cell line often used as biotransformation model to study inhibition and induction by drug candidates *in vitro*

(Brandon *et al.*, 2006; Harmsen *et al.*, 2008). It expresses PXR, CYP3A4 and CYP2B6 with no polymorphisms present and these enzymes are inducible with rifampicin and 1 α ,25-dihydroxyvitamin D₃, respectively (Brandon *et al.*, 2006; Gupta *et al.*, 2008). Furthermore, in a study by Harmsen *et al.* (2008), it was stated that of immortalized human cell lines used in biotransformation studies, the traditionally used hepatic HepG2 cell line was significantly outperformed and found inferior to the LS180 cell line as a model to study CYP3A4 induction. Gupta *et al.* (2008) also found that the LS180 cell line is a valuable model to study inhibition of CYP3A4. For these reasons it is believed that the LS180 cell line is an ideal candidate to serve as a 3D *in vitro* biotransformation model.

In some 3D cell culture techniques cells are grown as aggregates, called spheroids, in either dynamic or static systems. This includes self-aggregation systems, liquid overlay systems, spinner flasks and rotating micro-gravity bioreactors, hanging drop systems, using microcarrier beads, scaffolds, collagen, hydrogel and Matrigel™ supports (Lin & Chang, 2008; Breslin & O'Driscoll, 2013; Page *et al.*, 2013; Soldatow *et al.*, 2013, Wrzesinski & Fey, 2015). Each of these systems has advantages and drawbacks that should be assessed individually before deciding on an appropriate method for a specific application. The dynamic rotating micro-gravity bioreactors developed by Wrzesinski and Fey (2012a) provide a simple method capable of mass production of relatively large cell spheroids, with better cell differentiation and recovery to levels of physiologically (*in vivo*) relevant biochemical characteristics. These spheroids are capable of data generation that correlates well with *in vivo* data. This 3D cell culturing method requires specialized equipment, but nonetheless offers dynamic control of culturing conditions with little batch to batch variation (Fey & Wrzesinski 2012a; Wrzesinski *et al.*, 2014; Tvardovskiy *et al.*, 2015, Wojdyla *et al.*, 2015, Aucamp *et al.*, 2017).

Biomaterials able to mimic the extracellular matrix have been investigated since the early 1980's. Hydrogels such as the anionic polymer alginate, which is obtained from brown seaweed, can mimic the extracellular matrix of body tissues fairly well as it is biocompatible, with relatively high diffusion rates of macromolecules, has a low toxicity, is cost effective and biodegradable under normal physiological conditions (Gombotz & Wee, 2012; Lee & Mooney, 2012). Biomedical applications of alginate range from wound healing to delivery of bioactive agents, cell transplantation as well as tissue engineering and three-dimensional cell culturing (Murua *et al.*, 2008; Lee & Mooney, 2012). The aim of this study was to create a new three-dimensional cell culture model based on LS180 cells, by combining the dynamic rotating micro-gravity bioreactor technique with the micro-encapsulation technique using sodium alginate.

2. Materials and Methods

2.1 Materials

Sodium alginate, Poly-L-Lysine and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma (Johannesburg, South Africa), calcium chloride dihydrate ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$) and sodium chloride (NaCl) from UnivAR (SAARCHM, Johannesburg, South Africa). Phosphate buffered saline (PBS) was purchased from Whitehead Scientific (Cape Town, South Africa).

2.2 Culturing LS180 cells

The human colorectal adenocarcinoma cell line, LS180 (American Type Culture Collection cat. no. CL-187™, ATCC, Manassas, VA), was cultured using standard tissue culture conditions in high glucose Dulbecco's Modified Eagle's Medium (DMEM) (5.5g glucose/l) (Hyclone, Merck, Johannesburg, South Africa). Medium was supplemented with 1% non-essential amino acids (Lonza, Whitehead Scientific, Cape Town, South Africa); 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, Johannesburg, South Africa); 1% penicillin/streptomycin (Lonza, Whitehead Scientific, Cape Town, South Africa), 1% Glutamine (Lonza, Whitehead Scientific, Cape Town, South Africa) and 1% Amphotericin B (Biochrom, Randburg, South Africa). Cultures were incubated in a humidified atmosphere at 37°C, 5% CO_2 and 95% humidified air in a Thermo Scientific CO_2 incubator, with growth medium exchanged every second day. Upon reaching 60% confluency, the cells were sub-cultured by scraping the cells from culturing flasks.

2.3 Bioreactor setup

Bioreactors were prepared by filling the water chambers (Figure 1C) with distilled water and adding approximately 8 ml growth medium to the cell chamber (Figure 1B). The bioreactors were placed onto a drive-unit (BAM v 4.6 CelVivo, Denmark) in an incubator to rotate and equilibrate overnight at 37°C and 5% CO_2 . Prior to the transfer of the spheroids to the cell chamber of the bioreactor, all growth medium was removed, and 10 mL fresh medium was added.

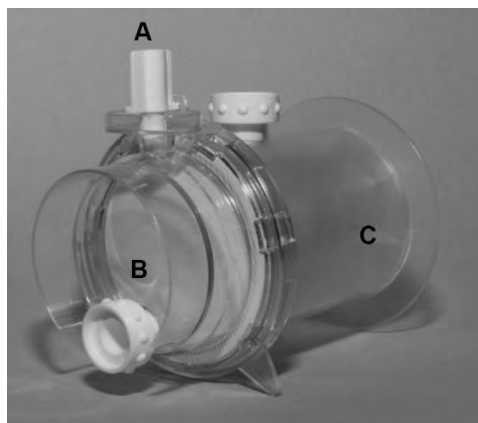


Figure 1: Photograph of a bioreactor developed by Wrzesinski and Fey (Fey & Wrzesinski, 2012b, USA PA 61/423,145, 2010) used to grow the LS180 spheroids, showing the top opening (A), cell chamber (B) and water chamber (C).

2.4 Spheroid formation using AggreWell™ plates

2.4.1 Seeding cells into AggreWell™ 400 plates

LS180 cells were seeded onto AggreWell™ 400 plates (Stemcel Technologies, Canada), at seeding densities of 1.2×10^3 , 1.6×10^3 , 1.8×10^3 , 1.4×10^4 , 1.6×10^4 and 1.8×10^4 cells per well. A cell suspension of 70% confluent LS180 cells was obtained by means of scraping. Upon complete detachment of the cells the cell suspension was treated with 3 ml FBS and 5 ml warm growth medium, the cell suspension was agitated extensively with a pipette to ensure complete detachment and de-agglomeration of the cells to form a suspension consisting of single cells. Cells in the suspension were counted by means of a hemocytometer and Trypan blue (Sigma Aldrich, Johannesburg, South Africa) staining. The AggreWell™ 400 plate was rinsed once with 1 ml pre-warmed growth medium. The cell suspension was subsequently diluted and seeded onto the AggreWell™ 400 plate, and the volume was adjusted with pre-warmed growth medium to a final volume of 2 ml. The plate was then centrifuged at $120 \times g$ for 3 min. After centrifugation, the wells were inspected by means of light microscopy to ensure even distribution of the cells in the wells. The plate was placed in the incubator at 37°C and 5% CO_2 for a period of 24 hours. After 24 hours, spheroids were removed from the AggreWell™ 400 plate using a pipette (Fey & Wrzesinski 2012b, Wojdyla *et al.*, 2015, Aucamp *et al.*, 2017). Spheroids were collected within a Petri dish and inspected by means of light microscopy.

2.4.2 Coating spheroids seeded in AggreWell™ 400 plates

LS180 cells were seeded on AggreWell™ 400 plates at a seeding density of 1.2×10^3 cells per well. After removing the spheroids from the AggreWell™ 400 plates, the spheroids were “coated” with one of the following: fetal bovine calf serum at 50% v/v for 5 min or 100% v/v for 5 min; EDTA at 2.5 mM, 5 mM and 10 mM for 5 min; and Poly-L-lysine at 0.01% v/v for 5 min, 0.007% v/v for 10 min, 0.004% v/v for 20 min and 0.0005% v/v for 30 min.

2.4.3 Seeding cells into AggreWell™ 800 plates and coating

LS180 cells were seeded in the AggreWell™ 800 plate as described in section 4.3.1. Cells were seeded densities of 8.0×10^3 , 1.2×10^4 and 1.6×10^4 , and the centrifugal force was increased to $240 \times g$ for 6 min. Cells were also seeded with 30% v/v 5 mM EDTA, and formed spheroids were alternatively also “coated” with 5 mM EDTA for 10 min after removal from the AggreWell™ 800 plate.

2.5 Spheroid formation using sodium alginate encapsulation

2.5.1 Preparation of sodium alginate and cross-linker

Sodium alginate solution of 0.6%, 0.7%, 0.8%, 1.0%, 1.5%, 2% and 5% w/v, respectively, were prepared in phosphate buffered saline and autoclaved after preparation. The cross-linker solutions, which consisted of 50 mM CaCl_2 and 150 mM NaCl, were prepared in distilled water and filtered with a 0.8/0.2 μm Acrodisc® PF Supor® membrane syringe filter (PALL, Midrand, South Africa) to sterilize the solution.

2.5.2 Preparation of LS180 cell suspension for spheroid formation

A cell suspension was prepared from 80% confluent LS180 cells through trypsination. This was done, since scraping the cells made it difficult to obtain a single cell suspension. Cells were only trypsinized once before seeding. Cells were washed twice with 10 ml PBS followed by the addition of 3 ml Trypsin-EDTA (Lonza, Whitehead Scientific, Cape Town, South Africa) for 5 min at 37°C. Following trypsination, the cell suspension was treated with 3 ml FBS and 5 ml warm growth medium. The cell suspension was centrifuged for 5 min at $140 \times g$ (EBA 21 Hettich centrifuge, Tuttlingen, Germany) to remove the trypsin-containing media. Growth medium (5 ml) was added and the pellet was re-suspended. The cell suspension was then counted using a Sceptre cell counter (Merck Millipore, Johannesburg, South Africa) and 60 μm sensor tips (Merck Millipore, Johannesburg, South Africa). The cell suspension was subsequently diluted to densities of 200, 400, 600, 800, 1,200, 1,500 and 2,000 cells per 1 μl . Following dilution, the cell suspension was again centrifuged at $140 \times g$ for 5 min, the supernatant was removed and the pellet gently re-suspended in 1 ml of the prepared sodium alginate solutions at 37°C.

2.5.3 Preparation of sodium alginate encapsulated spheroids

The LS180 cells, imbedded in the sodium alginate solution, were pipetted as 1 μ l droplets onto square blocks covered with hydrophobic paraffin film, as illustrated in Figure 2. After a set amount was pipetted onto the blocks, a volume of either 0.5 μ l or 1 μ l of the 50 mM CaCl_2 , 150 mM NaCl cross-linker solution was added to each droplet. The petri dish was then covered and incubated at 37°C for 5, 10 or 20 minutes. Spheroids were collected into growth medium after incubation and placed in the bioreactors. The speed of each bioreactor was initially set between 14 and 16 rotations per minute (rpm).

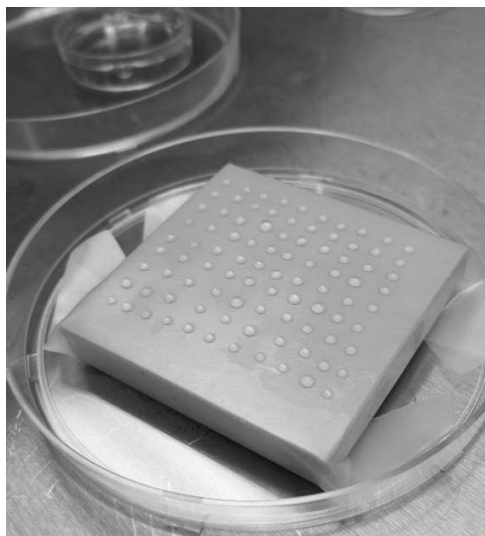


Figure 2: LS180 cells encapsulated in 2% w/v sodium alginate solution on a square block, treated with 50 mM CaCl_2 and 150 mM NaCl cross-linker solution prior to transfer to the bioreactor.

2.6 Encapsulated LS180 cell spheroid maintenance

The day the spheroids were transferred to the bioreactors, is referred to as day 1. The growth medium was exchanged after three days of culturing during the first week, followed by medium exchange every second day thereafter. Upon growth medium exchange, the bioreactor was removed from the drive unit and the spheroids were allowed to settle at the bottom of the bioreactor and the plug at the top of the cell chamber (see Figure 1A) was removed. Approximately 90% of the spent growth medium was removed by using a 10 ml syringe and 21'G needle. Growth medium was slowly replaced, taking care not to disrupt the spheroids, after which the plug at the top of the cell chamber was replaced. The plug cup was washed with 70% v/v ethanol and the bioreactor was placed back onto the drive unit in the incubator. Rotation speed was adjusted daily to keep the spheroids suspended in essentially a 'stationary orbit' (i.e. to prevent the spheroids from settling down). Photomicrographs of the developing spheroids were taken on day 1, 7, 13, 21 and 28 using a

Nikon Eclipse TS100 light microscope (Nikon Instruments, Tokyo, Japan) and a DFK 72AUC02 USB 2.0 color industrial camera (The Imaging Source, Bremen, Germany).

3 Results and Discussion

The initial method called for the culture of LS180 cells in spheroids by means of only the dynamic rotating micro-gravity bioreactors developed by Wrzesinski and Fey (2012a). Various seeding densities of the LS180 cells were seeded on AggreWell™ 400 plates, ranging from 1.2×10^3 to 1.8×10^4 cells per well using methods previously published (Fey & Wrzesinski 2012a; Tvardovskiy *et al.*, 2015, Wojdyla *et al.*, 2015, Aucamp *et al.*, 2017). A higher seeding density produced more compact and rounded spheroids than those from a lower seeding density, however, all spheroids produced in this manner seemed to clump into one big spheroid, or disintegrate into single cells within minutes of being placed within the bioreactors.

Based upon these findings, it was attempted to alter the polarity of the outermost cells by “coating” the spheroids after formation in the AggreWell™ 400 plates. Various solutions to achieve this can be found in the literature: fetal bovine calf serum, ethylenediaminetetraacetic acid (EDTA) and Poly-L-lysine (Mazia *et al.*, 1975; Yavin & Yavin, 1974; Lin & Sun, 1980; Khademhosseini *et al.*, 2004; Gupta *et al.*, 2008). By exposing the spheroids to 5 mM EDTA, the spheroids “clumping” only started at a much later stage compared to coating with all other solutions. The results suggested the combination of “coating” of the spheroids with 5 mM EDTA, an increased seeding density, centrifugation time and force at which the AggreWell™ 400 plate is centrifuged was a distinct improvement in the method to form viable spheroids.

The cells were also seeded in the AggreWell™ 800 plate, as this produces bigger spheroids. LS180 cells were seeded at densities of 8.0×10^3 , 1.2×10^4 and 1.6×10^4 , and the centrifugal force was increased to $240 \times g$ for 6 min. LS180 cells were also seeded with 30% v/v 5 mM EDTA, and formed spheroids were incubated with 5 mM EDTA after removal from the AggreWell™ 800 plate for 10 min to “coat” them.

The results indicated that spheroids formed from a higher seeding density and centrifuged at higher centrifugal force for longer, produced more rounded, compact and dense spheroids. By coating the formed spheroids, the time before clumping started was still further extended. When forming spheroids in the AggreWell™ 800 plate exposed to 5 mM EDTA, spheroids formed beautifully, but disintegrated into a single cell suspension upon removal them from the AggreWell™ 800 plate.

Hydrogels as a means to form spheroids prior to placement of the formed spheroids into the rotating bioreactors was then considered. Sodium alginate concentrations between 0.6 and 1.5% w/v produced encapsulated spheroids, but the gel was not firm enough and the spheroids were damaged during transfer to the bioreactors. LS180 cell seeding densities ranging between 200 and 1,200 cells per 1 μ l, produced spheroids with severely dispersed cells and low cellular density (as seen in Figure 3), while at densities between 1 500 and 2 000 cells per 1 μ l cells were more evenly distributed with an increased cellular density (as shown in Figure 4A). It was also determined that 0,5 μ l cross-linker, allowed to polymerize for 5 min, was optimal as 10 min or longer caused the gel to dry out.

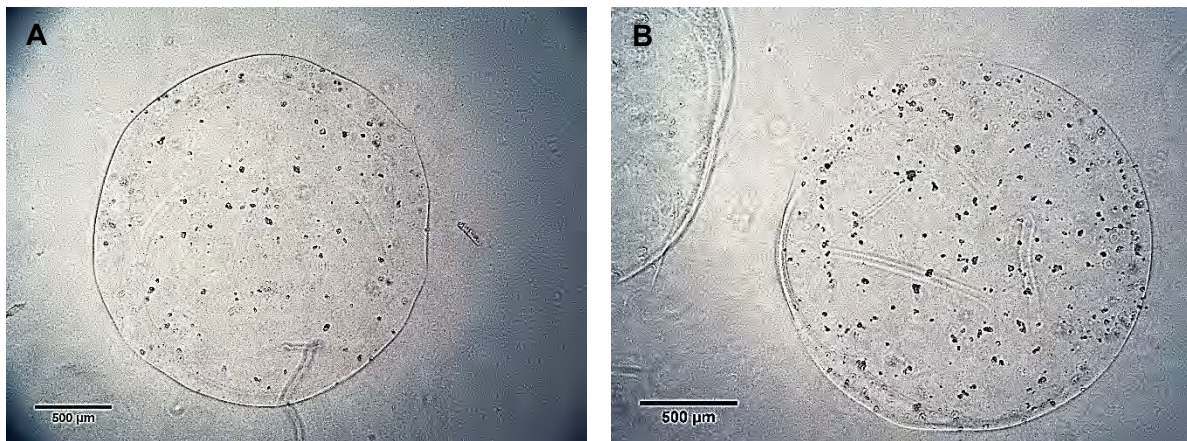


Figure 3: Photomicrographs illustrating LS180 spheroids directly after encapsulation at different seeding densities, namely 200 cells per 1 μ l (A); and 800 cells per 1 μ l (B) (4x magnification and 22°C).

Figure 4 shows photomicrographs of developing LS180 spheroids encapsulated in 2% w/v sodium alginate directly after seeding on day 1 with 1,500 cells/ μ L (A), day 7 (B), day 13 (C) and day 28 (D) after transfer to the bioreactors.

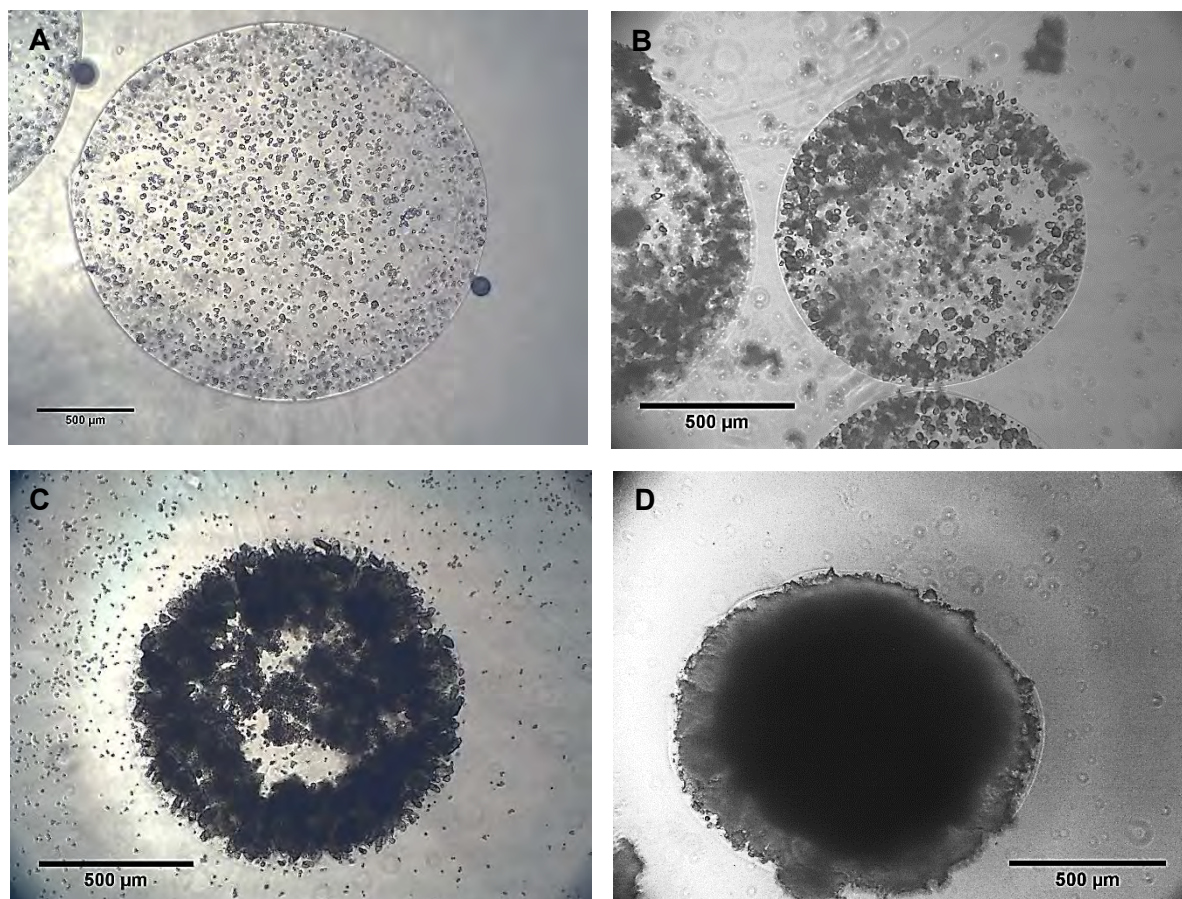


Figure 4: Photomicrographs illustrating LS180 spheroid development at different time points, namely on day 1 directly after encapsulation (A), day 7 after being transferred to rotating bioreactors (B), day 13 (C) and day 28 (D) (4x magnification and 22°C).

The encapsulated spheroids developed slowly for the first 10 days after transfer to the rotating bioreactors. Thereafter, they developed at a quicker pace and the rotation speed had to be adjusted frequently to compensate for the increasing spheroid weight as shown in Table 1. After 21 days' incubation in the rotating bioreactors, the spheroids appeared compact, optically dense and the rotation speed needed minimal adjustment suggesting slowed growth. In Figure 5A, 21 day old LS180 cell spheroids can be seen in a bioreactor, and Figure 5B illustrates the spheroids within a petri dish prior to imaging with a microscope. Macroscopically, the encapsulated LS180 spheroids appeared compact, dense, well rounded (spherical) with a relatively even edge and of equal size. The rotation tempos presented in Table 1 also illustrates the fairly reproducible size of the spheroids, as the speeds of the replicate rotating bioreactors were very consistent.

Table 1: Rotation speeds for two replicate bioreactors containing the encapsulated LS180 spheroids, as adjusted over time.

Day	Rotations per minute in Bioreactor 1	Rotations per minute in Bioreactor 2	Comments
0	15	15	Spheroids placed in rotating bioreactors.
1	15	15	Removed bubbles formed in bioreactors.
2	15	15	N/A
3	15,3	15,2	N/A
4	15,4	15,4	Culture medium exchanged, bubbles removed from bioreactors.
5	15,7	15,8	N/A
6	15,8	15,8	N/A
7	16,0	16,0	Culture medium exchanged, bubbles removed from bioreactors, photomicrographs taken.
8	16,2	16,2	Spheroids becoming more visible.
9	16,8	16,8	Culture medium exchanged, bubbles removed from bioreactors.
10	17,7	17,5	Spheroids visibly more dense.
11	18,8	18,8	Culture medium exchanged, bubbles removed from bioreactors.
12	19,0	19,0	N/A
13	21,0	21,0	Culture medium exchanged, bubbles removed from bioreactors, photomicrographs taken.
14	23,0	23,0	N/A
15	23,0	23,0	N/A
16	23,3	23,3	N/A
17	23,8	23,8	Culture medium exchanged, bubbles removed from bioreactors, uneven spheroids removed.
18	24,0	24,0	N/A
19	24,5	24,5	Culture medium exchanged, bubbles removed from bioreactors.
20	24,5	24,5	N/A

21	25	25	Culture medium exchanged, bubbles removed from bioreactors, photomicrographs taken, spheroids clearly visible, dense and compact.
22	25	25	N/A
23	25,6	25,4	N/A
24	25,6	25,5	N/A
25	26,2	26,0	Culture medium exchanged, bubbles removed from bioreactors.
26	26,0	26,0	N/A
27	26,0	26,0	N/A
28	26,0	26,0	Photomicrographs taken.

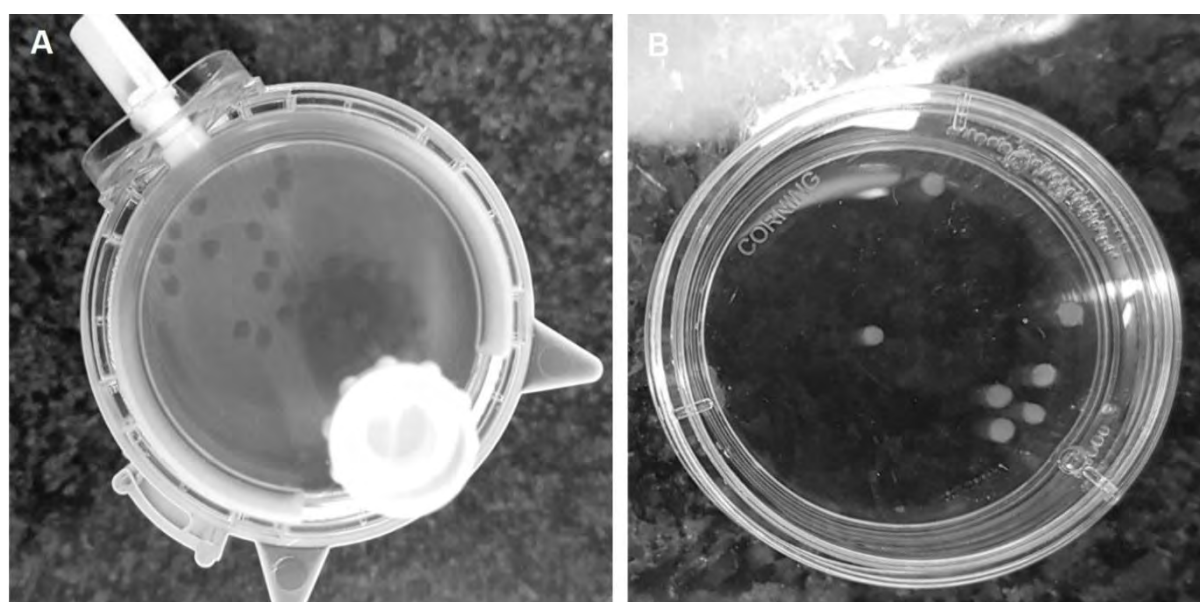


Figure 5: Photographs of 21 day old LS180 cell spheroids, encapsulated in 2% w/v sodium alginate and cross-linker solution in a rotating bioreactor (A), and within a petri dish prior to imaging (B).

4 Conclusion

Following the methods applied and various parameters adjusted, the method employing the following parameters were most ideal for creating sodium alginate encapsulated cell spheroids, namely 2% w/v sodium alginate, a seeding density of 1,500 cells per 1 μ l and exposure to 0.5 μ l cross-linker for 5 min. This method allows us to construct LS180 encapsulated spheroids with the potential to be employed as a novel long term cultured cell

method. This model has to be fully characterized to determine viability, CYP450 enzyme expression and correlation to the *in vivo* situation. This will allow the model to be used as an *in vitro* pre-clinical biotransformation model for the elucidation of drug metabolism, herb-drug and drug-drug interactions and to study the induction and/or inhibition of the cytochrome P450 enzyme family.

Acknowledgements

This work was supported by the National Research Foundation (NRF) of South Africa [Grant number 91460]; the South African Medical Research Council (MRC) [Self-Initiated Research grant] and DrugMode A/S and MC2 Biotek (Denmark). Opinions expressed, and conclusions arrived at, are those of the author and are not necessarily to be attributed to the NRF or the MRC.

Conflict of interest

The authors declare there are no conflict of interest.

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Table S1: Seeding and treatment variations and combinations screened to establish encapsulated LS180 spheroids.

Seeding density (x 10 ³ /μL)	LS180 cell suspension preparation	AggreWell™ plate type	Centrifugal force	Treatment	Observations
1.2	Scraping	400	120 x <i>g</i> 5 min	N/A	Nice spheroids, spheroids disintegrate or clump.
1.6	Scraping	400	120 x <i>g</i> 5 min	N/A	Nice spheroids, spheroids disintegrate or clump.
1.8	Scraping	400	120 x <i>g</i> 5 min	N/A	Nice spheroids, spheroids disintegrate or clump.
14.0	Scraping	400	120 x <i>g</i> 5 min	N/A	Nice spheroids, more compact, spheroids disintegrate or clump.
16.0	Scraping	400	120 x <i>g</i> 5 min	N/A	Nice spheroids, more compact, spheroids disintegrate or clump.
18.0	Scraping	400	120 x <i>g</i> 5 min	N/A	Nice spheroids, more compact, spheroids disintegrate or clump.
1.2	Scraping	400	120 x <i>g</i> 5 min	50% Fetal bovine serum, 5 min	Nice spheroids, spheroids disintegrate or clump within minutes.
1.2	Scraping	400	120 x <i>g</i> 5 min	100% Fetal bovine serum, 5 min	Nice spheroids, spheroids disintegrate or clump.
1.2	Scraping	400	120 x <i>g</i> 5 min	2.5mM EDTA, 5 min	Nice spheroids, spheroids disintegrate or clump within an hour.

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1.2	Scraping	400	120 x <i>g</i> 5 min	5mM EDTA, 5 min	Nice spheroids, spheroids disintegrate or clump within an hour.
1.2	Scraping	400	120 x <i>g</i> 5 min	10mM EDTA, 5 min	Nice spheroids, spheroids disintegrate or clump within an hour.
1.2	Scraping	400	120 x <i>g</i> 5 min	0.01% Poly-L-lysine, 5 min	Nice spheroids, spheroids disintegrate or clump within minutes.
1.2	Scraping	400	120 x <i>g</i> 5 min	0.007% Poly-L-lysine, 10 min	Nice spheroids, spheroids disintegrate or clump within minutes.
1.2	Scraping	400	120 x <i>g</i> 5 min	0.004% Poly-L-lysine, 20 min	Nice spheroids, spheroids disintegrate or clump within minutes.
1.2	Scraping	400	120 x <i>g</i> 5 min	0.0005%, Poly-L-lysine, 30 min	Nice spheroids, spheroids disintegrate or clump within minutes.
8.0	Scraping	800	260 x <i>g</i> 6 min	Seeded in medium containing 30% v/v 5mM EDTA, coated after seeding with 5 mM EDTA, 10 min	Spheroids disintegrate after removal from AggrWell™800 plate.
12.0	Scraping	800	260 x <i>g</i> 6 min	Seeded in medium containing 30% v/v 5mM EDTA, coated after seeding with 5 mM EDTA, 10 min	Spheroids disintegrate after removal from AggrWell™800 plate.
16.0	Scraping	800	260 x <i>g</i> 6 min	Seeded in medium containing 30% v/v 5mM EDTA, coated after seeding with 5 mM EDTA, 10 min	Spheroids disintegrate after removal from AggrWell™800 plate.
8.0	Scraping	800	260 x <i>g</i> 6 min	Coated after seeding with 5 mM EDTA, 10 min	Nice spheroids, spheroids clump within hour after removal.

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12.0	Scraping	800	260 x g 6 min	Coated after seeding with 5 mM EDTA, 10 min	Nice spheroids, spheroids clump within hour after removal.
16.0	Scraping	800	260 x g 6 min	Coated after seeding with 5 mM EDTA, 10 min	Nice spheroids, spheroids clump within hour after removal.
0.2 cells per μ l	Trypsinized	N/A	N/A	0.6% Sodium alginate, 0.5 or 1 μ l 50 mM CaCl ₂ -150 mM NaCl cross-linker for 5, 10 or 20 min	Gel too soft, optimal time for crosslinker is 5 min otherwise gel dries out at 0.5 μ l per droplet. Cells too dispersed
0.2	Trypsinized	N/A	N/A	0.7% Sodium alginate, 0.5 μ l 50 mM CaCl ₂ -150 mM NaCl cross-linker for 5 min	Gel too soft, cells too dispersed
0.2	Trypsinized	N/A	N/A	0.8% Sodium alginate, 0.5 μ l 50 mM CaCl ₂ -150 mM NaCl cross-linker for 5 min	Gel too soft, cells too dispersed
0.2	Trypsinized	N/A	N/A	1.0% Sodium alginate, 0.5 μ l 50 mM CaCl ₂ -150 mM NaCl cross-linker for 5 min	Gel too soft, cells too dispersed
0.2	Trypsinized	N/A	N/A	1.5% Sodium alginate, 0.5 μ l 50 mM CaCl ₂ -150 mM NaCl cross-linker for 5 min	Gel too soft, cells too dispersed
0.2	Trypsinized	N/A	N/A	2% Sodium alginate, 0.5 μ l 50 mM CaCl ₂ -150 mM NaCl cross-linker for 5 min	Gel perfect, cells too dispersed
0.2	Trypsinized	N/A	N/A	5% Sodium alginate, 0.5 μ l 50 mM CaCl ₂ -150 mM NaCl cross-linker for 5 min	Gel may be too firm, this may influence diffusion rates. Cells too dispersed

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0.4	Trypsinized	N/A	N/A	2% Sodium alginate, 0.5 μ l 50 mM CaCl ₂ -150 mM NaCl cross-linker for 5 min	Cells too dispersed
0.6	Trypsinized	N/A	N/A	2% Sodium alginate, 0.5 μ l 50 mM CaCl ₂ -150 mM NaCl cross-linker for 5 min	Cells too dispersed
0.8	Trypsinized	N/A	N/A	2% Sodium alginate, 0.5 μ l 50 mM CaCl ₂ -150 mM NaCl cross-linker for 5 min	Cells too dispersed
1.2	Trypsinized	N/A	N/A	2% Sodium alginate, 0.5 μ l 50 mM CaCl ₂ -150 mM NaCl cross-linker for 5 min	Spheroids looking fuller, can add more cells
1.5	Trypsinized	N/A	N/A	2% Sodium alginate, 0.5 μ l 50 mM CaCl ₂ -150 mM NaCl cross-linker for 5 min	Cells perfectly dispersed
2.0	Trypsinized	N/A	N/A	2% Sodium alginate, 0.5 μ l 50 mM CaCl ₂ -150 mM NaCl cross-linker for 5 min	Spheroids may have too many cells

CHAPTER 8

FINAL CONCLUSIONS AND FUTURE RECOMMENDATIONS

1. FINAL CONCLUSIONS

The liver is responsible for over 500 metabolic functions, including biotransformation and elimination of xenobiotic substances such as drugs and/or herbal entities. Liver injury caused by drug candidates and herbal supplements can cause asymptomatic elevation of liver enzymes, which may transpire into liver cirrhosis, fibrosis and without treatment even acute liver failure. Clearly, pre-clinical safety and toxicity assessment of drug candidates and herbal supplements, and the influence thereof on hepatotoxicity, are of great importance. Various two-dimensional (2D) *in vitro* models are used in conjunction with *in vivo* models for the pre-clinical assessment of drug biotransformation and hepatotoxicity. However, notwithstanding their advantages, all of these models are afflicted by various shortcomings and restrictions. Conventional 2D cell culture models used to investigate pharmacokinetic interactions and hepatotoxicity can only provide limited information due to a lack of physiological relevance, which may result in inadequate or even erroneous conclusions, while the use of animal models in scientific research causes an ethical dilemma. In addition, experimental data obtained from animal models are not always successfully correlated to humans, and some side effects may not even be detectable. It is therefore clear that a need exists for new or alternative *in vitro* biotransformation and toxicity screening models, such as three-dimensional (3D) cell culture systems, which can more closely resemble the *in vivo* environment. Various 3D model systems are available, each offering specific advantages and limitations. The research question, cell type and equipment available determine which model is most suitable.

The primary aim of this study was to assess the suitability of the 3D HepG2/C3A cell line, cultured as spheroids by means of the microgravity bioreactor technique, as an alternative *in vitro* toxicity screening model. The effects of a crude aqueous extract of the commercially available herbal remedy *Xysmalobium undulatum* (Uzara) were evaluated in the HepG2/C3A spheroid model following acute and sub-chronic exposure. These effects were then compared to effects measured in traditional 2D cultures of the HepG2/C3A cell line, and results from an *in vivo* study employing the Sprague Dawley rat model.

Acute treatment with the crude Uzara aqueous extract in the 3D spheroid culture model resulted in a marked growth inhibition, in a dosage dependent manner, when the inability to utilize glucose and the reduction in spheroid surface area/biomass were taken into account. The protein data obtained from the 2D cell culture model showed a similar trend of growth

inhibition, albeit to a lower extent. Physiological parameters such as intracellular ATP levels and actively released AK levels were evaluated as indicators of cell viability and toxicity. Intracellular ATP and AK levels in the 3D model showed potential toxicity, but simultaneous recovery at the lowest three concentrations of the crude Uzara aqueous extract tested (i.e. 200, 350 and 500 mg/kg). The 750 mg/kg crude Uzara aqueous extract, however, appeared to be toxic since the intracellular ATP levels were below the levels seen for the untreated control group and the actively released AK levels increased towards the end of the treatment. Intracellular ATP levels in the 2D cell culture model were increased for all the crude Uzara aqueous extract treatment groups, and this only decreased towards the end of the experiment coinciding with a sharp increase in AK released. These results suggest toxicity of the Uzara water extract for all the experimental groups when compared to the untreated control group. The data from these experiments correlate well with published data for plant extracts containing cardenolides, such as the major active constituent, uzarin, present in the prepared crude Uzara aqueous extract (Krishna *et al.*, 2015). This study highlighted the superiority of the 3D spheroid model when evaluating acute herbal toxicity, as it provided results more physiologically relevant when compared to the data from the 2D cell model, which possibly inflated the potential toxicity of the crude Uzara aqueous extract.

Although it has widely been hypothesised that 3D cell culture models could potentially refine, reduce or even replace *in vivo* animal models in pre-clinical drug biotransformation and toxicity studies, validation of these 3D models' suitability for these applications is required. A sub-chronic 21-day *in vitro* hepatotoxicity study with the HepG2/C3A spheroid culture model was conducted where the spheroids were treated with *X. undulatum* crude water extract and valproic acid (positive control). The effects observed were subsequently compared to effects as a result of sub-chronic treatment of Sprague Dawley rats with the same model compound during an *in vivo* hepatotoxicity study. Data indicated growth inhibition of the 3D spheroids following treatment with both crude Uzara aqueous extract concentrations, as well as the known hepatotoxin valproic acid. Also, a loss of cell viability and increased cytotoxicity could be observed following valproic acid treatment, indicating hepatotoxicity as expected, and correlating well with previously published data in both 3D spheroid cultures and *in vivo* systems (Ahmed & Siddiqi, 2006; Lee *et al.*, 2008; Fey & Wrzesinski, 2013; Vitins *et al.*, 2014). The HepG2/C3A spheroid model also indicated the potential hepatotoxicity of the crude Uzara aqueous extract at both 200 and 250 mg/kg, and this effect was moderately concentration dependant. Similar results were seen following treatment of the *in vivo* Sprague Dawley model. Increased levels of AST, ALT and LDH could be observed following valproic acid treatment, with a slight increase in triglycerides, indicative of hepatic cellular damage which could result in hepatotoxicity. The 250 mg/kg crude Uzara aqueous extract-

treated group presented with slightly increased levels of AST, ALT, LDH and triglycerides, while the 200 mg/kg crude Uzara aqueous extract showed only a slight increase in LDH and triglycerides, also indicative of potential moderate hepatotoxicity.

The results from both the acute and sub-chronic studies confirmed the potential of the 3D HepG2/C3A spheroid model to effectively and reliably predict possible hepatotoxicity. This model represents a readily available *in vitro* model, capable of bridging the gap between current 2D *in vitro* screening models and *in vivo* animal models. The 3D model therefore presents the possibility of reducing costs and time in pre-clinical toxicity testing, as current 2D systems have low predictive value. Furthermore, the use of animal models can be reduced by establishment of a reliable *in vitro* model, and in certain instances even replaced, although animal models will probably not be completely removed from this process.

A collaborative effort with the department of Biochemistry at the North-west University also explore the potential of the HepG2/C3A spheroid culture model for applications other than toxicology. The possible use of 3D spheroid cell cultures to elucidate the origin and biological functions of cell-free DNA (cfDNA) was investigated, and the results indicated that: 1) spheroids produced cfDNA, 2) changes in spheroid growth and stability were effectively mirrored by cfDNA characteristics, and 3) cfDNA characteristics correlated with that of previous 2D cell culture and human plasma research. It was found that 3D spheroid cultures can potentially serve as effective, simplified, *in vivo*-simulating “closed-circuit” models, since putative sources of cfDNA are limited to only the targeted cells. In addition, cfDNA can also serve as an alternative or auxiliary marker for tracking spheroid growth, development and culture stability. This was the first study of its kind to our knowledge.

The secondary aim of this study was to establish a 3D spheroid model to effectively study biotransformation, as current models to assess drug biotransformation cannot always accurately predict *in vivo* biotransformation and elimination. To this end, the LS180 cell line was established as a 3D spheroid cell culture model by means of the microgravity bioreactor technique. Creating LS180 spheroids proved to be exceptionally challenging, and following several adaptations to the AggreWell™ plate aggregation method it was eventually combined with another 3D culturing method employing the hydrogel, sodium alginate. The following method and parameters were established to be most ideal for creating sodium alginate-encapsulated LS180 cell spheroids, namely 2% w/v sodium alginate, a seeding density of 1 500 cells per 1 µl, and exposure to 0.5 µl cross-linker for 5 min. This method enabled production of encapsulated LS180 spheroids, with potential to be employed as a novel, long-term cell culture method, the first of its kind to our knowledge.

Many research as well as review manuscripts have proposed that three-dimensional cell culturing offers the potential to revolutionise pre-clinical drug screening and the potential of this model to bridge the gap between *in vitro*, *in vivo* and clinical drug screening. By taking a holistic view of the results obtained from this study it is possible to say that we are much closer at obtaining these goals and proving these statements to be true. We have seen that the three-dimensional spheroid model has the ability to give a more true representation of toxicological responses when compared to traditional two-dimensional cell culturing techniques. We also showed the possibility of predicting toxicological responses *in vivo*, by comparing the results obtained to that of an animal model providing proof that this model may indeed bridge the current gap, offering relief to the use of animal models in research. We also proved that this model is not limited to toxicological research by exploring other cell lines in an attempt to establish a three-dimensional bio-transformation model. That this system and model can be used as a means to establish possible biomarkers has also been proven and offers still another application of this system that warrants further exploration and development.

2. FUTURE RECOMMENDATIONS

Three-dimensional cell culturing is a rapidly developing field, and has tremendous potential to revolutionise pre-clinical assessment of drug candidates and herbal compounds, as indicated by the results obtained in this study. The question remains whether it can be successfully employed in other scientific endeavours. The microgravity bioreactor technique has the potential, as seen with the LS180 model, to be applied to various other immortalised and primary cell lines, providing an opportunity to answer many more pressing research questions, such as mechanism elucidation in cancer treatment.

Furthermore, the potential of extracts from the medicinal plant Uzara as cancer treatment should be investigated, as the cardenolide uzarin present in Uzara has been shown to have different modes of action in normal cells compared to cancerous cells. It appears to stimulate proliferation, while inhibiting cell death in normal cells compared to growth inhibition and stimulation of apoptosis in cancerous cells.

And finally, the 3D LS180 model has to be fully characterized to determine viability, CYP450 enzyme expression and correlation to the *in vivo* situation. This will enable its use as an *in vitro* pre-clinical biotransformation model for the elucidation of drug metabolism, herb-drug and drug-drug interactions, and to study the induction and/or inhibition of the cytochrome P450 enzyme family.

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APPENDIX A

**AUTHOR GUIDELINES FOR JOURNAL EXPERT OPINION ON DRUG
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B.18. QUERIES

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Updated April 2016.

B.19. REFERENCES

Toxicology mechanisms and methods. 2016. Author guidelines. URL: <http://www.tandfonline.com/action/authorSubmission?show=instructions&journalCode=itxm2>
Date of access: 09November 2017

APPENDIX C

DATA NOT PUBLISHED IN MANUSCRIPT:

Toxicity and anti-proliferative properties of *Xyloxylosum undulatum* water extract during short term exposure to two-dimensional and three-dimensional spheroid cell cultures

C.1. INTRODUCTION

This appendix represents data not published in the research article entitled: "Toxicity and anti-proliferative properties of *Xyloxylosum undulatum* water extract during short term exposure to two-dimensional and three-dimensional spheroid cell cultures".

It provides data on glucose consumption, spheroid growth, intracellular ATP and AK release of all experimental groups over time not normalised to the control group.

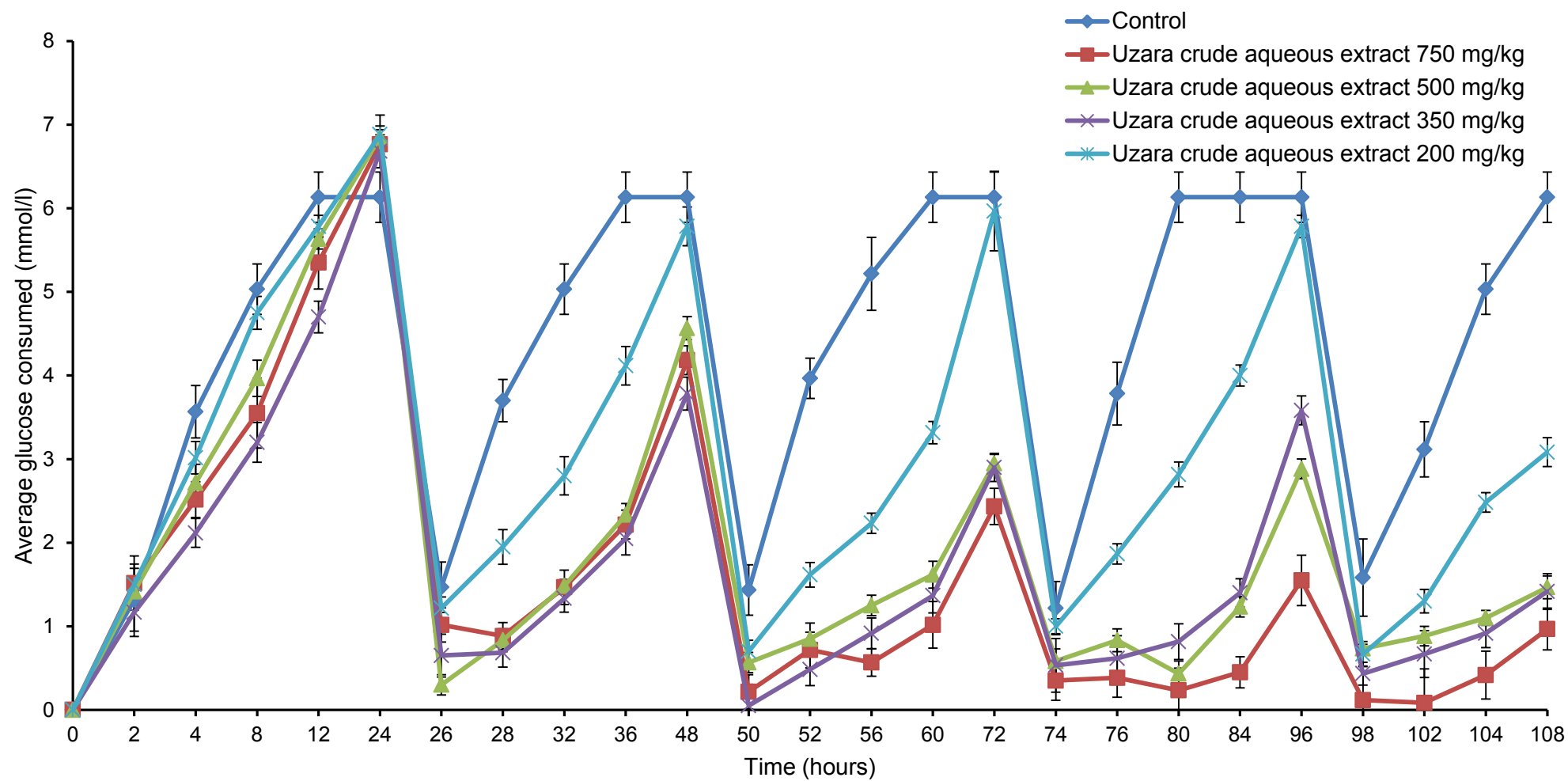


Figure C1: Average glucose consumed (mmol/l) over time ($n = 3$, Error bars = SD)

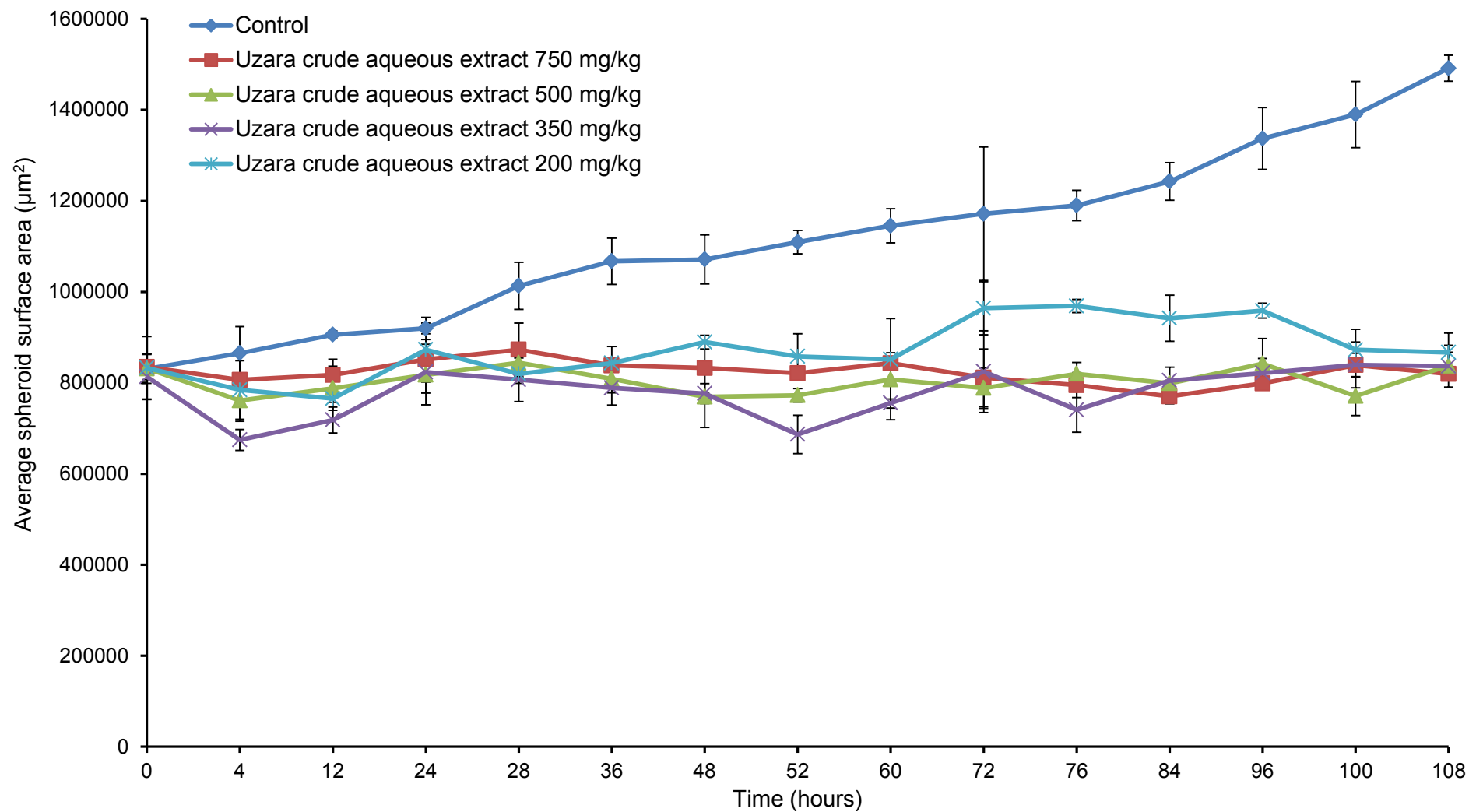


Figure C2: Average spheroid surface area (μm^2) over time ($n = 3$, Error bars = SD)

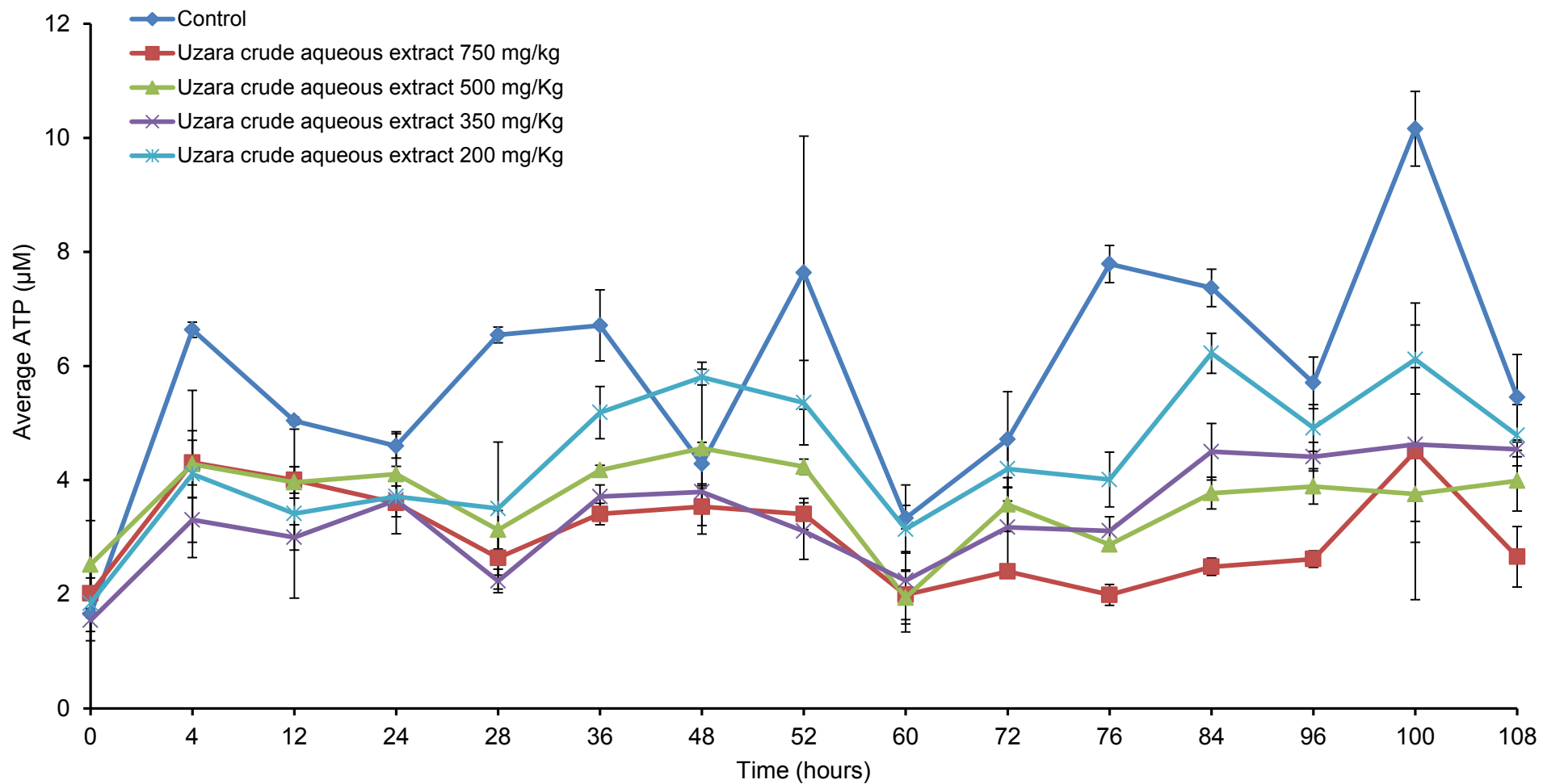


Figure C3: Average ATP (μM) over time ($n = 3$, Error bars = SD)

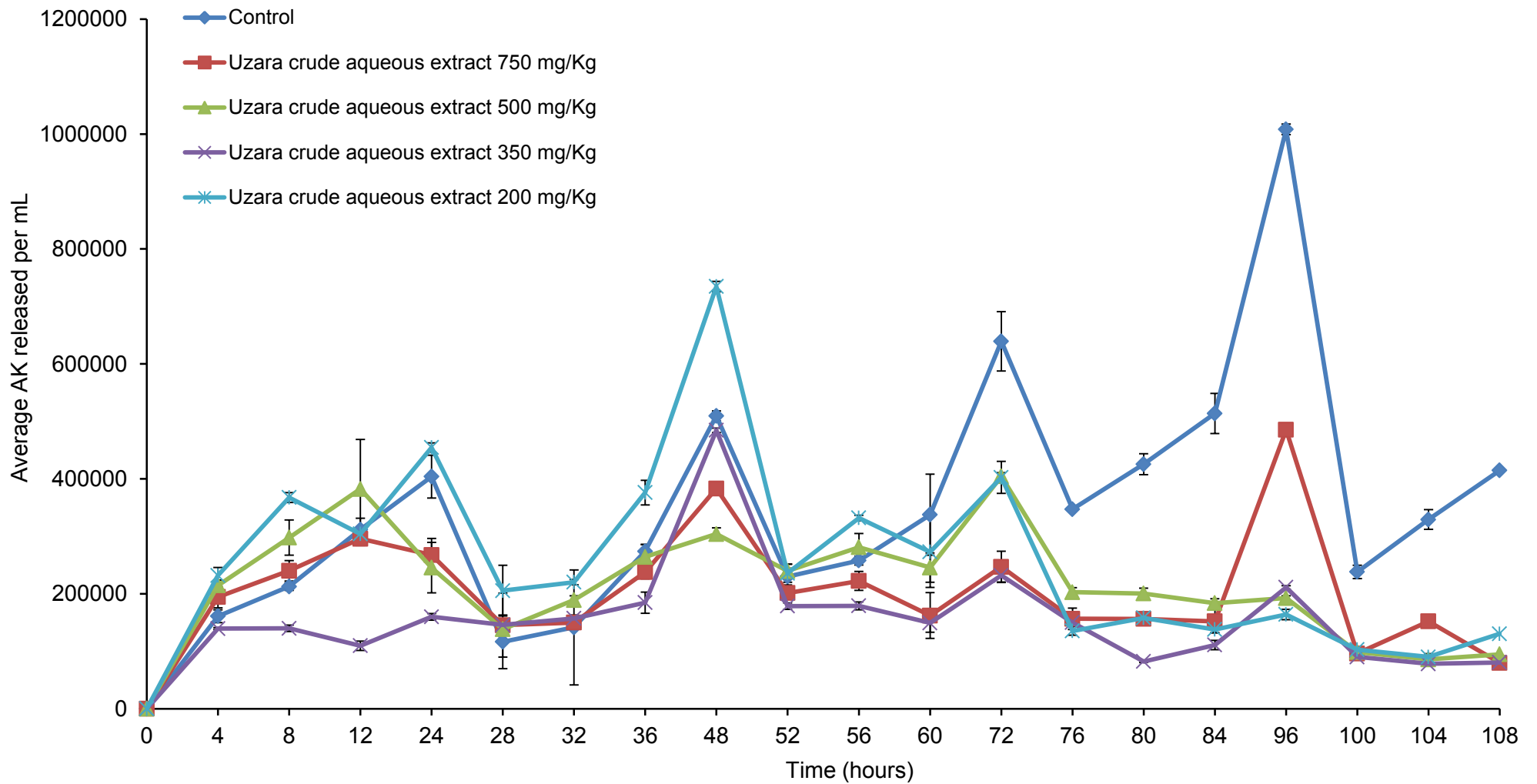


Figure C4: Average AK over time ($n = 3$, Error bars = SD)

APPENDIX D

**AUTHOR GUIDELINES FOR INTERNATIONAL JOURNAL OF
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D.1. INTRODUCTION

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(2) Reviews and Commentaries. These articles are by invitation only and provide the authors' views on a selected topic of interest to pharmacologists.

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discussion of the issues addressed in the Scientific Checklist.

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2. Are all tables and figures numbered and appropriately titled with descriptive legends that permit stand-alone interpretation? Are all data shown in the tables and figures also described in the Results section, discussed in the Discussion section and stated in the Conclusions?

Introduction

3. Is there a clear statement with background describing the hypothesis being tested by this study? Are the primary endpoints clearly stated?

Materials and Methods

4. Were human tissues or fluids used in this study? Were the experiments reviewed and approved by the Institutional review Board (IRB)?
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11. Are all group sizes approximately the same and clearly indicated in the text and/or in the tables and figures?
12. Were the criteria used for excluding any data from analysis determined prospectively

and clearly stated?

13. Was the investigator responsible for data analysis blinded to which samples/animals represent control and treatment groups?

14. Are there ported data displayed as means +/-standard deviation (SD)? Is the number of replicates of three or more independent experimental observations clearly indicated? Were post-hoc tests used to assess the statistical significance among means? Is the threshold for statistical significance (P value) clearly indicated?

Results

15. If western blots are shown, are the appropriate loading controls, replication data, and quantification and statistical analysis shown?

16. If PCR and RT-PCR are included, were MIQE guidelines followed? Was a reference standard (positive or negative controls) included in the study to validate the experiment?

Discussion

17. Are the primary conclusions and any secondary end points and their implications clearly stated?

18. Are the limitations of the current study or alternative interpretations of the findings clearly stated?

Conflict of Interest/Financial Support

19. Is a conflict of interest statement included in the manuscript?

20. Are all organizations providing funding for this work listed in Acknowledgements?

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All tables must be numbered consecutively in Arabic numerals and cited in the text in their order of appearance. Table titles should be brief and descriptive. Tables should appear individually on separate pages in the submitted version of the work, together with a legend that includes sufficient information about the experimental protocol and results so the reader does not have to refer back to the text to understand the experimental protocol and findings.

Tables should not have vertical lines, and the number of horizontal lines should be minimized.

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Citations in text

Ensure that every reference cited in the text is present in the reference list, and vice versa. Unpublished results and personal communications should not appear in the reference list, but may be indicated in the text.

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[dataset] [5] M. Oguro, S. Imahiro, S. Saito, T. Nakashizuka, Mortality data for Japanese oak wilt disease and surrounding forest compositions, Mendeley Data, v1, 2015. <http://dx.doi.org/10.17632/xwj98nb39r.1>.

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Authors asked to submit a revised version of the work for consideration must also supply a graphical abstract at that time. The graphical abstract, which will be displayed on the online Table of Contents, should provide a concise summary of the work in pictorial form designed to capture the attention of a wide audience and for compilation of databases. Graphical Abstract text should not exceed 30 words. The content of the graphical abstract should be kept within an area of 5 cm tall by 17 cm wide (landscape shape). Authors are encouraged to limit graphical abstracts to 189 pixels tall by 642 pixels wide to ensure the image and text will be legible when displayed online. Authors must supply the graphic separately as an electronic file. See <http://www.sciencedirect.com/science/journal/00062952/94/2forexamplesofgraphicalabstracts>.

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In addition, you can link to relevant data or entities through identifiers within the text of your manuscript, using the following format: Database: xxxx (e.g., TAIR: AT1G01020; CCDC: 734053; PDB: 1XFN).

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The journal encourages authors to create an AudioSlides presentation with their published

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Interactive plots

This journal enables you to show an Interactive Plot with your article by simply submitting a data file.

D.4. AFTER ACCEPTANCE

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D.5. REFERENCES

Biochemical Pharmacology. 2014. Author guidelines. URL:
<https://www.elsevier.com/journals/biochemical-pharmacology/0006-2952/guide-for-authors>
Date of access: 10 November 2017.

APPENDIX E

DATA NOT PUBLISHED IN MANUSCRIPT:

HepG2/C3A spheroid cultures as a screening tool for sub-chronic hepatotoxicity studies compared to an *in vivo* model

E.1. INTRODUCTION

This appendix represents data not published in the research article entitled: “HepG2/C3A spheroid cultures as a screening tool for sub-chronic hepatotoxicity studies compared to an *in vivo* model.”

It provides data on intracellular ATP and AK release of all experimental groups over time not normalised to the control group.

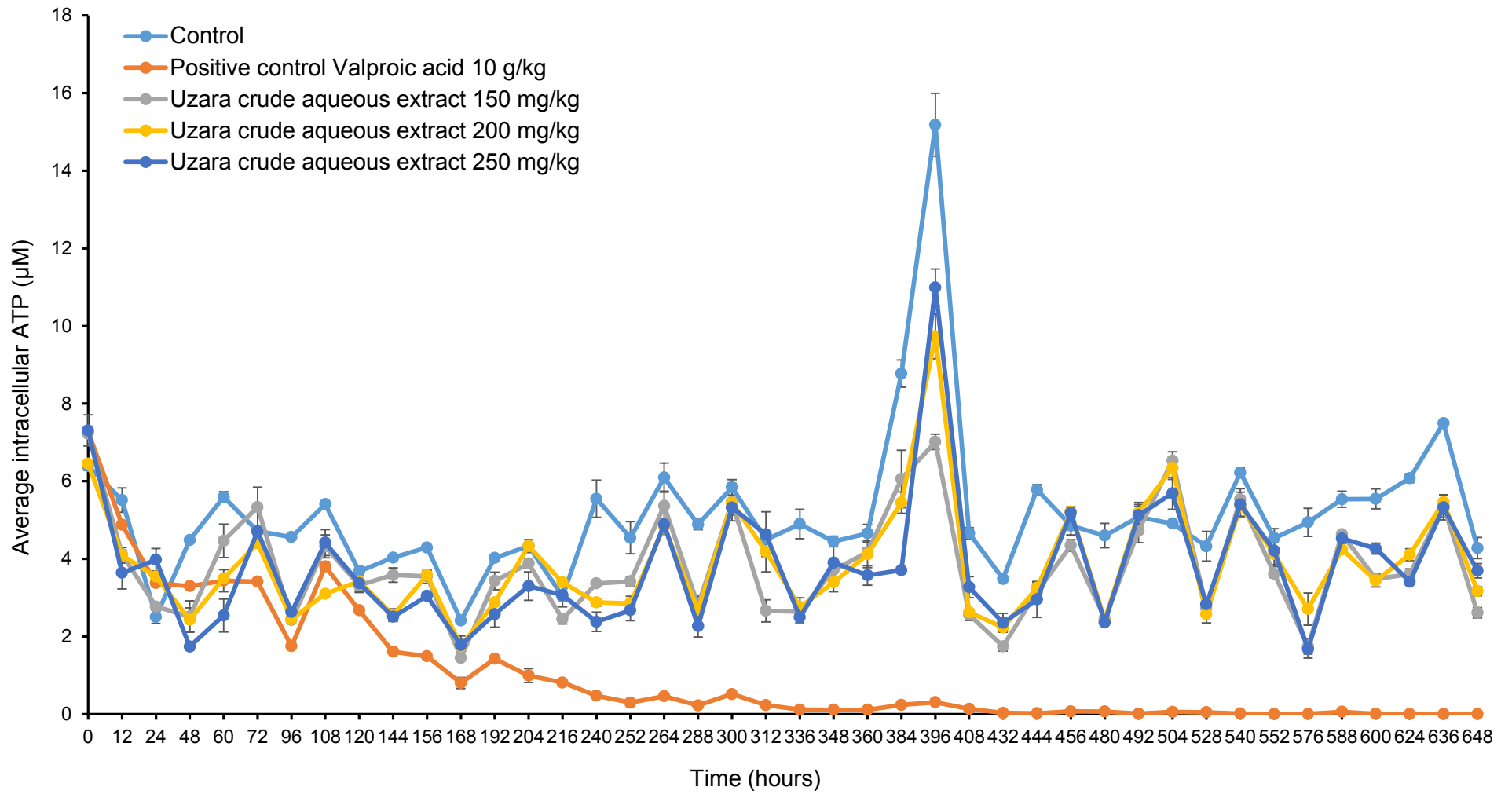


Figure E1: Average intracellular ATP (μM) over time ($n = 3$, Error bars = SD)

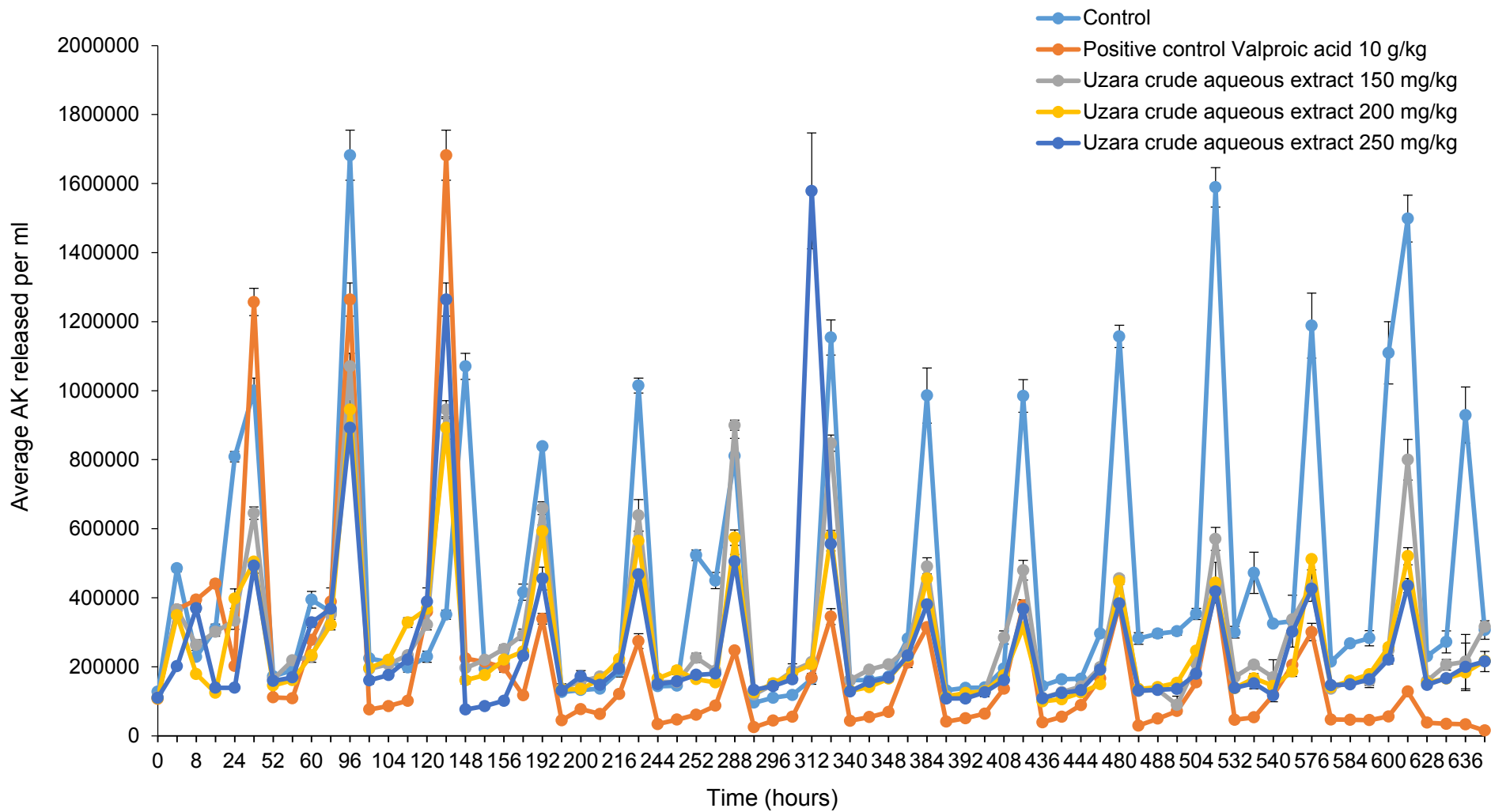
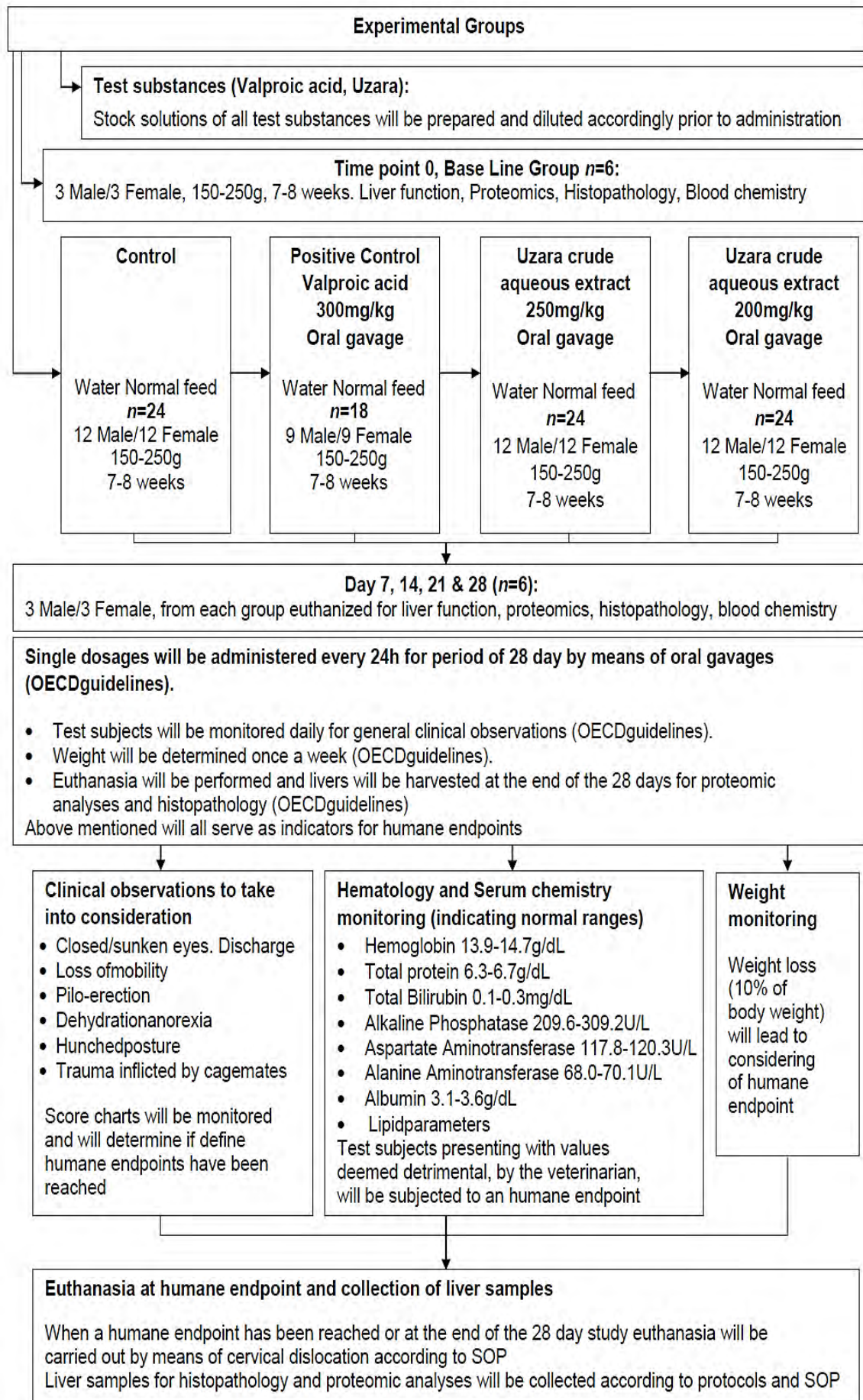


Figure E2: Average AK release per ml over time ($n = 3$, Error bars = SD)

APPENDIX F

DESIGN OF *IN VIVO* STUDY



APPENDIX G

ETHICAL APPROVAL



Private Bag X6001, Potchefstroom
South Africa 2520

Tel: +2718 299-1111/2222
Web: <http://www.nwu.ac.za>

SCIENTIFIC RESEARCH COMMITTEE: CENTRE
OF EXCELLENCE FOR PHARMACEUTICAL
SCIENCES
Tel: +2718 299 4035
Fax: +2787 231 5432
Email: sias.hamman@nwu.ac.za

To whom it may concern,

RE: RECOMMENDATION BY SCIENTIFIC RESEARCH COMMITTEE FOR THE CONTINUATION OF IN VIVO STUDY

The adverse events which occurred in the positive control group in the *in vivo* study of C. Calitz (NWU-00276017-S5) were reviewed by members of the Scientific Research Committee of Pharmacen™ (sub-programme Drug Delivery) on 11 October 2017.

Title of study: "Establishing three-dimensional cell culture models to measure bio-transformation and toxicity"

The committee considered the recommendations made during the consultation with members of the adverse events committee, as well as detailed literature regarding the following:

- The effective dose (ED₅₀) of valproic acid
- The lethal dose (LD₅₀) of valproic acid
- The half-life of valproic acid
- Dosing frequency in this study
- The non-linear pharmacokinetics of valproic acid
- The number of animals available
- The time available
- The accumulation of valproic acid in the blood over time
- Blood levels of previous *in vivo* animal studies using valproic acid
- Whether the data collected can be published
- If the group will still serve as a valid control (sufficient liver toxicity with elevated blood chemistry values of certain markers)
- Financial implications

Taking all of this information into account, the committee unanimously decided to recommend the following course of action:

1. Allow time for recovery of the group until Monday 16/10/2017
2. Continue with one concentration, namely 300 mg/kg, based on an informed decision after taking all the factors listed above into consideration
3. Continue using PBS to prepare the valproic acid solution, and check pH prior to administration
4. Continue with all healthy test subjects left in the positive control group
5. Maintain increased monitoring

In the event of further adverse effects resulting in the introduction of humane endpoints or unforeseen deaths as a result of toxicity due to the valproic acid administration at the newly recommended dose, the group should be terminated. In such case, other options should be considered to ensure publication of the work.

Yours sincerely,

A handwritten signature in black ink, appearing to read 'J H Hamman'. The signature is written in a cursive style with a large, looped initial 'J'.

PROF J H HAMMAN (Chair of the Scientific Research Committee)

APPENDIX H

CERTIFICATES OF ANALYSES FOR UZARA AND VALPROIC ACID

Afrinatural Holdings

23 Pappas Ave, P.O. Box 2121
 Hermanus 7200, South Africa
 Tel: 086 100 1978 Fax: 06 587 1842
 International +27 28 316 4550
 e-mail info@afriatural.com



Brings you the rich knowledge
 and wisdom of Africa

CERTIFICATE OF ANALYSIS

Xysmalobium undulatum (Uzara) - Milled:

Radix (400 micron)

Batch number: XU174
 Manufacturing date: Oct 2014
 Re-test date: Oct 2017

<u>Characteristic</u>	<u>Specification</u>	<u>Result</u>
Appearance	Beige to light brown	
Odour & Taste	Typical	
Moisture	< 12%	
Sieve test	95% < 400 micron	
Foreign matter	< 2%	
TLC	Compare to reference	
Heavy Metals		
Lead	< 3 mg / Kg	
Cadmium	< 1 mg / Kg	
Mercury	< 0.1 mg / Kg	
Arsenic	< 3 mg / Kg	
Microbiological		
TMA	< 1 000 000 CFU's / g	
Yeast & Moulds	< 10 000 CFU's / g	
E. coli	Absent	
Staph. Aureus	Absent	
Salmonella	Absent	

WA Joubert

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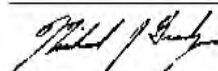
Certificate of Analysis

Product Name:

Valproic acid sodium salt - ≥98%

Product Number:	P4543	(CH ₃ CH ₂ CH ₂) ₂ CHCOO ⁻ Na ⁺
Batch Number:	MKCB2699V	
Brand:	SIGMA	
CAS Number:	1069-66-5	
MDL Number:	MFC00078604	
Formula:	C ₈ H ₁₅ NaO ₂	
Formula Weight:	166.19 g/mol	
Quality Release Date:	28 SEP 2016	
Recommended Retest Date:	SEP 2018	

Test	Specification	Result
Appearance (Color)	White	White
Appearance (Form)	Powder	Powder
Solubility (Color)	Colorless	Colorless
Solubility (Turbidity)	Clear	Clear
200 mg plus 4 mL, H ₂ O		
Purity (GC)	≥ 98 %	100 %
Titration by HClO ₄	≥ 98 %	100 %
Recommended Retest Period		
2 Years		



Michael Grady, Manager
Quality Control
Milwaukee, WI US

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APPENDIX I

**AUTHOR GUIDELINES FOR THE INTERNATIONAL JOURNAL OF
BIOCHEMISTRY AND CELL BIOLOGY**

I.1. INTRODUCTION

Authors are urged to use the Preparation checklist for illustrations, see the paragraph Electronic Artwork in this document. Manuscripts should contain evidence of submission of nucleic acid sequences to appropriate databanks. Manuscripts should contain a word count. Should the Editor request a revision, a revised version of the manuscript should be submitted within eight weeks. After this period, the article will be seen as a new submission.

Types of articles

(see Format Requirements)

The journal publishes

- Short communications (up to 3,000 words)
- Regular articles (no invitation required, up to 5,000 words)
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Word counts are exclusive of references.

Contact details for submission

For questions on the submission and reviewing process, please contact Joanna Kargul, Managing Editor at ijbcb@ucl.ac.uk.

Submission checklist

You can use this list to carry out a final check of your submission before you send it to the journal for review. Please check the relevant section in this Guide for Authors for more details.

Ensure that the following items are present:

One author has been designated as the corresponding author with contact details:

- E-mail address
- Full postal address

All necessary files have been uploaded:

Manuscript:

- Include keywords
- All figures (include relevant captions)
- All tables (including titles, description, footnotes)
- Ensure all figure and table citations in the text match the files provided

- Indicate clearly if color should be used for any figures in print Graphical Abstracts/Highlights files (where applicable) Supplemental files (where applicable)

Further considerations

- Manuscript has been 'spell checked' and 'grammar checked'
- All references mentioned in the Reference List are cited in the text, and vice versa
- Permission has been obtained for use of copyrighted material from other sources (including the Internet)
- A competing interests statement is provided, even if the authors have no competing interests to declare
 - Journal policies detailed in this guide have been reviewed
 - Referee suggestions and contact details provided, based on journal requirements

For further information, visit our Support Center.

I.2. BEFORE YOU BEGIN

I.2.1. Ethics in publishing

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I.2.2. Declaration of interest

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All authors should have made substantial contributions to all of the following: (1) the conception and design of the study, or acquisition of data, or analysis and interpretation of data, (2) drafting the article or revising it critically for important intellectual content, (3) final approval of the version to be submitted.

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Divide your article into clearly defined and numbered sections. Subsections should be

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If there is more than one appendix, they should be identified as A, B, etc. Formulae and equations in appendices should be given separate numbering: Eq. (A.1), Eq. (A.2), etc.; in a subsequent appendix, Eq. (B.1) and so on. Similarly for tables and figures: Table A.1; Fig. A.1, etc.

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Although a graphical abstract is optional, its use is encouraged as it draws more attention to the online article. The graphical abstract should summarize the contents of the article in a concise, pictorial form designed to capture the attention of a wide readership. Graphical abstracts should be submitted as a separate file in the online submission system. Image size: Please provide an image with a minimum of 531 × 1328 pixels (h × w) or proportionally more. The image should be readable at a size of 5 × 13 cm using a regular screen resolution of 96 dpi. Preferred file types: TIFF, EPS, PDF or MS Office files. You can view Example Graphical Abstracts on our information site. Authors can make use of Elsevier's Illustration Services to ensure the best presentation of their images and in accordance with all technical requirements.

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Immediately after the abstract, provide a maximum of 6 keywords, using American spelling and avoiding general and plural terms and multiple concepts (avoid, for example, 'and', 'of'). Be sparing with abbreviations: only abbreviations firmly established in the field may be eligible. These keywords will be used for indexing purposes.

I.3.11. Abbreviations

Define abbreviations that are not standard in this field in a footnote to be placed on the first page of the article. Such abbreviations that are unavoidable in the abstract must be defined at their first mention there, as well as in the footnote. Ensure consistency of abbreviations throughout the article.

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Collate acknowledgements in a separate section at the end of the article before the

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I.3.13. Formatting of funding sources

List funding sources in this standard way to facilitate compliance to funder's requirements:

I.3.13.1. Funding

This work was supported by the National Institutes of Health [grant numbers xxxx, yyyy]; the Bill & Melinda Gates Foundation, Seattle, WA [grant number zzzz]; and the United States Institutes of Peace [grant number aaaa]. It is not necessary to include detailed descriptions on the program or type of grants and awards. When funding is from a block grant or other resources available to a university, college, or other research institution, submit the name of the institute or organization that provided the funding. If no funding has been provided for the research, please include the following sentence: This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

I.3.13.2. Units

Follow internationally accepted rules and conventions: use the international system of units (SI). If other units are mentioned, please give their equivalent in SI.

I.3.13.3. Nomenclature and units

Follow internationally accepted rules and conventions: use the international system of units (SI). If other quantities are mentioned, give their equivalent in SI. You are urged to consult IUPAC: Nomenclature of Organic Chemistry for further information.

I.3.13.4. Math formulae

Please submit math equations as editable text and not as images. Present simple formulae in line with normal text where possible and use the solidus (/) instead of a horizontal line for small fractional terms, e.g., X/Y. In principle, variables are to be presented in italics. Powers of e are often more conveniently denoted by exp. Number consecutively any equations that have to be displayed separately from the text (if referred to explicitly in the text).

I.3.13.5. Footnotes

Footnotes should be used sparingly. Number them consecutively throughout the article. Many word processors can build footnotes into the text, and this feature may be used. Otherwise, please indicate the position of footnotes in the text and list the footnotes themselves separately at the end of the article. Do not include footnotes in the Reference list.

I.3.13.6. Artwork

Image manipulation

Whilst it is accepted that authors sometimes need to manipulate images for clarity, manipulation for purposes of deception or fraud will be seen as scientific ethical abuse and will be dealt with accordingly. For graphical images, this journal is applying the following policy: no specific feature within an image may be enhanced, obscured, moved, removed, or introduced. Adjustments of brightness, contrast, or color balance are acceptable if and as long as they do not obscure or eliminate any information present in the original. Nonlinear adjustments (e.g. changes to gamma settings) must be disclosed in the figure legend.

Electronic Artwork General points:

- Make sure you use uniform lettering and sizing of your original artwork.
- Save text in illustrations as 'graphics' or enclose the font.
- Only use the following fonts in your illustrations: Arial, Helvetica.
- Number the illustrations according to their sequence in the text.
- Use a logical naming convention for your artwork files.
- Provide captions to illustrations separately.
- Produce images near to the desired size of the printed version.
- Submit each figure as a separate file.

A detailed guide on electronic artwork is available on our website:
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You are urged to visit this site; some excerpts from the detailed information are given here.

Formats

Regardless of the application used, when your electronic artwork is finalised, please 'save as' or convert the images to one of the following formats (note the resolution requirements for line drawings, halftones, and line/halftone combinations given below):

EPS: Vector drawings. Embed the font or save the text as 'graphics'.

TIFF: Color or grayscale photographs (halftones): always use a minimum of 300 dpi. TIFF:

Bitmapped line drawings: use a minimum of 1000 dpi.

TIFF: Combinations bitmapped line/half-tone (color or grayscale): a minimum of 500 dpi is required. If your electronic artwork is created in a Microsoft Office application (Word, PowerPoint, Excel) then please supply 'as is'.

Please do not:

- Supply files that are optimised for screen use (e.g., GIF, BMP, PICT, WPG); the resolution is too low;
- Supply files that are too low in resolution;
- Submit graphics that are disproportionately large for the content.

Preparation checklist for illustrations

Please ensure that molecular weight markers are included in all immune blot and gel figures. For comparative studies, all the protein bands must originate from the same gel. Bar graphs must include standard errors or standard deviations. Please avoid over-complicated coding patterns for bars. Micrographs must include scale bars. Appropriately sized numbers, letters, and symbols should be used so they are no smaller than 2.4 mm (10 pt) in size after reduction to a single column width (7.84 cm or 3.085 inches), or a 2-column width (16.5 cm or 6.5 inches). Superscript and subscript characters are included in this rule. Numbers, letters, and symbols used in multi-paneled figures must be consistent. Figures should preferably be drawn to occupy a single column width (7.84 cm or 3.085 inches). Occasionally, the figures may occupy a double column width if such size enhances presentation. Produce images near to the desired size of the printed version. Make sure that the lettering is such that when the figure is in print, the size of the font is the equivalent to, or slightly larger than the text of the journal. Axis labels should be printed with an initial capital letter followed by lower case lettering with units in open brackets. Axis line width should be uniform and 1 pt in thickness. Column and line diagrams should not be boxed or include gridlines. Use a logical naming convention for your artwork files, and supply a separate listing of the files and the software used.

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Please make sure that artwork files are in an acceptable format (TIFF (or JPEG), EPS (or PDF), or MS Office files) and with the correct resolution. If, together with your accepted article, you submit usable color figures then Elsevier will ensure, at no additional charge, that these figures will appear in color online (e.g., ScienceDirect and other sites) regardless of whether or not these illustrations are reproduced in color in the printed version. For color

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Further information on the preparation of electronic artwork.

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I.3.13.7. Figure captions

Ensure that each illustration has a caption. Supply captions separately, not attached to the figure. A caption should comprise a brief title (not on the figure itself) and a description of the illustration. Keep text in the illustrations themselves to a minimum but explain all symbols and abbreviations used.

I.3.13.8. Tables

Please submit tables as editable text and not as images. Tables can be placed either next to the relevant text in the article, or on separate page(s) at the end. Number tables consecutively in accordance with their appearance in the text and place any table notes below the table body. Be sparing in the use of tables and ensure that the data presented in them do not duplicate results described elsewhere in the article. Please avoid using vertical rules and shading in table cells.

I.3.13.9 References

Citation in text

Please ensure that every reference cited in the text is also present in the reference list (and vice versa). Any references cited in the abstract must be given in full. Unpublished results and personal communications are not recommended in the reference list, but may be mentioned in the text. If these references are included in the reference list they should follow the standard reference style of the journal and should include a substitution of the publication date with either 'Unpublished results' or 'Personal communication'. Citation of a reference as 'in press' implies that the item has been accepted for publication.

Reference links

Increased discoverability of research and high quality peer review are ensured by online links

to the sources cited. In order to allow us to create links to abstracting and indexing services, such as Scopus, CrossRef and PubMed, please ensure that data provided in the references are correct. Please note that incorrect surnames, journal/book titles, publication year and pagination may prevent link creation. When copying references, please be careful as they may already contain errors. Use of the DOI is encouraged.

A DOI can be used to cite and link to electronic articles where an article is in-press and full citation details are not yet known, but the article is available online. A DOI is guaranteed never to change, so you can use it as a permanent link to any electronic article. An example of a citation using DOI for an article not yet in an issue is: VanDecar J.C., Russo R.M., James D.E., Ambeh W.B., Franke M. (2003). Aseismic continuation of the Lesser Antilles slab beneath northeastern Venezuela. *Journal of Geophysical Research*, <https://doi.org/10.1029/2001JB000884>. Please note the format of such citations should be in the same style as all other references in the paper.

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As a minimum, the full URL should be given and the date when the reference was last accessed. Any further information, if known (DOI, author names, dates, reference to a source publication, etc.), should also be given. Web references can be listed separately (e.g., after the reference list) under a different heading if desired, or can be included in the reference list.

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There are no strict requirements on reference formatting at submission. References can be in any style or format as long as the style is consistent. Where applicable, author(s) name(s), journal title/book title, chapter title/article title, year of publication, volume number/book chapter and the pagination must be present. Use of DOI is highly encouraged. The reference style used by the journal will be applied to the accepted article by Elsevier at the proof stage. Note that missing data will be highlighted at proof stage for the author to correct. If you do wish to format the references yourself they should be arranged according to the following examples:

Reference style

Text: All citations in the text should refer to:

1. Single author: the author's name (without initials, unless there is ambiguity) and the year of publication;
2. Two authors: both authors' names and the year of publication;
3. Three or more authors: first author's name followed by 'et al.' and the year of publication.

Citations may be made directly (or parenthetically). Groups of references should be listed first alphabetically, then chronologically. Examples: 'as demonstrated (Allan, 2000a, 2000b, 1999; Allan and Jones, 1999). Kramer et al. (2010) have recently shown'

List: References should be arranged first alphabetically and then further sorted chronologically if necessary. More than one reference from the same author(s) in the same year must be identified by the letters 'a', 'b', 'c', etc., placed after the year of publication.

Examples:

Reference to a journal publication:

Van der Geer, J., Hanraads, J.A.J., Lupton, R.A., 2010. The art of writing a scientific article. *J. Sci. Commun.* 163, 51–59.

Reference to a book:

Strunk Jr., W., White, E.B., 2000. *The Elements of Style*, fourth ed. Longman, New York.

Reference to a chapter in an edited book:

Mettam, G.R., Adams, L.B., 2009. How to prepare an electronic version of your article, in: Jones, B.S., Smith, R.Z. (Eds.), *Introduction to the Electronic Age*. E-Publishing Inc., New York, pp. 281–304.

Reference to a website:

Cancer Research UK, 1975. Cancer statistics reports for the UK. <http://www.cancerresearchuk.org/aboutcancer/statistics/cancerstatsreport/> (accessed 13 March 2003).

Reference to a dataset:

[dataset] Oguro, M., Imahiro, S., Saito, S., Nakashizuka, T., 2015. Mortality data for Japanese oak wilt disease and surrounding forest compositions. *Mendeley Data*, v1. <https://doi.org/10.17632/xwj98nb39r.1>.

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Journal names should be abbreviated according to the List of Title Word Abbreviations.

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Supplementary material such as applications, images and sound clips, can be published with your article to enhance it. Submitted supplementary items are published exactly as they are received (Excel or PowerPoint files will appear as such online). Please submit your material together with the article and supply a concise, descriptive caption for each supplementary file. If you wish to make changes to supplementary material during any stage of the process, please make sure to provide an updated file. Do not annotate any corrections on a previous version. Please switch off the 'Track Changes' option in Microsoft Office files as these will appear in the published version.

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Below are a number of ways in which you can associate data with your article or make a statement about the availability of your data when submitting your manuscript. If you are sharing data in one of these ways, you are encouraged to cite the data in your manuscript and reference list. Please refer to the "References" section for more information about data citation. For more information on depositing, sharing and using research data and other relevant research materials, visit the research data page.

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In addition, you can link to relevant data or entities through identifiers within the text of your manuscript, using the following format: Database: xxxx (e.g., TAIR: AT1G01020; CCDC: 734053; PDB: 1XFN).

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This journal supports Mendeley Data, enabling you to deposit any research data (including raw and processed data, video, code, software, algorithms, protocols, and methods) associated with your manuscript in a free-to-use, open access repository. Before submitting your article, you can deposit the relevant datasets to Mendeley Data. Please include the DOI of the deposited dataset(s) in your main manuscript file. The datasets will be listed and directly accessible to readers next to your published article online. For more information, visit the Mendeley Data for journals page.

I.4.3. Data in Brief

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I.4.4. MethodsX

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I.5. ARTICLE ENRICHMENTS

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The journal encourages authors to create an AudioSlides presentation with their published article. AudioSlides are brief, webinar-style presentations that are shown next to the online article on ScienceDirect. This gives authors the opportunity to summarize their research in their own words and to help readers understand what the paper is about. More information and examples are available. Authors of this journal will automatically receive an invitation e-mail to create an AudioSlides presentation after acceptance of their paper.

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Antibody Data is the reference application linking to information about the antibodies mentioned in the article, based on the NIF Antibody Registry. Authors are encouraged to include relevant antibody identifiers in their articles (e.g. Antibody Registry: AB_878537 or RRID: AB_878537) if appropriate.

I.5.3. Interactive plots

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data file.

I.5.4. Virtual Microscope

The journal encourages authors to supplement in-article microscopic images with corresponding high resolution versions for use with the Virtual Microscope viewer. The Virtual Microscope is a web based viewer that enables users to view microscopic images at the highest level of detail and provides features such as zoom and pan. This feature for the first time gives authors the opportunity to share true high resolution microscopic images with their readers. Authors of this journal will receive an invitation e-mail to create microscope images for use with the Virtual Microscope when their manuscript is first reviewed. If you opt to use the feature, please contact virtualmicroscope@elsevier.com for instructions on how to prepare and upload the required high resolution images.

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The International Journal of Biochemistry and Cell Biology. 2017. Author guidelines. URL: <https://www.elsevier.com/journals/the-international-journal-of-biochemistry-and-cell-biology/1357-2725/guide-for-authors> Date of access: 12 October 2017.

APPENDIX J

AUTHOR GUIDELINES FOR THE JOURNAL OF CELL BIOLOGY

J.1. AIMS AND SCOPE

The Journal of Cell Biology (JCB) is a broad journal that publishes original findings on all aspects of cell biology. We consider papers reporting new cellular or molecular advances in any areas of basic cell biology as well as papers that describe applied cell biology in a variety of systems including, but not limited to, immunology, neurobiology, metabolism, virology, developmental biology, and plant biology. We welcome all submissions that describe new findings of significant interest to cell biologists, regardless of the experimental approach.

If you are wondering whether your manuscript is appropriate for JCB, please feel free to contact the editorial office at cellbio@rockefeller.edu and we will respond to your presubmission inquiry as quickly as possible.

J.2. CRITERIA

To warrant publication in JCB, a manuscript must provide novel and significant insight into a cellular function or process. Insights may be significant because they are of great interest to a subset of cell biologists or because they offer an advance that is of intrinsic interest to a broad cell biological audience.

J.3. MANUSCRIPT TYPES

- **Articles** present a comprehensive analysis providing novel and significant mechanistic insight into an area of interest to our general readership. Articles are fully documented reports of original research that are as concise as possible without compromising the documentation of results. Articles should be no more than 40,000 characters (not including spaces, methods, or references), with up to 10 figures and/or tables. Articles may have up to five supplemental items and references are unlimited.
- **Reports** offer definitive observations of outstanding interest that have the potential to open up new avenues of research. Reports describe cutting-edge findings of immediate interest to a wide readership. Reports are no more than 20,000 characters (not including spaces, methods, or references), with up to five figures and/or tables. Reports may have up to three supplemental items and references are unlimited.
- **Tools** describe new methods or datasets (e.g., screens, systems-wide analyses, or computational modeling) of immediate value and broad utility to the cell biology community. Papers presenting methods should describe a technological advance of broad/general interest that permits the interrogation of cell biological problems in ways previously impossible and include novel cell biological insight as a proof of principle. For datasets,

authors must provide a compelling proof of principle that analysis of the dataset yields novel cell biological insights. Tools are no more than 40,000 characters (not including spaces, methods, or references), with up to 10 figures and/or tables. Tools may have up to five supplemental items and references are unlimited.

J.4. FRONT MATTER FORMATS

Most front matter in JCB is commissioned by the editors. However, we do accept proposals for the following formats.

- **Reviews** are peer-reviewed, comprehensive overviews on a topic of interest to the broad JCB readership. Reviews are usually 4,000–6,000 words in length and include three to four figures/tables. The number of references is unlimited.
- **Spotlights** highlight primary research articles published in JCB or a recent issue of another journal. Most Spotlights are commissioned, but proposals will be considered. Spotlights are 1,500 words in length, with no more than 15 references and one figure/table.
- **Viewpoint** articles put forth original models and hypotheses on cutting-edge cell biological research. This format is intended to stimulate discussion and/or the development of new research. These forward-looking articles should build on recent advances in a given field, and hypotheses should rest on published data. Viewpoints are 1,500–2,000 words in length, with no more than 15 references and one figure/table.

J.5. EDITORIAL PROCESS

At JCB, all editorial decisions on research manuscripts are made through collaborative consultation between in-house professional scientific editors and the academic editorial board. The final decision lies with the academic editors. We strive to provide exceptional service by ensuring timely, objective, and rigorous decision making.

Upon submission, manuscripts are reviewed by at least one in-house scientific editor and one member of the editorial board for general suitability and strength of advance. An initial decision whether to peer review the paper is typically reached within three to four days.

If sent for full review, the manuscript is refereed by leading scientists active in the relevant field regardless of their membership on the JCB editorial board. All reviewers have the opportunity to see and comment on each other's reports, ensuring that peer review is objective and balanced. Decisions after review are communicated jointly by the academic and professional editors.

Following review, we will encourage resubmission if revisions seem feasible within three to four months. We provide clear, detailed decisions that describe exactly what would be needed for publication in JCB. We will not reassess novelty when you resubmit your revision, even if related work has been published. We re-review manuscripts only when necessary. In many cases, an academic editor will make the final decision on a resubmission without further expert review. Our policy is to only allow a single round of major revision.

If revisions will likely take longer than three to four months, we typically advise authors to submit elsewhere. However, authors are free to appeal and request the opportunity to resubmit to JCB at a later time.

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J.6. EDITORIAL BOARD

The JCB editorial board is comprised of over 100 leading scientists from across the breadth of cell biology. Editorial board members are responsible for making timely, thoughtful, and objective editorial decisions on manuscripts in their research area. Editorial board members are renewed every two years. All editorial board members are added and renewed by the Executive Editor and Editor-in-Chief.

In addition to the editorial board, JCB has a team of 10 academic editors who, in addition to making editorial decisions, also contribute to discussions regarding editorial policy and editorial board appointments. These editors are identified on the journal masthead and website. They serve three-year terms. Editors are added and renewed by the Executive Editor and Editor-in-Chief.

The JCB Editor-in-Chief and Executive Editor are appointed by the Executive Director of The Rockefeller University Press.

J.7. REVIEWER GUIDELINES

Reviewers are to contact the editor to discuss any potential conflicts of interest prior to accepting an invitation to peer review a manuscript. Reviewers are asked to comment on the level of conceptual advance and broad interest, as well as the technical rigor, statistical analysis, and presentation of the manuscript. We ask that reviewers provide references

where possible when describing overlap between the current manuscript under consideration and past work done in the field, particularly when such overlap is suggested to undermine the novelty of the work under consideration. Reviewers will have the opportunity to see each other's comments, and modify their own comments in response, before a decision is made on a manuscript.

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We feel consistency in peer review is important; therefore, we request that reviewers commit to reviewing future versions of the manuscript if needed.

J.8. EDITORIAL POLICIES

J.8.1. Duplicate publication

When submitting a manuscript, the authors should affirm that no similar manuscript (including book chapters) is or will be under consideration for publication elsewhere (other than as an abstract that is less than 400 words in length and contains no figures). Any unpublished articles that are related to or could be perceived to overlap with the submitted manuscript must be included for evaluation by the editors and reviewers. Doctoral theses or dissertations are not regarded as prior publications.

J.8.2. Preprint policy

Posting of manuscripts to a community preprint server by the author does not preclude consideration for publication. Authors who post their work as a preprint should identify the preprint server and include the accession number or DOI during submission. Upon publication, authors should request that the community preprint server acknowledge that the work has been published and that the journal reference (including a DOI link to the published article) be included. See Reference Guidelines for formatting of citations to manuscripts posted to preprint servers. Citation of a previous preprint version of the manuscript under consideration should be included as a footnote.

J.8.3. Animal and human studies

All animal and human studies must be conducted in compliance with relevant local guidelines, such as the US Department of Health and Human Services Guide for the Care and Use of Laboratory Animals or MRC guidelines, and must be approved by the authors' Institutional Review Board(s). A statement to this effect with the name of the approving IRB(s) must be included in the Materials and methods section. All investigations with human subjects must be conducted according to the principles expressed in the Helsinki Declaration and must include a statement that informed consent was obtained from all subjects. We strongly encourage authors to use the appropriate Reporting Guidelines for their study type.

J.8.4. Data integrity and plagiarism

All accepted manuscripts will go through a plagiarism and image screening check prior to publication. We use Crossref Similarity Check to detect for textual similarity with other publications, including instances of self-plagiarism.

Images should be minimally processed and accurately reflect the original data. We understand that image processing may be necessary and is appropriate in most instances. Our screening process examines the following: whether any specific feature within an image has been enhanced, obscured, moved, removed, or introduced; whether dividing lines are added between juxtaposed images taken from different parts of the same gel or from different gels, fields, or exposures; whether adjustments of brightness, contrast, or color balance have been applied to the entire image and that adjustments do not enhance, erase, or misrepresent any information present in the original, including the background. We also look for duplicated images within the manuscript; any reuse of images, including control data, across multiple figures should be explicitly stated and justified in the legend. Nonlinear adjustments (e.g., changes to gamma settings) must be disclosed in the figure legend or Materials and methods section.

If figure resolution or quality is insufficient for proper image screening, we will request the original data. Failure to locate original data upon request during the editorial or production process will cause delays with your manuscript. In the event that inappropriate image processing is identified prior to publication, our editors will contact the authors to discuss further. In most instances, we can resolve the issue and move forward with publication. In more serious cases where inappropriate image processing obscures or changes the conclusions of the manuscript, we may be forced to revoke acceptance.

We investigate all instances of alleged scientific misconduct identified in our published papers (including, but not limited to, plagiarism, inappropriate data processing, and duplicate publication). Depending on the outcome of our investigation, we may publish a correction, ask authors to retract their paper, or publish an editorial statement of concern.

In instances where we are considering revoking acceptance, retracting a published article, or issuing an editorial statement of concern, we will contact the corresponding author's institution during the course of our investigation. As Committee on Publication Ethics (COPE) members, we abide by COPE guidelines in managing investigations of possible misconduct.

J.8.5. Materials and data sharing

As a condition of publication, authors must make protocols and unique materials (including, but not limited to, cloned DNAs; antibodies; bacterial, animal, or plant cells; and viruses) described in our published articles freely available upon request by researchers, who may use them in their own laboratory only. All materials must be made available on request and without undue delay. If researchers are having difficulty obtaining materials from the authors of a published article, they should contact the journal's editorial office.

We encourage all authors to plan for the long-term storage and sharing of all original data underlying their manuscript. All datasets included in the manuscript must be available from the date of online publication, and the source code for all custom computational methods, apart from commercial software programs, must be made available either in a publicly available database or as supplemental materials hosted on the journal website. Numerous resources exist for data storage and sharing (see Data Deposition), and authors should choose the most appropriate venue based on their data type and/or community standard. If no appropriate specific database exists, we encourage authors to deposit their data to an appropriate publicly available database.

J.8.6. Confidentiality

All manuscript details, reviewer identities, and comments to the editors are considered privileged information and will never be disclosed to third parties. Manuscripts will be transferred only with author approval (see Transfer policy). RUP journals adhere to the Committee on Publication Ethics (COPE) Code of Conduct and Best Practice Guidelines for Journal Editors.

J.8.7. Conflict of interest

We take guidance from the National Institutes of Health and National Science Foundation in determining how to define a perceived conflict of interest. Reviewers and editors are asked to disclose any potential conflicts of interest prior to evaluating a manuscript. To avoid potential conflicts of interest, individuals should recuse themselves from evaluating a manuscript if any of the following points apply:

- The author is at the same research organization or university
- The author is a recent collaborator or trainee (less than five years), family member, or a close personal friend
- The reviewer/editor, his/her immediate family, or a close professional associate has a financial or vested interest in the manuscript

J.9. TRANSFER POLICY

J.9.1. Transfer within The Rockefeller University Press (RUP)

Authors have the option to transfer their manuscripts between The Journal of Cell Biology, The Journal of Experimental Medicine, and The Journal of General Physiology. Both peer-reviewed and non-peer-reviewed papers can be transferred. Manuscripts must no longer be under consideration before transfer is allowed. Authors choosing to transfer their manuscript may use the instructions and link provided in the editorial decision letter or may contact the editorial office directly. For peer-reviewed papers, all reviewer identities and reports will be automatically transferred; confidential comments to the editors will not be transferred. (Reviewers can opt out of allowing their identities to be transferred.) Editors at the journal selected for transfer will aim to make a decision quickly and will seek additional advice only when necessary. Please contact the journal office to opt out of having your manuscript considered for transfer to another RUP journal.

J.9.2. Transfer outside RUP

RUP is committed to limiting time spent in peer review and reducing the burden placed on reviewers and will confidentially send reviewer reports and identities upon request (confidential comments to editors will not be sent). Reviewers can opt out of allowing their identities to be transferred. Journals should contact the editorial office at RUP to submit a request for reviewer reports and identities.

We welcome submissions that include reviewer comments from another journal. The full comments of all reviewers, name of the journal, and a detailed point by point response must be provided. Although we don't require that manuscripts be revised to address the reviewers' comments prior to submission, you are welcome to do so. We accept format-neutral first submissions, so there is no need to reformat your manuscript. The manuscript and reviewer comments will be assessed by our editors; we may reach out to you or the original journal for additional information or solicit further expert advice if needed. Once we've reached a decision, we will provide a detailed letter that explains either the revisions that would be required for acceptance or our decision to decline publication. Please contact the journal office with any questions about transferring your manuscript.

J.10. OPEN ACCESS

Prior to publication, all authors should check with their funding agency to ensure they are in full compliance with access requirements. All final published content of RUP journals will be automatically posted on PubMed Central and UK PubMed Central, where it will be available to the public no later than six months after the publication date. Please see the Sherpa Romeo website or the RUP Copyright page for details on our copyright and self-archiving policies.

Authors have the option to instead pay a single article charge of \$5,000 in order to make their article open access immediately upon publication under CC-BY license. Wellcome or COAF funding recipients please note: this option must be chosen if you intend to have Wellcome/COAF cover your open access fees.

Authors will be invoiced for publication or open access fees after publication of their article. Open access is verified prior to invoicing. RUP is unable to process cancellations, refunds, or returns of open-access fees after publication.

J.11. MEDIA POLICY

All accepted papers and manuscripts under consideration are strictly embargoed until the date of publication. Authors are free to talk with journalists one week prior to online publication, provided any information exchanged is embargoed until 9:00 AM US Eastern Time on the date of publication. Any questions or issues regarding our prepublication media policy should be directed to the RUP Director of Communications and Marketing, Rory Williams (rory.williams@rockefeller.edu).

J.12. SUBMISSION GUIDELINES

J.12.1. Presubmissions

Presubmission inquiries as to the suitability of a manuscript are welcome and can be sent via our online manuscript submission system. We typically respond within two days. Presubmission inquiries are intended for informal feedback on whether the scope of a manuscript is appropriate for JCB. Manuscripts will not be formally evaluated in full unless submitted via the usual process.

For authors interested in presubmission manuscript preparation support services, RUP suggests Editage. Use of this service is not mandatory for publication, does not guarantee peer review or acceptance, and you are not obliged to submit your manuscript to one of our journals if you choose to use the service.

J.12.2. Preparing for submission to JCB

JCB accepts format-neutral first submissions. Manuscripts do not need to be formatted according to specific journal guidelines at this stage. To submit via our online system, you will need the following files and information:

J.12.3. Manuscript

You may submit your manuscript as a single PDF or as separate files. Please ensure the title page includes all authors and affiliations. A full title, abstract, and short running title are required for submission. JCB accepts submission of Articles, Reports, and Tools. If you would like to submit a Review, Viewpoint, or Spotlight for consideration, please contact the journal office at cellbio@rockefeller.edu.

J.12.4. Cover letter

Authors should provide a cover letter describing the conceptual advance of their work, related or competing papers in press or consideration elsewhere, financial conflicts of interest, and whether submission is in response to a presubmission inquiry. Authors may request a specific editor, suitable reviewers, and up to three reviewer exclusions. Requests to exclude certain individuals will be honored when possible and if the grounds for exclusion are reasonable. If the original data in the manuscript are archived in a repository and accessible for review by editors and reviewers, please state this in your letter. If you would like the editors to consider peer reviewer comments from another journal, please

include this information in your letter (as described in our Transfer policy). The cover letter will not be shared with reviewers.

J.12.5. Corresponding author

The corresponding author is responsible for the integrity of all data and text described within the manuscript; archiving all data related to the manuscript and providing original data upon request; adherence to all journal policies, including disclosure of related work from all authors; appropriate author contribution descriptions and conflict of interest disclosures from all authors; getting approval from all authors for the contents of the manuscript and submission; and checking accuracy of the proofs. In some instances, it may be appropriate to have more than one corresponding author who would share the above responsibilities.

Even in cases of co-corresponding authors, one individual must be identified within our online manuscript submission system as the primary corresponding author. This individual will be the point of contact at all times with the JCB editorial and production offices and will be responsible for appropriate materials, computer code, and data sharing with the community after publication, and acting as arbiter in instances of author disputes.

J.12.6. Funding and financial conflicts of interest

All authors must disclose any commercial affiliations or consultancies, stock or equity interests, or patent-licensing arrangements that could be considered a conflict of interest regarding the submitted manuscript. All conflicts of interest and sources of funding should be included in the Acknowledgments section of the manuscript. We use text mining of the Acknowledgments to identify funding data for deposition with CrossRef and information sharing with funders. Therefore, it is important to use full, accurate agency names and grant numbers.

J.12.7. Revised submissions

Manuscripts invited to resubmit must be formatted according to all journal guidelines. General guidelines are included below. Detailed guidelines on figure and character limits based on article type can be found in our Manuscript types section. Authors should provide high-resolution individual figure files when they resubmit their revised manuscripts.

J.12.8. Response to reviewers

Revised manuscript submissions must include a point by point response to the reviewers'

comments. The response file should detail how each comment was addressed experimentally and/or textually or why it could not be/was not addressed. The response to reviewers is shared with all reviewers. Authors may include data in their responses to the reviewers that they do not wish to include in the manuscript.

J.13. MANUSCRIPT ORGANIZATION AND FORMATTING

J.13.1. Title

The title should be less than 100 characters (including spaces). Make the title concise and accessible to a general readership. A condensed title of less than 50 characters (including spaces) is also needed.

J.13.2. Authors

Full author names must be provided along with their institutional affiliations where the work was done. Names, affiliations, and author order should be checked carefully before resubmission. If a change of address is imminent for any author, indicate the change and the date effective. Corresponding author(s) must be identified, with ORCID, email, and full mailing address.

J.13.3. Summary

Provide a short, ~40-word summary statement for the online JCB table of contents and alerts. This summary should describe the context and significance of the findings for a general readership; it should be written in the present tense and refer to the work in the third person.

J.13.4. Abstract

Abstracts must not exceed 160 words. The abstract should describe the relevant background, key results, and conceptual significance of the findings in a way that is accessible to a broad audience. Abstracts should not include references.

J.13.5. Introduction

The Introduction should provide sufficient background to make the article accessible to non-expert readers; it should indicate what hypotheses were tested and provide sufficient context to make the significance of the problem studied and the rationale for the experiments clear to a broad audience.

J.13.6. Results

The Results section describes the experiments performed and presents the findings observed. This section should be divided into subheadings. For short Reports, the Results and Discussion sections should be combined.

J.13.7. Discussion

The Discussion summarizes the conclusions that can be drawn from the results, as well as the implications of the research. It should place your findings in the general context of the field and discuss any limitations of the experimental approaches you used. Subheadings may be used.

J.13.8. Materials and methods

RUP journals are signatories of the NIH Reproducibility guidelines. There are no limits on the length of JCB Materials and methods sections. JCB does not permit supplemental Materials and methods. Materials and methods sections should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. JCB does not permit citations of non-peer-reviewed publications or unpublished data in the Materials and methods section. If you feel readers would benefit from a step-by-step description of a method employed in your paper we encourage authors to consider submitting a detailed protocol to Bio-protocol.

J.13.9. Reporting guidelines

We encourage all authors to utilize the EQUATOR network to identify appropriate reporting guidelines based on study type. Authors should refer to the BRISQ reporting guidelines for any study in which human biospecimens are used. For authors reporting animal research, we encourage use of the ARRIVE guidelines. For studies using cell lines, authors should report the source of the line, whether the line has been authenticated and how, and the mycoplasma contamination status.

J.13.10. Reagents

All antibodies, cell lines, animals, and tools used in the manuscript should be described in full, including accession numbers for materials available in a public repository such as the Resource Identification Portal. For unique reagents such as antibodies, siRNA, primers, or other probes, authors should provide the full epitopes or sequences in the manuscript.

Please list chemicals and scientific instruments used and their manufacturer.

J.13.11. Microscope image acquisition

The following information must be provided about the acquisition and processing of images: make and model of microscope; type, magnification, and numerical aperture of the objective lenses; temperature; imaging medium; fluorochromes; camera make and model; acquisition software; and any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstitutions, surface or volume rendering, gamma adjustments, etc.). If you export files from a microscope or other acquisition device, be sure to use consistent file formats (8 bit, 16 bit, etc.).

J.13.12. Statistical analysis

Authors should provide clear, detailed descriptions of their statistical analysis in the Materials and methods section and/or figure legends, including but not limited to the statistical test used, actual p-values, number of biological and technical replicates, measure of center, and measure of variability. If an editor or peer reviewer feels it necessary, we may request expert feedback specifically on statistical analysis within the manuscript.

J.13.13. Supplemental material

Supplemental material is limited to figures, tables, large datasets, and videos. JCB does not permit supplemental text other than figure and table legends. A short summary paragraph of all supplemental material must appear at the end of the Materials and methods section.

J.13.14. Acknowledgments

The Acknowledgments should recognize contributions from non-authors and disclose all funding sources and financial conflicts of interest. If there are no potential financial conflicts of interest, please add the following statement to the Acknowledgments section: "The authors declare no competing financial interests." (If potential conflicts are listed, the statement "The authors have no additional competing financial interests" should be added instead.)

J.13.15. Author contributions

A separate author contribution section is required following the Acknowledgments in all research manuscripts. All authors should be mentioned and designated by their full names. We encourage use of the CRediT nomenclature.

J.13.16. References

All in-text citations and references must be formatted according to the JCB Reference Guidelines. In-text citations should be Harvard style and listed alphabetically by first author's last name in the Reference section. There is no limit to the number of references cited in a manuscript. JCB strongly encourages authors to cite the primary literature rather than review articles throughout their manuscripts.

JCB discourages citation of "data not shown," "manuscript in preparation," "manuscript submitted," etc., in any section of the manuscript. If included, "personal correspondence" must be accompanied by a signed letter of permission and may only be cited in the text.

Preprints may be cited in the text and included in the Reference section. Appropriate formatting is described in the JCB Reference Guidelines. Note that citation of a previous preprint version of the manuscript under consideration should be included as a footnote.

J.13.17. Abbreviations

A term that does not appear on the JCB standard abbreviations list must be used at least three times in a paper to qualify as an abbreviation. Spell out the term on first mention, and follow it with the abbreviated form in parentheses. Supply a list of nonstandard abbreviations used in the paper, in alphabetical order, giving each abbreviation followed by its spelled-out version.

J.13.18. Figures

All submitted images must conform to the JCB Figure and Video Guidelines. Figures should be cited in numerical order in the text in the order they are mentioned. Figures should be prepared at publication size; whenever possible, figures will be reduced to a single-column width (85 mm). If you have any questions regarding figure preparation, please contact one of our in-house specialists at production@rockefeller.edu. All accepted figures will go through an image screening process prior to publication, as described in our Data integrity and plagiarism section.

J.13.19. Videos

All submitted videos must conform to the Figure and Video Guidelines. Videos must be cited both in the Results section and in the legends of any figures that contain video stills or images related to the video. Videos are peer reviewed with the manuscript. A maximum of 10 videos may be posted for each article.

J.13.20. Figure and video legends

All figures and videos must have titles and detailed legends. Figure legends should contain sufficient information so that readers can understand what the data show without needing to refer back to the text. All symbols, such as arrows, must be defined in the legend. Scale bar measurements must be included in the legend. Where appropriate, legends should include any statistical test used, actual p-values, number of biological and technical replicates, measure of center, and measure of variability.

Each video legend should describe what is being shown, the cell type or tissue being viewed (including relevant cell treatments, concentration and duration, or transfection), the imaging method (e.g., time-lapse epifluorescence microscopy), what each color represents, how often frames were collected, the frames/second display rate, and the number of any figure that has related video stills or images.

J.13.21. Tables

Include a table number and title for each table. Insert explanatory material and footnotes below the table, designating footnotes using lowercase superscript letters reading horizontally across the table. Supply units of measure at the heads of the columns. Abbreviations that are used only in a table should be defined in the table legend. Do not divide tables into subtables.

J.13.22. Nomenclature

JCB follows the abbreviations and other conventions of *Scientific Style and Format: The CSE Manual for Authors, Editors, and Publishers*. For chemical nomenclature, follow the Subject Index of Chemical Abstracts. Capitalize trade names and give manufacturers' names.

Gene names must be italicized. Authors must use the original name published for a gene unless they have obtained permission to rename the gene from the authors of the original study (or from a governing body such as, in the case of a yeast gene, the *Saccharomyces* Genome Database curator). Please follow the generally accepted rules for gene and protein nomenclature appropriate for the organism you are discussing.

Gene nomenclature guidelines for:

- humans (<http://www.genenames.org/about/guidelines>),
- rats and mice (<http://www.informatics.jax.org/mgihome/nomen/gene.shtml>),
- frogs (<http://www.xenbase.org/gene/static/geneNomenclature.jsp>),

- zebrafish
(<https://wiki.zfin.org/display/general/ZFIN+Zebrafish+Nomenclature+Guidelines>),
- *Drosophila* (<http://flybase.org/wiki/FlyBase:Nomenclature>),
- *C. elegans* (<http://www.wormbase.org#012-34-5>),
- *Arabidopsis* (<https://www.arabidopsis.org/portals/nomenclature/guidelines.jsp>) are available at the indicated sites.

J.13.23. Accepted manuscripts

Before we can accept your manuscript, the files and information below must be received by the editorial office.

J.14. MANUSCRIPT FILES

High-resolution, editable, individual figure files are required, preferably in the original file format in which they were created. The manuscript text must be provided as a DOC or DOCX file.

J.15. LICENSE TO PUBLISH

A license to publish must be signed by the primary corresponding author prior to acceptance. View our license to publish agreement for more details.

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J.18. COVER SUBMISSIONS

Authors whose manuscripts are accepted for publication may submit cover images and a brief legend for consideration. Images submitted should be at least 300 dpi at the size of publication (8.75" W x 11.25" H). Please submit cover suggestions via our online manuscript submission system when you upload final files prior to editorial acceptance.

J.19. PRODUCTION AND PROOFS

After acceptance, your manuscript will be sent to production for copy editing and preparation of the proofs. If you will be unavailable at any time while your manuscript is in production, please contact production@rockefeller.edu.

J.20. PROOFS

Corresponding authors will be emailed a link to proofs within two weeks after acceptance. Authors may choose to edit either the HTML or PDF versions of their proofs. Proofs must be returned within 48 hours.

J.21. DAILY PUBLICATION

Articles are usually published within 48 hours after the author returns corrected proofs. This can vary depending on the extent of requested changes. Publication date also depends on whether the article has a companion article or press coverage. On publication day, the articles are usually posted before 10AM EST. See our FAQ on daily publication for more details.

J.22. AUTHOR FEES

Authors will be charged a \$2,000 flat fee for all publication charges. An author's inability to pay these charges will not affect the publication of acceptable manuscripts.

Authors have the option to instead pay a single article charge of \$5,000 in order to make their article open access immediately upon publication under CC-BY license.

J.23. REPRINTS AND JOURNAL COPIES

You will have the opportunity to order paper reprints of your article, print copies of the issue in which your manuscript will appear, or cover posters when you return your proofs.

J.24. COPYRIGHT POLICY

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J.25. APPEALS

Authors wishing to appeal an editorial decision should do so through the online manuscript submission system by clicking on the “Submit Appeal” link in the relevant manuscript record. A rebuttal letter and—if appropriate—a point by point response to the reviewers should be provided. A decision is usually communicated to the authors within 10 days. Please note that editors prioritize handling new submissions that have not yet received a first decision over appeals. JCB does not allow multiple appeals on a manuscript.

J.26. CORRECTIONS AND REFUTATIONS

Corrections will be published at the discretion of the editors. Contact the journal office at cellbio@rockefeller.edu to request a correction to a published article.

JCB considers submissions aimed at refuting conclusions previously published in the journal. Such manuscripts should provide direct and definitive evidence that the conclusions in a previous JCB paper are incorrect. Please indicate in your cover letter that your manuscript is submitted as a refutation. The refutation will be peer reviewed, and the authors of the original work will be given the opportunity to submit a response for editorial consideration. Manuscripts that describe differing observations or draw different conclusions than past work through the use of new approaches or tools are a normal part of the scientific discourse and will not be considered as refutations. For more information, please contact the JCB editorial office at cellbio@rockefeller.edu.

J.27. REFERENCES

The Journal of Cell Biology. 2017. Author guidelines. URL: <http://jcb.rupress.org/foraDate> of access: 02 November 2017.

APPENDIX K

CERTIFICATE OF ANALYSIS SODIUM ALGINATE

SIGMA-ALDRICH

sigma-aldrich.com

3050 Spruce Street, Saint Louis, MO 63103, USA

Website: www.sigmaaldrich.com

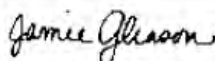
Email USA: techserv@sial.com

Outside USA: eurtechserv@sial.com

Certificate of Analysis

Product Name:
Sodium alginateProduct Number: W201502
Batch Number: MKBN7680V
Brand: ALDRICH
CAS Number: 9005-38-3
MDL Number: MFCD00081310
Quality Release Date: 27 FEB 2013

Test	Specification	Result
Appearance (Color)	Tan to Light Brown	Tan with an Orange cast
Appearance (Form)	Solid	Solid
Infrared spectrum	Conforms to Structure	Conforms
Loss on Drying	≤ 15.0 %	11.8 %
Viscosity	5.0 - 40.0 cps	12.0 cps
c = 1%, Water @ 25°C		
pH	5 - 8	5
c = 1%, Water @ 25°C		
Arsenic (As)	≤ 3.0 ppm	< 1.0 ppm
Cadmium (Cd)	≤ 1.0 ppm	< 1.0 ppm
Mercury (Hg)	≤ 1.0 ppm	< 1.0 ppm
Lead (Pb)	≤ 10.0 ppm	< 1.0 ppm

Jamie Gleason, Manager
Quality Control
Milwaukee, Wisconsin US

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