Synthesis, characterisation and properties of fulvic acid, derived from a carbohydrate

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Thesis submitted for the degree Doctor Philosophiae in Pharmaceutical Chemistry at the Potchefstroom Campus of the North-West University

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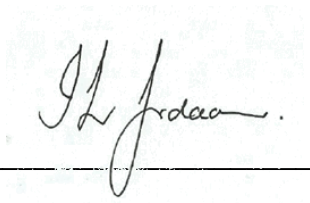
DECLARATION

This thesis is submitted in fulfilment of the requirements for the degree of Philosophiae Doctor in Pharmaceutical Chemistry, at Centre of Excellence for Pharmaceutical Sciences, North-West University.

I, Imelda Latitia Jordaan, hereby declare that this thesis with the title:

Synthesis, characterisation and properties of fulvic acid, derived from a carbohydrate

is my own work and has not been submitted at any other University either whole or in part.



Imelda Latitia Jordaan

Signed at Stellenbosch on the 22nd day of November, 2018

PREFACE

This study originated from my passion for pharmaceutical chemistry, the science of discovering new medicines. Working with fulvic acid for several years and noticing the pharmacological efficacy in human and animal health and the physicochemical properties has driven my curiosity to "unlock" the science in the chemical composition of CHD-FA. I believe that this study offers a new platform in biotechnology.

I could not have achieved this goal without the encouragement, support and assistance of:

Prof. A. Petzer, Prof. J.P. Petzer and Prof. P.J. Milne, my promotors, for their guidance, support and technical assistance. This study is an acknowledgement of their insight and immense knowledge in the field of pharmaceutical chemistry.

Dr. G. Jordaan, my mentor and husband, for his continuous support and motivation. His enthusiasm has driven me to ensure that this study presents a comprehensive understanding of CHD-FA and its potential use in natural medicine.

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FulHold Pharma Ltd. for their financial support to ensure that CHD-FA takes its rightful place in natural medicine.

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Amorie, my daughter for caring to ensure that the technical layout of the thesis and articles meets with the requirements of NWU and journal publishers. Her attention to detail in editing has ensured excellence in the standard and quality of this thesis.

Gerhard and Juriaan, my two sons who have always motivated and supported me. They believed that my work presents the pharmaceutical industry with a "gold standard" for the development of new and innovative medicines.

My parents for love and wisdom and for never failing to be there for support.

Most importantly my CREATOR, for strength, perseverance and providing each new day filled with opportunities. HIS grace has ensured the successful completion of this thesis.

Imelda L. Jordaan, November 2018.

ACKNOWLEDGEMENT

The author wishes to acknowledge the pioneering work of Mr. Rudolph Malan. Mr Malan is credited for being the founder of Carbohydrate-Derived Fulvic Acid (CHD-FA). This is his story:

Fulvic acid, derived from a carbohydrate source, sucrose, was discovered by Mr. Malan in 2005. He was involved for more than 2 decades as an expert technologist on wet oxidation processes in the production of oxihumic and oxifulvic acids. Mr. Malan assisted in the development and testing of a wet oxidation process reactor and to assist in a research and development project to oxidise waste material. In order to test the reactor required an energy source that would have the capacity to generate enough energy to start the exothermic reaction. Mr. Malan decided on a solution of sucrose and water and placed it with oxygen under increased temperature and pressure in the reactor. On successfully completing the testing of the technical capabilities of the reactor, the “end product” was drained and Mr. Malan immediately recognised the smell of the “end product” as fulvic acid. He realised instinctively that he had created a “brown fulvic acid” solution. His new invention was tested by the University of Pretoria and it was concluded that the wet oxidation of sucrose, water and oxygen at high temperature and pressures performed by Mr. Malan, yielded a pure form of fulvic acid. This was subsequently referred to as Carbohydrate-Derived Fulvic Acid (CHD-FA). Follow-up tests by Mr. Malan with fructose, dextrose and glucose have also produced fulvic acid, but at a much lower yield.

Mr Malan’s passion inspired me to seek answers on the chemistry of this complex and intriguing molecular structure.

ABSTRACT

Key terms: Carbohydrate-Derived Fulvic Acid, non-catalytic wet oxidation process, NMR, GC-MS, LC-MSMS, MALDI-TOF MS, supramolecular structure, backbone structures, anti-inflammatory, antimicrobial, antifungal, antiviral, antioxidant.

Introduction:

Human and animal health is constantly threatened by pathogenic microbes and the emergence of bacteria resistant to standard medicinal interventions has escalated the search for new and innovative pharmaceutical solutions. Natural medicines have received much attention in recent years as a potential answer to the problem of “super bugs”. Fulvic acid is a composition of organic acids found in nature and known for its anti-inflammatory, antimicrobial and antioxidant properties. Unfortunately, the detection of heavy metals embedded in the molecular structure of fulvic acids extracted from numerous environmental sources has rendered it unsafe for medicinal applications. A new invention by Fulhold Pharma Ltd to synthetically produce fulvic acid from sucrose, identified as Carbohydrate-Derived Fulvic Acid (CHD-FA), is a major international breakthrough in the production of a heavy metal free fulvic acid. CHD-FA is produced through a non-catalytic wet oxidation process and complies with standardised product specifications for molecular consistency and safety. CHD-FA has anti-inflammatory, antimicrobial and antioxidant therapeutic health benefits.

Purpose:

- To propose a theoretical model for the compound identified as the major constituent of CHD-FA.
- The identification of the backbone structures embedded in CHD-FA.
- To review the pharmacological properties of CHD-FA based on the composition of the backbone structures embedded in the molecular composition of CHD-FA’s cluster structure with the emphasis on anti-inflammatory, antibacterial, antifungal, antiviral and antioxidant properties.

Methods:

- A theoretical model, based on literature, was developed to describe the mechanisms involved in the non-catalytic wet oxidation process that has transformed sucrose into the major component (anhydrofulvic acid) of CHD-FA.

- Samples were collected for each of the different phases of the non-catalytic wet oxidation process, from the start-up of the reactor to the start of the exothermic reaction. Nuclear magnetic resonance spectroscopy (NMR) and liquid chromatography-tandem mass spectrometry (LC-MSMS) was used to analyse these samples to identify molecular changes during the various stages of this process.
- Gas chromatography-mass spectrometry (GC-MS), Fourier's transform infrared (FTIR) and NMR were used to provide general information on the mixture.
- Matrix-assisted laser desorption and ionisation time-of-flight mass spectrometry (MALDI-TOF MS) was used to desorb and ionise CHD-FA without fragmentation, in order to identify and measure the absolute molecular weight of the main component in CHD-FA.
- LC-MSMS was used to identify the most prominent backbone structures embedded in the CHD-FA molecular structure. These compounds were mainly identified by injecting a sample of CHD-FA in the LC-MSMS, identifying the major mass ions, generating empirical formulas associated with these peaks and then using software to predict molecular structures associated with these compounds.
- The clinical applications associated with the anti-inflammatory, antimicrobial, antifungal, antiviral and antioxidant properties of CHD-FA were assessed through a comprehensive literature review of the characteristics of the backbone structures in the molecular structure of CHD-FA.

Results:

Objective 1: The detailed description of the theoretical pathway for the synthesis of the major component of CHD-FA, namely molecular fulvic acid, has provided evidence that the non-catalytic wet oxidation synthetic process of sucrose to produce molecular fulvic acid is a one-pot synthesis process consisting of a myriad of chemical reactions in the reactor. Colour changes in the reactor solution have confirmed the theoretical pathway description of a step-by-step process. The colours changed progressively from a light yellow to a dark brown colour.

NMR and LC-MSMS analyses have confirmed that the colour changes demonstrated the transformation of sucrose into molecular fulvic acid. GCMS analysis revealed a concord between the structure of CHD-FA and penicillin-derivated fulvic acid. The MALDI-TOF MS identified 308 g/mol as the highest intensity peak with a natural abundance of 20.8 % in the spectrum and confirmed it as the most prominent component in CHD-FA. Batch-to-batch consistency of CHD-FA was recorded by chromatographic and spectroscopic data for more than 30 production runs over a four year period.

Objective 2: Similarities between the spectroscopic data of CHD-FA and literature data from environmental fulvic acids were indicated by FTIR and ^{13}C NMR. However, CHD-FA has unique characteristics which differentiate it from environmental fulvic acids. CHD-FA has more carboxyl, ester, amide and aliphatic carbons in its molecular structure compared to the fulvic acid reference standards from the International Humic Substances Society.

GC-MS confirmed the complexity of the molecular CHD-FA structure. The chromatogram overlay of CHD-FA and the reference standard, penicillin-derived fulvic acid (CAS 479-66-3), confirmed the presence of fulvic acid in CHD-FA.

The most prominent component of the molecular structure of CHD-FA shown by LC-MSMS spectrum is 7,8-dihydroxy-3-methyl-10-oxo-1*H*,10*H*-pyrano[4,3-b]chromene-9-carboxylic acid with the empirical formulae of $C_{14}H_{10}O_7$. This component is the dehydrated analogue of fulvic acid ($C_{14}H_{12}O_8$) indicative of a loss of a water molecule during sample preparation. The LC-MSMS shows molecular ion m/z 290 and 24 prominent peaks, which represents the key structures in CHD-FA. It is evident that CHD-FA is a cluster of organic compounds. 24 prominent peaks were characterised as the backbone structures embedded in CHD-FA. This, with reference to the molecular composition of CHD-FA, is the most significant finding of the present study.

Malic acid, maleic acid, levulinic acid, succinic acid, propenoic acid, phthalic acid, arabonic acid, itaconic acid, glucuronic acid, glutaric acid, benzene tri- and tetracarboxylic acids were identified as the backbone structures of CHD-FA. These backbone structures are interlinked with each other and with the parent structure via intermolecular bonding to form a cluster molecular structure.

Objective 3: A comprehensive literature review of the clinical properties of the backbone structures of CHD-FA has demonstrated anti-inflammatory, antibacterial, antifungal, antiviral and antioxidant properties. These properties are therefore embedded in CHD-FA.

Conclusion:

CHD-FA, derived synthetically from sucrose through a non-catalytic wet oxidation process, is a pure form of fulvic acid. CHD-FA has the same medicinal properties as penicillin-derived reference standard fulvic acid and fulvic acids derived from various environmental sources. The identification of the twenty four backbone structures embedded in the supramolecular structure of CHD-FA is evidence that CHD-FA is a cluster of organic structures. This cluster of organic structures is responsible for the unique characteristics of CHD-FA, which include anti-

inflammatory, antimicrobial and antioxidant properties. The batch-to-batch consistency demonstrated for the manufacturing of CHD-FA in this study offers much potential for the use of CHD-FA as a natural pharmaceutical compound in the development of natural medicines.

OPSOMMING

Sleutelterme: Koolhidraat-afgeleide fulviensuur, nie-katalitiese nat-oksidasie, KMR, GC-MS, LC-MSMS, MALDI-TOF MS, supramolekulêre strukture, ruggraatstrukture, anti-inflammatories, antibakteries, antifungus, antiviraal, antioksidant.

Die ontstaan van weerstandbiedende patogene teen standaard medikasie het die behoefte laat ontstaan vir die ontwikkeling van innoverende farmaseutiese oplossings vir hierdie probleem. Natuurlike medisynes word beskou as ’n moontlike oplossing in die behandeling van die sogenaamde “super bugs”. Fulviensuur is ’n mengsel van organiese sure wat bekend is vir sy anti-inflammatoriese, antimikrobiese en antioksidantiese eienskappe. Ongelukkig is daar swaarmetale teenwoordig in die molekulêre strukture van verskeie fulviensure wat uit verskillende bronne geïsoleer is, wat dit onveilig maak vir medisinale gebruik. Die sintetiese vervaardiging van fulviensuur uit suiker, bekend as koolhidraat-afgeleide fulviensuur (CHD-FA), is ’n nuwe ontdekking wat deur Fulhold Pharma Ltd.gemaak is, en word beskou as ’n ’n groot internasionale deurbraak in die vervaardiging van swaarmetaalvrye fulviensuur. CHD-FA word vervaardig deur ’n nie-katalitiese nat-oksidasie proses en die vervaardiging van CHD-FA lewer fulviensuur wat voldoen aan veiligheids- en kwaliteits-gestandardiseerde produkspesifikasies. Dit het gesondheidsvoordele wat berus op die anti-inflammatoriese, antimikrobiese en antioksidant eienskappe van CHD-FA.

Doelwitte:

- Die voorstelling van ’n teoretiese model vir die meganismes betrokke in die chemiese omskakeling van sukrose na CHD-FA tydens die nie-katalitiese nat-oksidasie proses.
- Die identifisering van die sleutelstrukture in CHD-FA.
- Die ontleding van die anti-inflammatoriese, antimikrobiese, antifungus, antivirale en antioksidant eienskappe van CHD-FA soos in die literatuur beskryf met die klem op aktiwiteite van die sleutelstrukture in CHD-FA.

Metode:

- ’n Teoretiese model is voorgestel om die meganisme vir die omskakeling van sukrose na CHD-FA te verduidelik.
- CHD-FA monsters is geneem tydens die opeenvolgende reaksies wat tydens die nie-katalitiese nat-oksidasie proses voorgekom het. Monsters is geneem tydens die aanvang van die proses in die reaktor tot en met die aanvang van die eksotermiese

reaksie. Die analise van die monsters is met kernmagnetieseresonansie (KMR) spektroskopie en vloeistof chromatografie-tandem massaspektrometrie (LC-MSMS) uitgevoer.

- Die chemiese en spektroskopiese eienskappe van CHD-FA is met gas chromatografie massaspektrometrie (GC-MS), KMR en LC-MSMS ontleed.
- Fourier-transformasie infrarooi spektroskopie (FTIR), KMR, GC-MS, LC-MSMS en matriks-ondersteunde laser desorpsie en ionisering tyd-vlug massa-spektrometrie (MALDI-TOF MS) is aangewend vir die karakterisering van CHD-FA en die identifisering van die mees prominente sleutel strukture in CHD-FA.
- 'n Literatuur oorsig is gebruik om die anti-inflammatoriese, antimikrobiese, antifungus, antivirale en antioksidant eienskappe van CHD-FA te beskryf. Hierdie studie plaas die klem op die eienskappe van die individuele sleutelstrukture in CHD-FA.

Resultate:

Doelwit 1: Die omvattende beskrywing van die nie-katalitiese nat-oksidasie sintetiese proses het bewys dat die omskakeling van sukrose na CHD-FA 'n eenpotstelsel proses is wat uit verskeie opeenvolgende chemiese reaksies bestaan. Hierdie reaksie word deur opeenvolgende kleurveranderinge van liggeel tot donkerbruin in die reaktoroplossing gedemonstreer. KMR en LC-MSMS analise het die afbraak en omskakeling van sukrose na 'n supramolekulêre fulviensuurstruktuur, CHD-FA, bevestig.

Die GC-MS analise het getoon dat fulviensuur in CHD-FA identies is aan die penisillien-afgeleide fulviensuur. Die MALDI-TOF MS het bevestig dat die mees prominente struktuur van CHD-FA 'n molekulêre massa van 308 g/mol het en dit is 20.8 % van CHD-FA.

Ooreenstemmende chromatografiese en spektroskopiese profiele het getoon dat meer as 30 CHD-FA bondelprodukte wat oor 'n tydperk van 4 jaar vervaardig is, soortgelyk aan mekaar is.

Doelwit 2: FTIR en KMR het ooreenkomste tussen CHD-FA en fulviensure wat uit natuurlike bronne ontgin word geïdentifiseer. CHD-FA het egter unieke eienskappe wat dit onderskei van fulviensuur wat uit die omgewing verkry is. KMR het getoon dat karboksiel, ester, amied en alifatiese koolstowwe in 'n hoër mate teenwoordig is in CHD-FA as in die verwysingstandaarde van die Internasionale Humus Substanse Vereniging.

GC-MS het die kompleksiteit van die molekulêre CHD-FA struktuur bevestig. Chromatografiese vergelyking met die verwysingsstandaard, penisillien-afgeleide fulviensuur (CAS 479-66-3), het bevestig dat fulviensuur met 'n empiriese formule van $C_{14}H_{12}O_8$ in CHD-FA teenwoordig is.

GC-MS het verder ook ’n defragmenteringspatroon vir die molekulêre struktuur van CHD-FA geïdentifiseer. Die fragmentasie patrone toon ’n konstante massaverlies wat op ’n gekoördineerde struktuur met ’n polimeer karakter dui.

Die mees prominente komponent in die CHD-FA struktuur soos deur die LC-MSMS geïdentifiseer, is 7,8-dihidroksie-3-metiel-10-okso-1*H*,10*H*-pirano[4,3-b]chromeen-9-karboksielsuur met ’n empiriese formule van $C_{14}H_{10}O_7$. Hierdie komponent is die gedehidreerde analoog van fulviensuur ($C_{14}H_{12}O_8$) wat dui op die moontlike verlies van ’n watermolekule gedurende die voorbereiding vir analise. Die LC-MSMS spektrum toon ’n molekulêre ioon m/z 290 en 24 prominente pieke wat die sleutelstrukture in CHD-FA verteenwoordig. Hierdie inligting was die mees beduidende bevinding van hierdie studie oor die molekulêre struktuur van CHD-FA.

Die sleutelstrukture van CHD-FA is appelsuur, maleïensuur, levuliensuur, suksiensuur, akrielsuur, ftaalsuur, araboniese suur, itakonsuur, glukoroonsuur, glutaarsuur, aromatiese tri- en tetra-karboksielsure. Hierdie komponente word deur middel van intermolekulêre bindings met die primêre komponent en met mekaar verbind om sodoende die supramolekulêre struktuur van CHD-FA te vorm.

Doelwit 3: ‘n Omvattende literatuuroorsig toon dat die sleutelkomponente in CHD-FA oor anti-inflammatoriese, antimikrobiese, antifungus, antivirale en antioksidantiese eienskappe beskik. Hierdie komponente is dus gesamentlik verantwoordelik vir die kliniese eienskappe van CHD-FA.

Gevolgtrekking:

CHD-FA is ’n suiwer fulviensuur wat deur die omskakeling van sukrose na CHD-FA gedurende ’n sintetiese nie-katalitiese nat-oksidasie proses geproduseer word. CHD-FA toon ooreenstemmende karakteristieke eienskappe met penisillien-afgeleide fulviensuur en fulviensuur wat uit natuurlike hulpbronne ontgin word.

CHD-FA het ’n supramolekulêre struktuur wat deur vier-en-twintig prominente sleutel-karboksielsure gekenmerk word. Die teenwoordigheid van hierdie sleutelstrukture in ’n saamgestelde supramolekulêre eenheid bepaal die kliniese eienskappe van CHD-FA wat anti-inflammatoriese, antibakteriese, antifungus, antivirale en antioksidant aktiwiteite insluit.

Die huidige studie het bewys dat CHD-FA ’n natuurlike farmaseutiese aktiewe bestanddeel is vir gebruik in die vervaardiging van natuurlike medisyne.

THESIS LAYOUT

This PhD thesis is a compilation in article format as approved by the North-West University, Potchefstroom. The main body of the thesis (methodology and experimental data) are presented in three manuscripts. The manuscripts are currently submitted for publication in international peer-review journals.

Chapter 1 is the introduction to the study and provides a concise motivation to the background of the problem statement, study hypothesis and aims of this study.

Chapter 2 is a comprehensive literature review of the origin, synthesis, character and properties of fulvic acid and provides the background to the referenced literature in the three manuscripts.

The bibliography for Chapters 1 and 2 is presented at the end of each chapter.

Chapters 3, 4 and 5 are the three manuscripts respectively and contain the key findings of this study. The manuscripts are presented in the required format prescribed by *Instructions for Authors* as outlined on the website of each respective journal.

Chapter 3 presents the “ready for submission” manuscript for publication in *ChemEngineering* published by MDPI entitled “Identification of the major constituent of CHD-FA and a theoretical model for the mechanism by which molecular fulvic acid is formed from sucrose through a non-catalytic wet oxidation process.”

Chapter 4 presents the “ready for submission” manuscript for publication in *Molecules* published by MDPI entitled: “Characterisation of Carbohydrate-Derived Fulvic Acid and identification of the backbone structures embedded in fulvic acid structure.”

Chapter 5 presents the “ready for submission” manuscript for publication in the *International Journal of Molecular Sciences* published by MDPI entitled: “Carbohydrate-Derived Fulvic Acid (CHD-FA) is a unique supramolecular fulvic acid with biological properties”

The bibliography for Chapters 3, 4 and 5 are provided separately for each manuscript.

Chapter 6 is the final chapter of the thesis and is a summary of discussions and concludes the study as a whole, incorporating the data presented in the three manuscripts.

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ABBREVIATIONS

µl	microlitre
5-HMF	hydroxymethyl-2-furfural
ACP	acid phosphatase activities
AKP	alkaline phosphatase activities
API	active pharmaceutical ingredient
ASI	acne severity index
ATP	adenosine triphosphate
BCFA	brown coal fulvic acid
BSTFA	[N,O-Bis(trimethylsilyl)trifluoroacetamide]
C NMR	carbon-13 nuclear magnetic resonance
C=O	carbonyl
CAS	chemical abstracts service
CBZ	carbamazepine
CD	IL-2 receptor alpha chain
CD4	T-cell count
CHCA	α-cyano-4-hydroxycinnamic acid
CHD-FA	Carbohydrate-derived Fulvic Acid
CHIKV	chikungunya virus
cm-1	frequency
-COOH	carboxyl
COX	cyclooxygenases
CR3	Neutrophils express receptors
CS	chondroitin sulphate
D_2O	deuterium oxide
DNA	deoxyribonucleic acid
Da	dalton
DEN2	dengue virus type 2
DENV2	Dengue virus
DPPH	diphenyl-2-picryl-hydrazyl
EHEC	enterohemorrhagic
e.g.	for example
EMP	Embden–Meyerhof–Parnas pathway
F0210	formulated wellness drink
FMLP/CB	N-formyl-methionyl-leucyl-phenylalanine / cytocalasin B
FTIR	Fourier transform infrared spectroscopy
g/mol	gram per molar mass
GC-MS	Gas chromatography-mass spectrometry
GMP	good manufacturing practice
H	hydrogen
H NMR	Hydrogen-1 nuclear magnetic resonance
H4btec	1,2,4,5- benzenetetracarboxylic acid
Hep3B	hepatoma liver cell line

HIV	Human Immunodeficiency virus
HL60	leukemia cell line
HMP	hexose monophosphate shunt pathway
HMW	high molecular weight
hPiV3	human parainfluenza virus
HTLV-1	Human T-Cell Lymphotropic virus
IC_{50}	half maximal inhibitory concentration
IFN-β	interferon-β
IgM	immunoglobulin M
IHSS	International Humic Substances Society
IL-10	interleukin 10
IL-6	interleukin 6
IL-8	interleukin 8
IR	infra-red
IRG1	immuneresponsive gene 1
K_3NOSepiLMW	O-sulphated heparin-like semi-synthetic polymer
kHz	kiloherts
KMR	kern magnetiese resonansie spektroskopie
LBEA	Lobry de Bruyn-Alberda van Ekenstein
LcCL	lucigenin-enhanced chemiluminescence
LC-MS	liquid chromatography mass spectrometry
LC-MSMS	liquid chromatography tandem mass spectrometry
LC-QTOF-MS	liquid chromatography coupled with quadrupole time-of-flight mass spectrometry
LDH	lactate dehydrogenase
$LiTaO_3$	lithium tantalate
LLC-MK2	Rhesus monkey kidney cells
LMW	low molecular weight
LNCaP	lymph node carcinoma of the prostate
LPS	lipopolysaccharide
m/z	mass-to-charge ratio
MALDI-TOF MS	matrix-assisted laser desorption and ionisation time-of-flight mass spectrometry
MAP	microtubule associated protein
MCF-7	Michigan Cancer Foundation-7
MCFAs	medium-chain fatty acids
mg/g	milligram per gram
MHz	megahertz
Mi, m/z	molecular mass
MIC	minimum inhibitory concentration
MMP9	matrix metalloproteinase
Mn	number average molecular weight
MPO	myeloperoxidase
MRSA	Methicillin Resistant *Staphylococcus aureus*
MS	mass spectrometer
Mw	weight average molecular weight
Mw/Mn	polydispersity

-N=	tertiary amine
NADPH	nicotinamide adenine dinucleotide phosphate hydrogen
NF-κb	nuclear factor kappa B
-NH-	secondary amine
$-NH_2$	primary amine
Ni	peak integration
NIST	National Institute of Standards and Technology
NMR	nuclear magnetic resonance spectroscopy
NOE	Nuclear overhauser effect
NSAIDs	non-steroidal anti-inflammatory drugs
O_2	oxygen
OAs	organic acids
OFA	oxifulvic acid
-OH	hydroxyl
pH	potential of Hydrogen
PHA	phytohaemagglutinin
pKa	acid dissociation constant
PMA	pharbol myristate acetate
ppm	parts per million
PTGS2	prostaglandin endoperoxide synthase 2
PXR	pregnane X receptor
QTOF	quadrupole-time-of-flight
RBCs	red blood cells
RDA	redundency analysis
ROS	reactive oxygen species
SEC-FTICR-MS	Fourier-transform ion cyclotron resonance mass spectrometry
SECQTOFMS	size exclusion chromatography coupled with quadrupole time-of-flight mass spectrometry
SN1	nucleophilic substitution reaction 1
SN2	nucleophilic substitution reaction 2
STZ	streptozotocin
SUCNR1	succinate receptor 1
TCA	tricarboxylic cycle
TEAC	trolox equivalent antioxidant capacity
TMCS	trimethylchlorosilane
TMCS	trimethylchlorosilane
TNF-α	tumor necrosis factor-α
UPLC	ultra performance liquid chromatograph
UV	ultraviolet
UV-Vis	ultraviolet-visible spectroscopy
VAS	visual analogue scale
VnmrJ	Start vnmrj
µg/ml	microgram per millilitre

CHAPTER 1: INTRODUCTION

1.1 BACKGROUND TO THIS STUDY – FROM NATURE TO SCIENCE

1.1.1 HISTORICAL DEVELOPMENT

Fossil records dating back sixty thousand years ago present irrefutable evidence that the history of medicine and the evolution of mankind are interwoven. Considerable knowledge and experience in the preparation, selection and identification of medicinal compounds were accumulated and developed over thousands of years from “clinical trials” by trial and error and by sharing information from one generation to the next (Zhang *et al*., 2013). This is demonstrated by the discovery of numerous pharmaceutical documents in the Ebers Papyrus (2900 B.C.) and medical records from Mesopotamia (2600 B.C.) dating back to ancient civilisations (Cragg & Newman, 2005). These documents are evidence of the use of plants for medicinal purposes by pre-historic humans (Shi *et al*., 2010; Fabricant & Farnsworth, 2001). Documented in the Ebers Papyrus, an Egyptian pharmaceutical record, are over seven hundred plant-based drugs ranging from gargles, pills and infusions to various ointments. The Mesopotamia medical records depicted on clay tablets in cuneiform are also the oldest records of natural medicinal products, suggesting that the development of medicine from natural sources is the oldest form of healthcare (Yuan *et al*., 2016). Documented in these records are oils from Cypressus sempervirens (Cypress) and Commiphora species (myrrh) still used today for treating coughs, colds and inflammations (Cragg & Newman, 2005). The medicinal use of willows dates back to 6000 years ago when ancient civilisations used willow tree extracts to treat pain, inflammation and musculoskeletal conditions (Jones, 2011; Kluwer, 2008).

Natural pharmaceuticals were the only “drug-based” healing remedies available to mankind (Jones, 2011) prior to the introduction of aspirin in 1899 (Cordell, 2015).

1.1.2 MODERN MEDICINE

The first synthetic drug, chloral hydrate, was discovered and introduced in 1869 as a sedative-hypnotic (Jones, 2011). It had a bitter taste and irritated the gastric mucosa and simple chemical process was developed to improve palatability and the end result, acetylsalicylic acid, is presently known as Aspirin®, the first blockbuster drug of modern medicine (Jones, 2011).

Francois Magendie, one of the founders of modern pharmacology, pioneered the development of morphine (Chavarria, 2017). Morphine forms the basis for the characterisation of opium and

nicotine and many other narcotics (Chavarria, 2017). The first of the barbiturate family of drugs entered the pharmacopoeia at the start of the twentieth century (Jones, 2011).

1.1.3 ANTIBIOTICS

The discovery of antibiotics was one of the most significant events in medical history. The first antibiotic, penicillin, was discovered by Alexander Flemming in 1928. It took more than a decade to introduce penicillin as a treatment for bacterial infections (Chopra, 2000). Penicillin marked the beginning of the "golden era" for antibiotics (Chopra, 2000). The first commercially available antibacterial product, prontosil, a sulfonamide was developed by the German biochemist Gerhard Domagk in the 1930s (Sneader, 2001).

Antibiotics have transformed the development of pharmaceutical chemistry and saved millions of lives to become one of the keystones of modern medicine (Gould & Ball, 2013). The dramatic successes achieved with antibiotic treatments have led to the view that antibiotic-based chemotherapy heralded the complete conquest of infectious diseases (Chopra, 2000). It was advocated that their discovery has "added a decade" to the life expectancy of humans (McDermott & Rogers, 1982). The optimistic mood was reflected by the historic statement of the US Surgeon General when testifying to the US Congress in 1959 that: *"The time has come to close the book on infectious diseases"* (Chopra, 2000).

Antibiotics still remain the treatment of choice (Magiorakos *et al.,* 2011) and are still presented as the most and often only effective medicinal treatment against bacterial infections to date (Zhitnitsky *et al.*, 2017). They are broadly used in clinical treatments, farm animal feeds and crop protection (Zhitnitsky *et al.*, 2017) but their excessive and injudicious use has led to high incidences of drug and multi-drug resistant bacterial strains (Magiorakos *et al.,* 2011), causing the antibiotic pipeline to "dry up" at the beginning of the 21st century (Spellberg & Gilbert, 2014). Few novel antibiotics are being developed at present, leading to a shortage of new antibiotics being introduced in healthcare (Spellberg & Gilbert, 2014).

1.1.4 ANTIBIOTIC RESISTANCE

Over prescription of antibiotics has become a real threat to global health (Ricke, 2003) and in 2012, the US: Food and Drug Administration (FDA) stated that: "*Misuse and overuse of antimicrobial drugs create selective evolutionary pressure that enables antimicrobial resistant bacteria to increase in numbers more rapidly than antimicrobial susceptible bacteria and thus increases the opportunity for individuals to become infected by resistant bacteria."* (FDA, 2012a).

Félix Martí-Ibáñez, a well-known physician, psychiatrist and author stated almost seventy years ago in 1955: "*Antibiotic therapy, if indiscriminately used, may turn out to be a medicinal flood that temporarily cleans and heals, but ultimately destroy life itself*" (Harbath & Samore, 2005; McFayden, 1979).

Resistance to nearly all antibiotics that were developed (Kok *et al*., 2015; Ventola, 2015) has, many decades after the first patients were treated with antibiotics, previously the organisms were the threat, now resistance are the threat to global health (Ventola, 2015). Bacterial resistance to antibiotics was first noticed in the 1940s when it became clear that the duration in antimicrobial benefit appeared limited (Harbarth & Samore, 2005).

Multi-drug resistant organisms popularly known as "super bugs" are micro-organisms, predominantly bacteria, which have developed resistance to one or more classes of antimicrobial agents. The increasing threat to global health posed by their multiple antibiotic resistance characteristics remains a serious concern to health authorities and symbolise one of the most dangerous threats in modern history (Chopra, 2000; Khan & Siddiqui, 2014; McDermott & Rogers, 1982).

An estimated seven hundred thousand people die annually from drug resistant microbial infections, a figure that is projected to increase to about 10 million by 2050 (Hrvatin, 2017; Walsh, 2015). Currently, at the dawn of the 21st Century, antimicrobial resistance has developed against every class of antimicrobial drug and it appears to be spreading into new clinical niches (Harbarth & Samore, 2005), suggesting that the "magic bullet" has come back to hit us (Kok *et al*., 2015).

An Expert Workshop co-sponsored by the World Health Organization, Food and Agricultural Organization and World Animal Health Organization respectively, have concluded that "*there is clear evidence of adverse human health consequences due to resistant organisms resulting from non-human usage of antimicrobials. These consequences include infections that would not have otherwise occurred, increased frequency of treatment failures (in some cases death) and increased severity of infections."* (WHO, 2003).

The FDA, in banning certain extra label uses of cephalosporin antimicrobial drugs in certain food producing animals, stated that: "*In regard to antimicrobial drug use in animals, the Agency considers the most significant risk to the public health associated with antimicrobial resistance to be human exposure to food containing antimicrobial-resistant bacteria resulting from the exposure of food-producing animals to antimicrobials*." (FDA, 2012b).

1.1.5 SEARCH FOR ALTERNATIVES

The worldwide increase in bacterial infections associated with morbidity has underscored the need to implement new and novel approaches to antibiotic resistance (Howard *et al.,* 2003). Alternatives to antibiotics include bacteriophage therapy and predatory bacteria (Allen, 2017). It appears that none of these alternative treatments were able to consistently demonstrate efficacy against bacteria comparable to that of antibiotic treatment (Dwivedia *et al*., 2016).

A worldwide incentive to discover new antibiotics from novel bioactive natural products was initiated to prompt leading pharmaceutical companies to initiate Natural Product Discovery programmes, aiming to reduce the spreading of bacterial and fungi infectious diseases (Dias *et al*., 2012). Unfortunately, these programmes did not result in the expected production of new and alternative medicines, possibly caused by financial constraints, availability of natural products, intellectual capital, etc. (Ngo *et al*., 2013; Zhu *et al*., 2012).

The twentieth and early years of the twenty-first centuries were characterised by dramatic advances in every aspect of medicine, culminating in the description of diseases at molecular level, which has changed various patterns in the provision of healthcare (Weatherall & Weatherall, 2014). However, the multi-layered complexities of diseases and an ever changing environment of sick people have ensured that clinical medicine, despite numerous advances in the biomedical sciences during the past two centuries, still remains a mixture of applied science and the art of healing (Weatherall & Weatherall, 2014).

1.1.6 NATURAL MEDICINE

The pharmaceutical industry is facing new challenges as the impact of infectious diseases over the next twenty years will influence the increase of microbial resistance against antibiotics (Jones *et al*., 2008). Developing new antibiotics may further increase the likelihood of microbial resistance (Hrvatin, 2017). This phenomenon has created an urgent need to increase efforts in developing novel and innovative medicines against the threat of microbial resistance (Dwivedia *et al*., 2016; Yuan *et al*., 2016), leading to a substantial interest in pharmaceutical compounds developed from natural sources (Galm & Shen, 2007).

Natural medicine developed from environmental sources, also referred to as complementary or alternative medicine (Kok *et al*., 2015) offers merits over other forms of medicine in the: 1) discovery of lead compounds and drug candidates; 2) examining drug-like activity; 3) exploring physicochemical, biochemical, pharmacokinetic and toxicological characteristics (Zhang *et al*., 2013). Despite the fact that many of the larger pharmaceutical companies decommissioned their

Natural Product Discovery programmes between 1990s 2000 (Dias *et al*., 2012), natural product chemistry methodologies that were developed have enabled the potential for the discovery of a vast array of bioactive secondary metabolites from terrestrial and marine sources (Dias *et al*., 2012). The unique diversity in chemical structures and biological activities (Galm & Shen, 2007) suggest that natural medicines have incomparable advantages over chemical medicines (Yuan *et al*., 2016; Zhang *et al*., 2013).

The use of humic substances in traditional medicines (Baatz, 1988; Lent, 1988) and research on humus-soil organic matter, formed by the physical, chemical and microbiological transformation (humification) of organic biomolecules (Brzozowski *et al*. 1994; Mund-Hoym, 1981; Peña-Méndez *et al*., 2004) have led to reports of antiviral, profibrinolytic, anti-inflammatory and estrogenic properties (Peña-Méndez *et al*., 2004; Rizon, 2016; Van Rensburg, 2015; Yamada *et al*., 1998).

1.1.6.1 Humic substances

Numerous humic substance based pharmacological drugs including Shilagen, Diabecon 400, Geriforte, Pilex, Rumalava, Humex® and Salhumin® gel are commercially available (Schepetkin *et al.,* 2002) as anti-inflammatory, antibacterial, antitoxic, antiulcerogenic, antiarthritic and antiallergic agents (Salz 1974; Schepetkin *et al.,* 2002).

1.1.6.2 Fulvic acid

Fulvic acids, often referred to as “naturally occurring” organic acids (Thurman & Malcom, 1981), are oxidised fragments of larger humic substances (Hayes, 1998). They are clusters of acidic organic polymers (Thurman & Malcom, 1981) and the carboxyl structural complexity determined by the environmental diversity including rich composting soil (Ogner & Schnitzer, 1971; Waksman, 1938), peat (Peschel and Wildt, 1988), sediment (Schnitzer, 1969; Levine, 1989), shilajit (Schepetkin *et al*., 2002), coal (Kalhoro *et al*., 2014) and aquatic sources (McKnight *et al.*, 1991) from which they are sourced (Gregor, *et al.,* 1989). The chemical composition of fulvic acid is also influenced by the specific location in a geographical region (Luo, 2012) and the depth of the vertical soil layers (Qu, 2010) from which they are sourced.

Analytical assessment techniques provide bulk information on humus chemical structures (Zhao *et al*., 2012) but no single or combined procedures have succeeded in analysing the chemical composition of fulvic acid (Abbt-Braun *et al*., 2004; Frimmel, 1998; Leenheer & Croué, 2003). It is described as an acidic organic polymer cluster (Thurman & Malcom, 1981) with a highly complex supramolecular nature (Abbt-Braun *et al*., 2004; Samios *et al*., 2005; Yang *et al*., 1993). Fulvic

acid is characterised by carboxylic acids, phenols and hydroxyl functional groups (Yang *et al*., 1993).

1.1.6.3 Synthetic fulvic acid

The synthesis of humic substances dates back to 1786 when Achard (1786) used an alkali substance to extract a dark brown liquid material from a natural peat source. This was followed by the work of Henri Braconnot, a French chemist and pharmacist who discovered that mixtures combining starch and sucrose with various acid solutions formed a dark precipitate with the same appearance and physical characteristics reported by Achard (1786) for soil humic substance (Waksman, 1938). Braconnot (1819) identified his synthetic produced substances as artificial ulmin.

The early pioneering work of Muller in 1839 reported by Cromarthy (2004) focused on the synthesis of cellulose-derived humic substances. It was assumed that humic acids are derived from polysaccharides based on the presence of furan in humic substances (Cromarthy, 2004).

Coal, formed over millennia from rich humic substances are characterised by humic acid furan structures (Cromarthy, 2004). The natural process of coal oxidation to form humic acids is accelerated through the chemical oxidation of coal to produce oxihumate, a semi-synthetic potassium rich humic acid formulation (Cronje, 1988; Cronje *et al*., 1991). The advantage of a wet oxidation manufacture process is that fulvic acid is also extracted in the process (Cromarthy, 2004).

The use of a carbohydrate source in the synthesis process of humic substances was first reported by Malguti (1835). He discovered that glucose synthesis produced humic substances with the same physical appearance and characteristics as previously reported by Henri Braconnot in 1819.

1.1.6.4 Carbohydrate-Derived Fulvic Acid – a new invention

A non-catalytic synthesis process with sucrose produced a heavy metal free fulvic acid referred to as Carbohydrate-Derived Fulvic Acid (CHD-FA). It has antimicrobial and anti-inflammatory properties (Sabi *et al.,* 2011; Sherry *et al*., 2012). The intellectual property, manufacturing process and several applications of this invention are patented by PfeinsmitH Ltd, a subsidiary of Fulhold Pharma Ltd. and various company insignia are registered trademarks (Loxton *et al.,* 2012).

1.2 PROBLEM STATEMENT

Humic substances form chemical complexes with metals and hydrophobic organic pollutants in natural environments have mostly unidentifiable structures (De Paolis & Kukkonen, 1997). Fulvic acids in soil, contaminated with heavy metals (Rahim *et al*., 2016) impose a health risk when used in pharmaceutical formulations (Peña-Méndez *et al*., 2004). These sources are not suitable for addressing the need for safe and effective natural medicines discussed in the introduction, Chapter 1.1 of this study.

A new invention, CHD-FA, developed from the synthesis of a carbohydrate source, sucrose, is a heavy metal free fulvic acid. The various phases in the non-catalytic oxidative degradation of sucrose to form CHD-FA have not been identified previously.

The question pertaining to batch-to-batch consistency in molecular characteristics of fulvic acid synthesised from natural sources has not been addressed in scientific literature.

The individual chemical components embedded within the CHD-FA structure responsible for the anti-inflammatory, antimicrobial and antioxidant properties have not been identified previously.

It is not possible to present CHD-FA as a safe and effective pharmaceutical ingredient in the manufacturing of natural medicines without addressing the following:

1. Can a pathway for the synthesis of one of the constituents of CHD-FA from sucrose through a non-catalytic wet oxidation process be proposed?

2. Can the backbone structures of CHD-FA be identified and characterised?

3. Which compounds embedded in the CHD-FA structure contributes to the clinical properties of CHD-FA?

1.3 STUDY OBJECTIVES

Objective 1:

To present a theoretical model for the mechanism by which sucrose is transformed synthetically through a non-catalytic oxidation process to one of the constituents of CHD-FA.

Objective 2:

The identification of the backbone structures embedded in CHD-FA.

Objective 3:

To review the clinical properties of CHD-FA based on the composition of the backbone structures embedded in the molecular composition of CHD-FA's cluster structure with regards to their anti-inflammatory, antibacterial, antifungal, antiviral and antioxidant properties.

1.4 HYPOTHESIS

It is hypothesised that one of the constituents of CHD-FA, synthesised from sucrose, water and oxygen through a controlled non-catalytic wet oxidation process, is a pure form of fulvic acid. It is also hypothesised that the characteristics of CHD-FA are similar to the fulvic acid reference standards from the International Humic Substances Society (IHSS). CHD-FA is a highly complex mixture of compounds that are bound intermolecularly to form a supramolecule. This supramolecular structure possesses of biological properties.

This study may form a platform for future biotechnological medicinal research for the development of natural medicines.

1.5 METHODOLOGY

This thesis is a composition of three clearly defined separate studies. The aims, methodology and outcomes of each study related to problem statements presented in Chapter 1.2. The methods used were:

- A theoretical model was developed to describe the mechanisms involved in the non-catalytic wet oxidation process that has transformed sucrose into the major constituent of CHD-FA, molecular fulvic acid.
- CHD-FA samples were collected for each of the different phases of the non-catalytic wet oxidation process, from the start-up of the reactor to the start of the exothermic reaction. Nuclear magnetic resonance spectroscopy (NMR) and liquid chromatography-tandem mass spectrometry (LC-MSMS) was used to analyse these samples to identify molecular changes during the various stages of this process.
- Gas chromatography-mass spectrometry (GC-MS), NMR and LC-MSMS were used to identify the chemical and spectroscopic properties of CHD-FA.
- Matrix-assisted laser desorption and ionisation time-of-flight mass spectrometry (MALDI-TOF MS) was used to desorb and ionise CHD-FA without fragmentation, in order to identify and measure the absolute molecular weight of the main component in CHD-FA.

- LC-MSMS was used to identify the most prominent backbone structures embedded in the CHD-FA molecular structure.
- The clinical applications associated with the anti-inflammatory, antimicrobial, antifungal, antiviral and antioxidant properties of CHD-FA was assessed through a comprehensive literature review of the characteristics of the backbone structures in the molecular structure of CHD-FA.

1.6 OUTCOMES

The outcomes of the study objectives, summarised below, are presented and discussion in chapters 3, 4 and 5.

Objective 1:

Identification of the major constituent of CHD-FA and a theoretical model for the mechanism by which molecular fulvic acid is formed from sucrose through a non-catalytic wet oxidation process.

A comprehensive and detailed illustrative theoretical model was developed to describe the myriad of sequential chemical stages in the non-catalytic wet oxidation synthetic process of sucrose to molecular fulvic acid, the major constituent of CHD-FA. The mechanism by which a *Saccharum officinarum* (sugar cane) solution was transformed, offers an understanding of the physical and chemical changes of sucrose decomposition at high temperatures and high pressure. It resulted in a pH reduction which promoted the formation of various carboxylic acids. LC-MSMS and NMR data was used to propose a mechanistic pathway for the transformation of sucrose into glucose and fructose during the chemical stage of the non-catalytic wet oxidation process. This was followed by the oxidative thermal degradation of firstly fructose and then glucose into low molecular weight carboxylic acids as demonstrated by the LC-MSMS and ^{1}H NMR spectra.

GC-MS confirmed the complexity of the molecular CHD-FA structure. The chromatogram overlay of CHD-FA and penicillin-derived fulvic acid (CAS 479-66-3) indicated the presence of fulvic acid in CHD-FA.

The molecular weight of the parent structure in CHD-FA was identified by MALDI-TOF MS as 308 g/mol. MALDI-TOF MS identified the number average molecular weight (M_n) of CHD-FA's molecular structure as 437.7 ± 264.7 Da and the weight average molecular weight (M_w) as 487.1 ± 244.5 Da respectively. Fulvic acids are complex mixture of molecular species with different molecular weight and functional groups. This would contribute to deviation in the results and the assumption that CHD-FA is a complex supramolecular structure.

The characteristics of CHD-FA showed similarity to the characteristic profile of fulvic acid used as reference standards by IHSS (Thorn *et al.,* 1989) and demonstrated conclusively that fulvic acid was synthesised from sucrose through a non-catalytic wet oxidation process.

Objective 2:

Characterisation of Carbohydrate-Derived Fulvic Acid and identification of the backbone structures embedded in the fulvic acid structure.

LC-MSMS was used to identify the backbone structures of CHD-FA.

Similarities between CHD-FA and literature data obtained for environmental fulvic acids were demonstrated by FTIR and ^{13}C NMR. However, CHD-FA has unique characteristics which differentiate it from environmental fulvic acids. CHD-FA has more carboxyl, ester, amide and aliphatic carbons in its molecular structure compared to the fulvic acid reference standards from the International Humic Substances Society.

The most prominent component of the molecular structure of CHD-FA was identified by LC-MSMS as 7,8-dihydroxy-3-methyl-10-oxo-1*H*,10*H*-pyrano[4,3-b]chromene-9-carboxylic acid with the empirical formula of $C_{14}H_{10}O_7$. This component is the dehydrated analogue of penicillin-derived fulvic acid ($C_{14}H_{12}O_8$). It is evident that CHD-FA is a cluster of organic compounds. 24 prominent peaks were characterised as the backbone structures embedded in CHD-FA. This, with reference to the molecular composition of CHD-FA, is a most significant finding of the present study.

Malic acid, maleic acid, levulinic acid, succinic acid, propenoic acid, phthalic acid, arabonic acid, itaconic acid, glucuronic acid, glutaric acid, benzene tri- and tetracarboxylic acids were identified as the backbone structures of CHD-FA. These backbone structures are interlinked with each other and with the parent structure via intermolecular bonding to form a cluster molecular structure.

Objective 3:

Carbohydrate-Derived Fulvic Acid (CHD-FA) is a unique supramolecular fulvic acid with biological properties.

A comprehensive literature review of the clinical properties of CHD-FA has demonstrated that the anti-inflammatory, antibacterial, antifungal, antiviral and antioxidant properties of CHD-FA are

related to the composition and characteristics of the individual backbone structures within the cluster molecular structure of CHD-FA.

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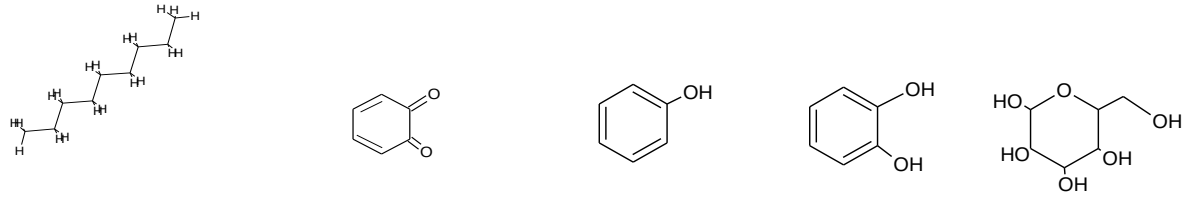
CHAPTER 2: LITERATURE OVERVIEW

2.1 FULVIC ACID FROM ENVIRONMENTAL SOURCES

The humic substance composition is the greatest carbon reservoir on earth (Eladia *et al.*, 2005; Kiprop *et al.*, 2013; Orsi, 2014) and the immense heterogeneity and polydispersity in the chemical composition of humic substances is directly related to their stochastic humificated nature (Hertkorn *et al.*, 2002). Scientists are unable to isolate and define a specific molecular structure for humic substances (Alvarez-Puebla *et al*., 2006; Orsi, 2014; Stevenson, 1982) and are forced to paraphrase it as a category of naturally occurring, biogenic, heterogeneous organic substances characterised by a high molecular weight and a yellow to black colour (MacCarthy *et al.*, 1990). Humic substances are macromolecular structures formed by functional groups and various aliphatic substance linkages to aromatic rings (Stevenson & Olsen, 1989). Functional groups include carboxyl (-COOH), aliphatic and phenolic, hydroxyl (-OH), primary amine ($-NH_2$), secondary amine (-NH-), tertiary amine (-N=) and carbonyl (C=O). Figure 2-1 illustrates the complexity in the chemical composition of humic substances (Orsi, 2014). The chemical characteristics of humic substances, identified by Malcolm (1985) are:

- extremely complex molecular structures,
- abundance of acidic, ester, and phenolic functional groups,
- predominance of aliphatic character,
- ability to fluoresce,
- refractory nature to microbial decay,
- ability to form complexes with metal ions.



Hydrocarbon quinone phenol catechol sugar

Figure 2-1. Chemical structures of the main molecular building blocks forming humic substance.

Fulvic acid is the smallest member of the heterogeneous humic substance “family” (Beckett *et al*., 1987; Buffle & Leppard, 1995; Khan & Schnitzer, 1971; Nimmagadda & McRae 2007; Ogner & Schnitzer, 1971; Senesi *et al*., 1989; Stevenson & Olsen,1989; Yu *et al*., 2013), identified as oxidised fragments of larger humic substances (Hayes, 1998). They are the reactive components of humic substances (Ivanov *et al*., 2016) and are biologically very active (EL-Table *et al*., 2017).

Fulvic acids are often referred to as naturally occurring organic acids (Thurman & Malcolm, 1981), representative of a far-processed stage in humification (Hertkorn *et al.*, 2002). Fulvic acids are found in a diversity of carboxyl environmental sources (Gregor & Powell, 1987) incuding rich composting soil (Ogner & Schnitzer, 1971; Waksman, 1926), peat (Peschel & Wildt, 1988), sediment (Schnitzer, 1969; Levine, 1989), shilajit (Schepetkin *et al*., 2002), coal (Kalhoro *et al*., 2014) and aquatic environments (McKnight *et al.*, 1991) with low and varied concentration levels (Hayes, 1998; Vandenbroucke *et al*., 1985). Fulvic acids are soluble under all pH conditions in acids and alkalines (Nimmagadda & McRae, 2007; Senesi *et al*., 1989; Stevenson & Olsen, 1989; Yu *et al*., 2013).

The chemical complexity is substantiated by its colour differences which vary from light yellow to brown (Buffle & Leppard, 1995) as illustrated by Stevenson (1982) in Table 2-1. Table 2-1 is also a demonstration of the characteristics and interactions between the different structures in the chemical content of humic substances.

Table 2-1. Chemical content of humic substances (Stevenson, 1982).

Humic substances (pigmented polymers)				
Fulvic acid		Humic acid		Humin
Light yellow	**Yellow brown**	**Dark brown**	**Grey-brown**	**Black**
increase in intensity of colour →				
increase in degree of polymerisation →				
2 000 — increase in molecular weight →				300 000?
45% — increase in carbon content →				62%
48% — decrease in oxygen content →				30%
1400 — decrease in exchange acidity →				500
decrease in degree of solubility →				

Fulvic acids are hydrophilic polymeric structures (Ogner & Schnitzer, 1971) and the aromatic hydroxyl-carboxylic acids are held together by hydrogen-bonding indicating that any weakening of these bonds will destroy the structure of fulvic acid (Ogner & Schnitzer, 1971). Earlier (“older”), humified fulvic acids have a high abundance of carboxyl and phenolic groups (Zhang *et al*., 2018) and the phenolic and carboxyl groups in their structural composition determine their properties (Dixon & Larive, 1997).

The difficulties faced by scientists to characterise the molecular structure of fulvic acid (Abbt-Braun *et al*., 2004; Bhutia, 2017; Samios *et al*., 2005; Yang *et al*., 1993) is related to the abundance of the polar functional groups in its structure (Dixon and Larive, 1997) and demonstrated by Day and Hansen (2007) who isolated more than 4,000 distinct compounds from only one Suwannee River fulvic acid sample. Characterising the molecular structure of fulvic acid is further complicated by significant variations in the location of it within a geographical area and in the depth of the vertical soil layers from where fulvic acid is sourced (Qu, 2010).

2.1.1 AQUATIC

Interest in characterising humic substances sourced from natural water has grown rapidly since the 1970s as a result of new technologies developed that enabled scientists to isolate reasonable quantities of humic materials from this source (MacCarthy *et al*., 1990). Preston and Schnitzer (1987) discovered that the molecular structure of fulvic acid is related to the diversity in aquatic sources and reported that the pH of aqueous solutions is determined by the positional shift of the carboxyl and phenolic carbons. It varied with the degree in the dissociation of these functional groups in the chemical composition of aquatic solutions. Table 2-2 is a summary of the characteristics reported for fulvic acid from various aquatic sources.

Table 2-2. Characteristics of fulvic acid extracted from aquatic sources.

SOURCE	CHARACTER	REFERENCE
Suwannee river	• Complex amphiphilic molecule • Not a mixture of a well-defined binary system.	Chaabana *et al*. (2016)
Suwannee river	• 43 % carboxyl-group structures contributed significantly to the strong-acid characteristics of fulvic acid and the remaining 57 % identified as aliphatic carboxyl groups in the molecular complex	Leenheer *et al*. (1995)
Antarctic lakes	• Low C:N atomic ratios (19-25) • Low contents of aromatic carbons (5-7 % of total carbon atoms) • More comparable to marine fulvic acids than to fresh water fulvic acids	McKnight *et al*. (1991)
Lakes in eastern Antarctica	• Notable differences in chemical composition and structure. • The distribution of nitrogen-containing compounds in the nitrogen-rich Antarctic fulvic acids differed significantly from each other. • Dissimilarities were also recorded for quantity and quality of the nitrogenous constituents	Farzadnia *et al*. (2017).
Lake Ontario	• Fulvic acid matter have acquired most of their trace metal	Nriagu & Coker

	burden in the overlying water	(1980)
Natural waters	• Alumina-based pillared clays absorbed up to five times the amount of fulvic acid that iron-based pillared clays could absorb.	Lacey *et al*. (1997)
Australian floodplain river and billabong	• Fulvic acids from the river and billabong had the same functional groups, however, the river fulvic acids had higher apparent *M*n (number average molecular weight) and *M*w values (weight average molecular weight) and were more polydisperse than the billabong fulvic acid. • Initial pH of the water samples and subsequent interaction with the resin may play a part in the final chemical composition of the fulvic acids.	McDonald *et al.* (2007)
German waterworks groundwater	• The fulvic and humic acids isolated from the bulk humic substances were low in nitrogen content and had low H/C atomic ratios. • The fulvic and humic acids had very similar elemental, spectroscopic and copper binding characteristics. • Over 70 % carbon in both the fulvic and humic acids was present in aromatic or aliphatic groups. • Competitive elemental binding studies indicated that Ca^{2+}, Mg^{2+}, Al^{3+} and Fe^{3+} do not effectively compete for copper binding sites on these compounds	Alberts *et al*. (1992)
Suwannee River	• The composition showed strong changes and a relative increase of the low molecular weight anions. • Molecules with a low oxidation state (low O/C ratio) and a high degree of unsaturation (low H/C ratio) was observed. • Structure of the fulvic acid molecules influenced their reactivity toward ozone. • Molecules with a more extended carbon skeleton and less carboxylate substituents showed higher reactivity.	These & Reemtsma (2005)

2.1.2 SEDIMENT

Chemical degradation experiments have shown that the chemical structure of organic matter extracted from bottom sediments in various aquatic systems have originated from adjacent soils (Whitby & Schnitzer, 1978). Erotion, because of its substantial soil run-off effect on river banks, agricultural land and water streams, carries with it inorganic and organic materials (Irie *et al*., 2013). This has raised the potential for extracting fulvic acid for commercial use from this source (Irie *et al*., 2013). However, Irie *et al*. (2013) reported that the extraction rate of fulvic acid from sediment was found to be much lower than extracting it from other sources, rendering sediment not a viable source for commercial purposes. Characteristics of fulvic acid extracted from sediment are summarised in Table 2-3.

Table 2-3. Characteristics of fulvic acid extracted from sediment sources.

SOURCE	CHARACTER	REFERENCE
Baiyangdian Lake sediments	Fulvic acid concentrations were higher in the lake outlet area. The results also showed that the degree of humification increased with an increase in depth	Yuan *et al.* (2013)
Joumine Reservoir in Tunisia	Fulvic acid extracted from the sediment had different characteristics compared to the fulvic acid from the sediment in the Japanese reservoir	Kawachi *et al*. (2012)
Marine sediments	Surface tension decreases with an increase in humic acid and fulvic acid concentration and both were found to be surface active materials with fulvic acid exhibiting the lowest surface tension.	Hayase & Tsubota (1983)
Lake Wuliangsuhai sediments	• Organic matter and fulvic acid affected the heavy metal adsorption onto sediments. • With the increasing fulvic acid addition, the adsorption percentage of heavy metals on both types of sediments showed gradually decreasing trends, with the order of Cu^{2+} >> Cd^{2+} > Zn^{2+} > Pb^{2+}. • When the fulvic acid content was more than 5 %, it became the governing factor on the decreasing adsorption percentage of heavy metals. • Fulvic acid additions showed significant negative and positive correlations with percentages of metals bound to carbonates and organic matter, respectively, since the fulvic acid addition increased the H^+ concentration of the system, in which H^+ could activate the metals to bind to carbonate from the sediments. As an organophilic weak element, the fraction percentage of Cd^{2+} bound to organic matter was the lowest with the minimal changes.	Li *et al*. (2016)

2.1.3 SOIL

Soil is the major source of fulvic acid in humic substances and the composition of humic substances play an important role in the physical and chemical properties of soil (Di *et al*., 2016; Wu *et al*., 2016). Table 2-4 is a brief summary of the characteristics of fulvic acid extracted from different soil sources.

Table 2-4. Characteristics of fulvic acid extracted from different soil sources.

SOURCE	CHARACTER	REFERENCE
Podzol soil	• Twenty-one phenolic and benzene-carboxylic acids were identified as methyl ethers and esters • Polymethoxy-benzene polycarboxylic acids with unknown identity were isolated.	Ogner & Schnitzer (1971)

Podzolic soil and peat, located in the S. Paulo state, Brazil	Peat had a higher carbon content than podzolic soil and the materials had a molecular weight range > 5000 with predominance in the fraction between 1500 and 5000	Toledo *et al*. (1985)
Soil from Israel and southern Italy	• The analytical characteristics of the humic and fulvic acids extracted from Israeli and Italian soils were generally similar to those of humic materials originating from soils formed under widely differing geographic and pedologic conditions. • There were some differences in the content and composition of inorganics in the fulvic acids which were reflected in functional group analyses.	Chen *et al*. (1978)
Japan volcanic ash soil	• Concentration of fulvic acids decreased slightly throughout the series (site 1 to site 3). • The findings of this study clearly demonstrate that humic acids, but not fulvic acids, significantly changed with decreasing aryl C content.	Limura *et al*. (2010)
Tropical forest soils	• Fulvic acid carbon and nitrogen contents were higher in the subtropical forest soil. • They did not increase in exact proportion to the organic matter contents. • Aliphatic groups were lower in humic acid of tropical soils while fulvic acids presented with few aromatic groups. These changes may be due to the differences in clay and moisture contents and vegetative cover.	Balagopalan & Jose (1994)
Arable soils (a red soil and an alluvial soil) from Taiwan	• Molecular weights of fulvic acids, especially those in the lower weight group, extracted from the sub-soil of the non-cultivated red soil were lower than those from the topsoil indicating a downward movement of low molecular weight fulvic acids due to leaching. • These leached acids were mainly C=O containing polymers with a molecular weight <1000 and were carboxyl-containing aliphatic chains, while those with a molecular weight >1000 were carboxyl-containing cyclic aromatics.	Chen & Wang (1992)
Podzol soil	• Twenty-one phenolic and benzene-carboxylic acids were identified as methyl ethers and esters • Polymethoxy-benzene polycarboxylic acids with unknown identity were isolated.	Ogner & Schnitzer (1971)
Soil layers of the same vertical profile in a Korean pine forest	• Particle morphologies of different fulvic acid fractions, including hydrophilic and hydrophobic fractions, were similar. • However, particle sizes and distributions of fulvic acid fractions from different soil layers at the same vertical profile did differ. • They were increased with the increasing of the soil depth.	Yu *et al*. (2013)
Chinese standard fulvic	• Carboxylic group-containing component decreased as a function of elution sequence according to Van Krevelen diagram, FTIR spectra, and ^{13}C NMR	Bai *et al*. (2015)

acid from Forest Soil	spectra. • The phenolic group-containing component increased and then decreased with elution sequence, and showed the greatest enrichment when using a buffer with an original pH of 9	
Agricultural soil from plastic greenhouse vegetable production in China	• The soil humic and fulvic acids were found to show inhibition activities against phytopathogenic fungi for the first time • Humic acid and fulvic acid could be distinctly separated from each other and cultivation years mainly determined the variation • The active fungicidal components in soil humic acid and fulvic acid decreased along with the extension of cultivation years, which made the soil suffer more risk to phytopathogenic fungi	Wu *et al*. (2016)

2.1.4 PEAT

Peat is an accumulation of partially decayed vegetation and unique to natural areas, peatlands, bogs, mires and moors (Joosten & Clarke, 2002). The Origin Peatland ecosystems are regarded as the most efficient carbon reservoirs on earth. Factors such as the geographic location of bogs, peculiarities of bog formation and differences in the elemental supply sources with respect to content and scattering in chemical elements determine the structural composition of peat (Dudare & Klavins, 2015). Correlation analysis between chemical elements in peat humic substances from different depth layers of Eipurs, Dzelve and Dizpurvs bogs have demonstrated significant differences between natural and anthropogenic peat accumulation zones (Dudare & Klavins, 2015). Table 2-5 is a summary of the characteristics of fulvic acid extracted from different peat sources.

Table 2-5. Characteristics of fulvic acid extracted from different peat sources.

SOURCE	CHARACTER	REFERENCE
200 year-old milled peat from Finland	Main products of all fulvic acid fractions (as shown by CuO oxidation) were 4-hydroxy-benzaldehyde and 4-hydroxycetophenone.	Hänninen *et al*. (1993)
Peat in eastern Anatolia, Turkey	Showed differences related to the source and climate, with phenolic-OH dominant for the different samples	(Turan *et al*. (2005).
Humified milled peat	• Aliphatic features in the peat fulvic acid structure are of carbohydrate origin • Phenolic structures of peat fulvic acid yielded a 6 % concentration in the ethyl acetate soluble fraction • A loss of 60 % of the fulvic acid during CuO oxidation due to the carbohydrate in the fulvic acid	Hänninen (1987)

Mechanochemical modification on the acid-base properties of fulvic acid peat	• Peat mechanochemical modification provides increased yield of fulvic acid and a particular modification of the structure that allows for a new set of fulvic acid physicochemical properties • Alkaline hydrolysis of peat in the process of mechanical activation has a significant influence on the acid-base properties of the functional groups of aromatic nature in fulvic acid. • Modified organic substances become strong detoxifying agents for detoxification of hetero toxic substances • Preparation can be recommended for the binding of heavy metals and polycyclic aromatic hydrocarbons.	Maltseva *et al.* (2014)
High-moor sphagnum peat with a low ash content	• Carboxyl groups at the aromatic rings of fulvic acids are most reactive, and this fact is responsible for their role in sorption and detoxification processes • The mechanical activation of peat decreased the acidic properties of fulvic acid • The pK values for the C*n*COOH and ArOH remained almost unchanged • Mechanical activation of peat increased the dissociation of carboxyl groups ArCOOH at aromatic rings in fulvic acid by 20 % • Structural rearrangement associated with a change in the amount of functional groups occurred in fulvic acid on the mechanical activation of peat • The amount of carboxyl and alcoholic OH groups in their composition increased • Change in the quantity of hydrogen atoms in the carbon skeleton of fulvic acid indicates an increase in the concentration of unsubstituted aliphatic structures	Ivanov *et al.* (2016)
Hungarian peat	• Average molecular mass of fulvic acids is about 2000 g/mol • The actual average molecular mass is dependent on the following conditions: temperature, pH and ionic strength	Aguilar *et al.* (2009)

2.1.5 SHILAJIT

Shilajit is a complex mixture of organic humic substances produced by the decomposition of plant materials and microbial metabolites that occur in the rock rhizospheres of mountains in China, Nepal, Pakistan Tibet, Ural, Baykal, Sayan, Caucasus, Altai, Kazakhstan and other mountain regions of the world (Schepetkin *et al*., 2002; Agarwal *et al*., 2007). It is a semi-hard brownish to black dark greasy resin with a distinctive coniferous smell and bitter taste (Schepetkin *et al*., 2002; Agarwal *et al*., 2007). Physical characteristics are variable and related to the origin of shilajit (Agarwal *et al*., 2007). Its unique composition as a phytocomplex structure is rich in fulvic acid (Schepetkin *et al*., 2002). Pre-clinical investigations in traditional medicinal applications have demonstrated that shilajit has potential for treating certain diseases (Agarwal *et al*., 2007;

Carrasco-Gallardo *et al*., 2012). Table 2-6 is a brief summary of the characteristics of fulvic acids extracted from different environmental shilajit sources.

Table 2-6. Characteristics of fulvic acid extracted from different shilajit sources.

SOURCE	CHARACTER	REFERENCE
Found in mountains of China, Nepal, Pakistan Tibet, Ural, Baykal, Sayan, Caucasus, Altai, Kazakhstan	• Main organic substances in water-soluble fraction of shilajit consist of fulvic acids • Infrared spectra of the samples in KBr pellets are very similar to each other in accordance with similar chemical nature of the fractions and sub fractions. • All the infra-red (IR) spectra contain strong band near 3400 cm^{-1} (vibrations of hydroxyl groups), show significant absorbance at 1630–1640 cm^{-1} (aromatic rings and/or carboxyl groups), 1400 cm^{-1} (deformational vibrations of hydroxyl groups), 1070–1150 cm^{-1} (C-O bonds), 540–545 cm^{-1} (very characteristic for aromatic rings). • NMR spectra show signals near 0.8 ppm (protons of methyl groups in the aliphatic chains) and 6.5–7.5 ppm (protons of aromatic rings). • The group of signals in the region 1.2–2.3 ppm testifies to the presence of CH_2 groups in aliphatic chains. • Weak resonance at 2.6 ppm can be related to methyl groups connected directly to the aromatic rings. • Intense signals at 3.4–4 ppm are rather interesting. These are the signals of protons at carbon atoms connected directly to strongly electron-acceptor atoms (for example, to oxygen).	Schepetkin *et al*. (2002)
Himalayas and Hindukush ranges of the Indian subcontinent	• Considering its unique composition as a phytocomplex, very rich in fulvic acid • Complex mixture of organic humic substances and plant and microbial metabolites occurring in the rock rhizospheres of its natural habitat.	Agarwal *et al.* (2007)

2.1.6 COAL

Humic substances have a strong correlation with coal (Waksman, 1938) as furan-containing compounds found in coal are characteristic of numerous humic sources (Marcusson, 1920; Gortner, 1916). Coal originates from ancient lush vegetation as humic deposits that have never turned into oil (Kalhoro *et al*., 2014). “Pure” fulvic acid fractions were extracted from Pakistan coal by Kalhoro *et al*. (2014) who crystallised the coal fulvic acid in water (2.45 % yield) before using Fourier-transform infrared (FT-IR) and UV-Vis spectroscopy to conclude that the obtained crystals isolated from coal is fulvic acid. Kalhoro *et al*. (2014) reported that the spectral fulvic acid

in coal is similar to fulvic acid fractions from other environmental sources. Table 2-7 is a summary of the characteristics of fulvic acid extracted from different coal sources.

Table 2-7. Characteristics of fulvic acid extracted from different coal sources.

SOURCE	CHARACTER	REFERENCE
Low-grade solid fossil fuels (brown coal and peat) brown coal humic	• Reactive groups are suitable for subsequent organic syntheses to obtain valuable coal chemical products • Absorption bands at 3419 cm^1 are due to the stretching vibrations of the hydroxyl groups that were identified. • A split band in the range of 2931-2885 cm^1 due to the stretching vibrations of aliphatic $–CH_2$ and $–CH_3$ groups. • An intense narrow band at 1664 $cm^{–1}$ due to the vibrations of the C=O group in $CH_3C(O)OR$ esters.	Yu, *et al*. (2013)
Oxifulvic acid	• 50 different compounds, most of which were carboxylic acids • Oxygenated straight chain or branched acids, in the hydroxy or keto form, containing up to six carbon atoms. • The oxy function was not limited to a specific position, i.e. it could be in α, β, etc. position. • Dicarboxylic aliphatic acids containing up to six carbon atoms. Oxygenated and unsaturated dicarboxylic acids were also detected. • The unsaturated acids may have been artefacts and possibly derived from the corresponding hydroxy acids under the strong dehydrating conditions for silylation. • Higher carboxylic acids (dodecanoic, palmitic and stearic acid). A common factor is that these acids contain an even number of carbons, in accordance with the natural occurrence. • Benzoic acid and its hydroxy derivatives and phthalic acid.	Bergh *et al.* (1997)
Pakistani coal	Pure fulvic acid fraction was recovered from coal fulvic acid by the crystallisation of the coal fulvic acid in water and yielded 2.45 %.	Kalhoro *et al*. (2014)
Weathered coal from Xinjiang in China	• The yield of the produced fulvic acid by this procedure was at most 26 % • The formed fulvic acid showed a higher content of oxygen and carboxyl groups than those extracted from the original weathered coal. • The phenolic hydroxyl group content was higher than those of products of the ozone oxidation of humic acid from the same weathered coal, reported previously.	Shinozuka *et al*. (2004)

The classic method for extracting fulvic acid from natural humus sources involves the utilisation of a strong base to isolate the alkaline-soluble materials in the humus source followed by acidifying the alkaline solution to separate the fulvic fraction after removal of the insoluble components

(Stevenson & Olsen, 1989). Fulvic acid is separated through adsorption on a non-ionic macro porous acrylic ester resin at an acidic pH (Lamar *et al*., 2014). The process is in accordance with the guidelines of the IHSS.

2.1.7 SYNTHESISED FULVIC ACID

Synthesis is the physical and chemical manipulation of different processes and involves a cascade of chemical reactions (Vogel *et al*., 1996). Synthetic processes are reproducible, reliable and purposefully constructed and executed (Vogel *et al*., 1996). A reactant is used to start the process which is yielded into a new composition.

The first attempt to produce a synthesised humic substance was performed by Malguti (1835). Malguti (1835) used a carbohydrate source, glucose, as the reactant. He conducted his work shortly after the discovery by Braconnot (1819) of a “dark brown” liquid material that was extracted from peat. Malguti (1835) found that the synthesis with glucose produced materials similar in physical appearance and characteristics to that of the humic substance Braconnot (1819) has extracted from peat. The discovery of urea, quinine, morphine and strychnine through experimental methods in practical chemistry during the late eighteenth and early nineteenth centuries have laid the foundation and provided the impetus for the emergence of organic synthesis in recent biological and medicinal developments (Nicolaou, 2014).

The first scientific evidence of a synthesised fulvic acid was published in 1935 by Oxford *et al*. (1935). *Penicillium griseofulvum* Dierckx was grown on a liquid media using glucose as the carbon source and sodium nitrate as the nitrogen source to form 6-methylsalicylic acid, gentisic acid, fumaric acid and mannitol. Oxford *et al.* (1935) discovered that glucose metabolism yielded a complex yellow phenolic acid structure with the empirical formula $C_{14}H_{12}O_8$. Oxford *et al*. (1935) proposed the name “fulvic acid” for the complex penicillin-derivated yellow solution. This compound isolated by Oxford *et al.* (1935) is a single, pure, simple organic molecule which is a secondary metabolite of the *Penicillium* and other fungal species.

Recently studies have proposed that fungal metabolites such as fulvic acid, citromycetin and fusarbin are biosynthesised through a common intermediate (Yamauchi *et al*. 1987). Yamauchi and his co-workers studied a known intermediate, enetrione and through this work, they were able to successful synthese fulvic acid from a synthetic intermediate (Yamauchi *et al.,* 1987). Yamauchi *et al.* (1987) were able to demonstrate the synthetic pathway of fulvic acid and anhydrofulvic acid.

2.1.7.1 Wet oxidation

Wet oxidation is a form of hydrothermal treatment of organic substances characterised by the oxidation of dissolved or suspended components in water where oxygen is the oxidising agent (Zimmermann, 1950). The chemistry is complex and involves numerous chain reactions caused by radicals of the organic compounds present in the reaction mixture (Bhargava *et al*., 2006). Wet oxidation has been used in the degradation of waste materials for many years (Zimmermann, 1950).

Coal has a pure organic origin (Jeffrey, 1915) and the methodology applied during a mild wet oxidation process to successfully extract a semi-synthetic potassium-rich humic acid from bituminous coal (Cronjé, 1988; Cronjé *et al*., 1991) was used to yield a synthetic coal derived fulvic acid from bituminous coal (Bergh *et al*., 1997; Gan *et al.,* 2007). However, the natural oxidative weathering of coal to form highly complex mineralised humic substances (Rausa *et al.,* 1994), suggest that a host of unidentified compounds is contained within fulvic acid derived from coal (Bergh *et al*., 1997).

Hardy (1955) presented the first conclusive evidence that various molecular structures are formed during the wet oxidation process of a carbohydrate source, sucrose, and demonstrated an inversion of sucrose and a decomposition of hexoses from the oxidation of a hot neutral sucrose solution by natural air oxygen. Šimkovic *et al*. (2003) discovered that the primary reaction during the thermal degradation process of sucrose was characterised by the splitting of glycosidic bonds to form sucrose derivatives at 185 °C. These derivatives were identified as sucrose isomers characterised by altered configurations on their pyranose rings and it was speculated that the derivatives could also contain various hydro-sucrose bonds which formed sucrose stereoisomers during the thermal degradation process. Theander *et al.* (1988) found that hydrothermolytic processes of carbohydrates formed aromatic compounds and speculated that aldol condensation was a primary route for the production of aromatic compounds from saccharides. Bhargava *et al.* (2006) suggested that the exothermic reaction of carbohydrate wet oxidation was thermally initiated by various catalytic reactions and that it involved the chemical removal of α-hydrogens from carboxylic acids by hydroxide and base-induced retro-aldol reactions.

2.1.8 CHARACTERISATION

Analytical techniques can provide scientists with bulk information on the chemical characteristics of molecular structures (Zhao *et al*. 2012). The complex molecular nature of fulvic acid suggests that a number of experimental techniques are required to characterise fulvic acid (Gamble, 1972).

The characterisation of fulvic acid was initiated by Berzelius, a Swedish scientist, during the 1830s and 1840s. He isolated “crenic” and “apocrenic” acids from water-soluble organic acids (Yakimenko, 2001). Berzelius was closely followed by Mulder who succeeded in isolating “nitrogen-free” crenic and apocrenic acid substances from soil and peat samples with empirical formulae $C_{24}H_{24}O_{16}$ and $C_{24}H_{12}O_{12}$, respectively (Yakimenko, 2001).

Oden, a German scientist who aimed to determine the molecular structure of humic substances was the first scientist to categorise humic substances into humus coal, humic acid, hymatomelanic acid and fulvic acid (Aiken *et al.,* 1985; Waksman, 1938). He worked between 1914 and 1919 and is accredited for being the first scientist to use the term fulvic acid (Aiken *et al.,* 1985; Waksman, 1938).

It was only in 1957, almost fifty years after the ground-breaking work of Olden that molecular scientists began to focus on characterising fulvic acid’s chemistry and its reaction with metal ions (Schnitzer, 1969). Various techniques ranging from simple methods such as elemental analysis, potentiometric titration and molecular weight identification techniques to powerful techniques such as NMR were applied but no single or a combination of these techniques could characterise the molecular structure of fulvic acid (Abbt-Braun, *et al*. 2004; Frimmel, 1998; Leenheer & Croué, 2003). Leenheer (2007) concluded that fulvic acid molecules are a diverse and complex aggregate of small molecules.

Baigorri *et al*. (2009) was able to identify the presence of smaller and more O-containing functional groups in the molecular structure of fulvic acid by using a combination of ultraviolet-visible spectroscopy (UV-Vis) and FTIR spectroscopy, ^{13}C NMR, thermogravimetric and pyrolysis GC-MS. Baigorri *et al*. (2009) discovered, by using multiple analysis techniques, that fulvic acid has a tendency to form molecular aggregates (hydrogen bridges, metal bridges, and hydrophobic interactions) when in a solution.

The work of Baigorri *et al*. (2009) is evidence that multiple analytical techniques are required when complex molecular structures such as fulvic acid is analysed.

2.1.8.1 Fourier Transform Infrared spectroscopy (FTIR)

FTIR spectroscopy is a qualitative analytical technique mainly used for the identification of specific functional groups within a chemical molecular structure (Wang *et al*. 2010). FTIR analysis is rapid, robust and inexpensive and has been extensively employed in characterising fulvic acids extracted from aquatic (Giovanela *et al*. 2004; Senesi *et al*. 1989) and soil sources (Neyrouda &

Schitzer, 1974; Watanabe & Kuwatsuka, 1992) and synthesised structures from waste (Baddi *et al*. 2004) and coal (Bergh *et al*., 1997; Kiprop *et al*. 2013).

2.1.8.2 Nuclear Magnetic Resonance (NMR) Spectroscopy

NMR is a popular technique used for the analysis of humic structures and is the preferred method used by the IHSS for characterising standard and reference fulvic acids (Thorn *et al*., 1989). Using NMR, fulvic acids were characterised as predominantly aliphatic (Wilson *et al*., 1987; Cook & Langford, 1998) and ^{13}C NMR has indicated that carbohydrate moieties play a major structural role in the chemical composition of fulvic acid (Cook & Langford, 1998). Utilising ^{13}C NMR, it was demonstrated that the majority of the weak acid protons in fulvic acid are aliphatic – OH structures and not phenolic alcohols (Cook & Langford, 1998).

Samples extracted from Suwannee River soil and peat standard fulvic and humic acids, Leonardite standard humic acid, Nordic aquatic reference fulvic and humic acids and Summit Hill soil reference humic acids characterised by Thorn *et al*. (1989) using ^{13}C and ^{1}H NMR exhibited five major humic substance spectra bands:

- aliphatic carbons; 0 to 60 ppm,
- hetero-aliphatic carbons; 60 to 90 ppm (primarily sp^3 hybridised carbons bonded to oxygens),
- aromatic carbons; 90 to 165 ppm,
- carboxyl carbons; 165 to 190 ppm,
- ketone and quinone carbons; 190 to 220 ppm.

Thorn *et al*. (1989) proposed that many spectroscopica bands of functional groups in the five major regions overlapped:

- methoxyl carbons occurring in the region from 50 to 62 ppm,
- acetal and hemiacetal carbons overlapped with aromatic carbons in the region from 90 to 110 ppm,
- olefinic carbons overlapped with aromatic carbons in the region from 110 to 150 ppm,
- lactone, ester, and amide carbons overlapped with the carboxyl carbons in the region from 165 to 190 ppm.

2.1.8.3 Gas Chromatography Mass Spectrometry (GC-MS)

GC-MS is suitable for adding depth to the study of the molecular structure of fulvic acid. It has been used successfully to identify carbohydrates, phenols, benzenes and lignin phenol molecular

structures in environmental fulvic acids (Baigorri *et al*. 2009; Ikeya *et al*. 2004; Neyrouda & Schnitzer, 1974; Nimmagadda & McRae, 2007; Schellekens *et al*. 2017; Xiaoli *et al*. 2008; Zhou *et al*. 1994).

Nimmagadda and McRae (2007) subjected fulvic acids isolated from Elliott soil, Suwannee River and Waskish peat to a selective reduction method and used GC-MS to characterise the "backbone" structures. Nimmagadda and McRae (2007) concluded that previously unreported compounds such as furan derived and simple aromatic and cyclic carboxylic acids were found and stated that GC-MS confirmed that fulvic acid has a supramolecular structure.

Nimmagadda and McRae (2007) found a higher carbohydrate level and greater amounts of higher molecular weight and heterocyclic compounds in soil fulvic acid compared to Suwannee River fulvic acid samples from IHSS. The reduction products of Suwannee River fulvic acid appear richer in straight chain aliphatic compounds (Nimmagadda & McRae, 2007).

2.1.8.4 Liquid Chromatography Tandem Mass Spectrometry (LC-MSMS)

The combination of liquid chromatography and mass spectrometry into a single, well-integrated LC-MS analytical technique has revolutionised the approach to the quantification and characterisation of complex organic molecules (Cappiello *et al*. 2008). It has become the most widely used chromatographic technique for characterising and separating humic substance molecular structures (Ryan, 2000). LC-MS is a quick and easy technique to differentiate, on a qualitative basis, between fulvic acid fractions extracted from different geographical regions (Mawhinney *et al*. 2009). The use of tandem mass spectrometry and stable isotope internal standards allows for the development of highly sensitive and accurate assays (Pitt, 2009). Pitt (2009) proposed that the most beneficial application of LC-MSMS is to characterise complex molecular structures. It is important to note that mass spectrometric determination is the only mass-specific measurement of fulvic acid's molecular weight (Leenheer *et al*. 2001).

Fulvic acids from Suwannee River, Pony Lake, Elliot Soil, Waskish Peat, and Nordic Reservoir were characterised by Mawhinney *et al*. (2009) using LC-QTOF-MS operative in a negative electrospray ionisation mode. Mawhinney *et al*. (2009) discovered that species containing larger amounts of hydrogens displayed a larger mass and were retained for longer on the LC column providing evidence of reduced polarity. A reduction in the degree of fragmentation was related to polar functional groups recorded by an increase in mass defect and retention times (Mawhinney *et al*., 2009).

2.1.8.5 Matrix-assisted Laser Desorption and Ionisation Time-of-Flight Mass Spectrometry (MALDI-TOF MS)

MALDI-TOF MS, a soft ionisation technique, is used extensively to analyse humic substances (Lyubomirova & Djingova, 2013). It desorbs and ionises the molecule to measure the absolute molecular weight without fragmentation of the molecule (Mugo & Bottaro, 2004).

The advantage of MALDI-TOF MS is the ability to accurately determine the molecular weight of fulvic acid (Liu, 1997; Mugo & Bottaro, 2004) and the molecular weight distribution of fulvic acid (Liu, 1997). Liu (1997) discovered between 9 and 40 components for the molecular weight (m/z) range between 200 and 900 Da for fulvic acid The average molecular weight for various fulvic acids were between 360–410 Da and the weight averages ranged between 370–420 Da (Liu, 1997).

Novotny and Rice (1995) found that the intensity peaks for fulvic acid in the region of 37–530 Da. Ion distributions obtained for the IHSS soil and IHSS Suwannee river fulvic acids by MALDI-TOF MS were in the regions of 600 m/z and 500 m/z respectively (Haberhauer *et al*. 2000), confirming the efficacy of MALDI-TOF MS to characterise complex molecular structures (Lyubomirova & Djingova, 2013; Mugo & Bottaro, 2004).

2.1.9 MOLECULAR STRUCTURE

Plant materials are humified into a “family” of organic acids (Day & Hansen, 2007) characterised by carbohydrates, amino acids, amino sugars (hydrophilic) and lignin-derived aromatic molecules (Zhu *et al*., 2013). Aromatic molecules include aromatic phenols, hydrocarbons, fats and nucleic acids (Zhu *et al*., 2013).

Fulvic acids are oxygen-rich molecules containing carbonyls, hydroxyls, phenols, quinones and semiquinones reactive functional groups (Aguilar *et al*., 2009; Bravo, 1998; Yoshino, 1998). Fulvic acids are characterised by aliphatic carbons, hetero-aliphatic carbons, olefinic carbons, aromatic carbons, carboxyl carbons and ketonic and aldehydic carbons (Thorn *et al.,* 1989). Khan and Schnitzer (1971) reported that 28 % of the molecular structure of fulvic acid comprises of phenolic acids, 19 % benzene carboxylic acids, 13 % alkanes and 40 % fatty acids and dialkyl phthalates.

Leenheer *et al.* (2001) stated that malic acid, hydroxyphenylacetic acid, phenyl malonic acid, pyromellitic acid, coumarin-3-carboxylic acid and chelidonic acid carboxylic acid structures are characteristic of the fulvic acid molecular structure. The most prominent fragments, succinic and

malonic acids accounts for 29 % and 17 % of the total fragment content respectively (Qin *et al.,* 2016).

The major degradation products of plant materials, benzene-carboxylic and phenolic acids with smaller amounts of aliphatic (mainly n-fatty) acids, are the “building blocks” (Neyrouda & Schnitzer, 1974) of the highly complex supramolecular structure of fulvic acid (Abbt-Braun *et al*. 2004; Bhutia, 2017; Buffle & Leppard, 1995; Hayes, 1998; Rodriguez *et al*., 2011; Samios *et al*. 2005; Senesi *et al.,* 1989; Yang *et al*. 1993). Leenheer *et al*. (2001), Nimmagadda and McRae (2007) and Cappiello *et al.* (2008) reported similarities in the backbone structures of fulvic acids isolated from Elliott soil, Suwannee river and Waskish peat. Fragments identified by Nimmagadda and McRae (2007) is illustrated in Figure 2-2.

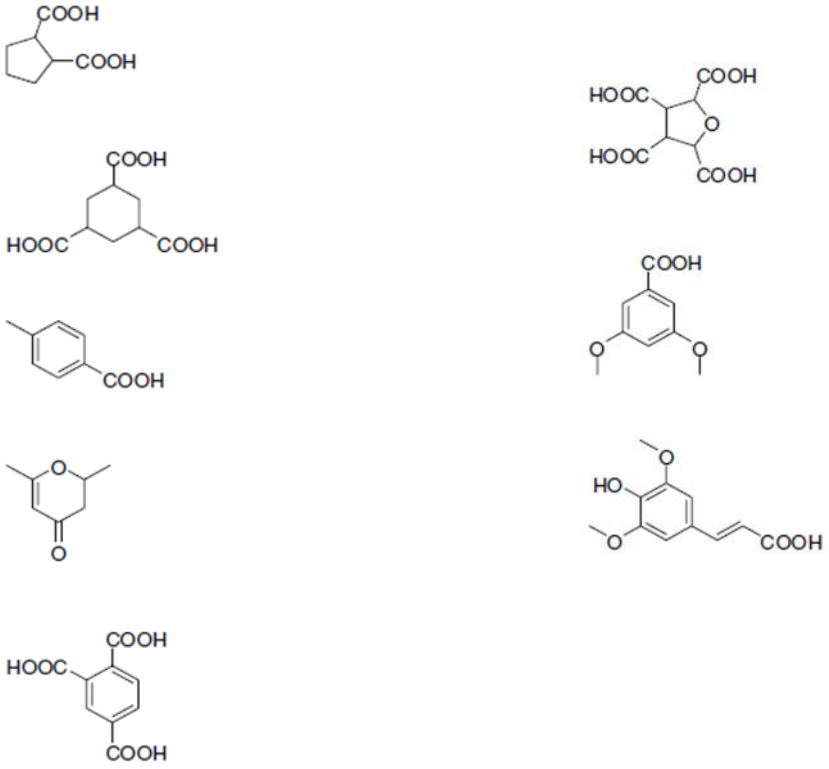


Figure 2-2. Proposed backbone structures of fulvic acid.

Figure 2-3 is an illustration of the theoretical structure of fulvic acid as suggested by Buffle (1977).

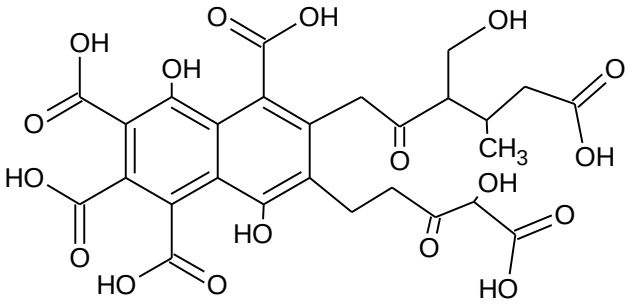


Figure 2-3. An illustration of the hypothetical model molecular structure of fulvic acid as proposed.

Every proposed molecular model for fulvic acid should be taken as the representation of a small portion of the real mix (Bruccoleri et al., 2001).

Stevenson (1982) compared the structural composition of fulvic acid extracted from aquatic sources to that of fulvic acids isolated from coal and soils and found that the composition of the molecular structures varied significantly. Stevenson (1982) concluded that molecular differences are related to the diversity of the environmental sources from which samples were extracted. Bhutia (2017) suggested that the empirical formula for a supramolecular fulvic acid is $C_{135}H_{182}O_{95}N_5S_2$ and hypothesised that fulvic acid is a: "*loose assembly of aromatic organic polymers with many carboxyl groups (COOH)*". Hiraide (1992) suggested that the ability of humic substances such as fulvic acid characterised by multiple sites for chemical reactions makes them relevant to numerous biochemical processes includes mineral weathering, nutrient bioavailability and contaminant transport playing a role in their structural composition.

Khanna *et al*. (2008) identified $C_{18}H_{23}O_{10}$ as the empirical formula for fulvic acid extracted from shilajit and Mawhinney *et al*. (2009) summarised the empirical formulae of fulvic acid extracts from various sources:

- Elliot soil $C_{18}H_{13}O_{11}$
- Nordic reservoir $C_{19}H_{17}O_{10}$
- Pony lake $C_{18}H_{29}O_{10}$
- Suwannee river $C_{19}H_{17}O_{10}$
- Wakish peat $C_{19}H_{17}O_{10}$

The complexity in the molecular structure of fulvic acid was confirmed by the work of These and Reemtsma (2005). They used size exclusion chromatography coupled with quadrupole time-of-flight mass spectrometry (SECQTOFMS) changes to characterise the molecular composition of ozonated fulvic acid isolated from the Suwannee River. These and Reemtsma (2005) found an increase in low molecular weight anions and concluded that extended mass spectrometry

demonstrated that the composition of the fulvic acid molecule was a significant factor in influencing its reactivity towards ozonation. These and Reemtsma (2005) showed that molecules with a more extended carbon skeleton and less carboxylate substituents had a higher reactivity towards ozonation and that some highly unsaturated fulvic acid molecules did not have measurable reactive structures.

Fulvic acid molecular size increase in succession from aquatic sources as the smallest molecular structure to soil and then to peat bog followed by lignite with coal having the largest molecular structure, demonstrating that the molecular size is a function of the source from which it is extracted (Beckett *et al*., 1987).

The molecular weight of fulvic acid can range from more than a 100 g/mol to ±1000 g/mol and more (Blach & Christman, 1963). Alvarez-Puebla *et al*. (2006) found that the carbon and oxygen contents, acidity and the degree of polymerisation changed with an increase in the molecular weight of fulvic acid. Reemtsma *et al*. (2008) verified these findings by identifying high molecular weight (HMW) fractions and low molecular weight (LMW) fractions in the molecular structure of fulvic acid. Fourier transform ion cyclotron resonance mass spectrometry (SEC-FTICR-MS) ranged between 200-700 Da. The HMW fractions were rich in carboxyl groups and more aromatic than the LMW structures (Reemtsma *et al*., 2008). Reemtsma *et al*. (2008) proposed that the shift in the relative frequency of ions from LMW to HMW fractions was in line with various interactive mechanisms and support the phenomenon that HMW fulvic acids are structurally developed from LMW fulvic acids. Reemtsma *et al*. (2008) concluded that HMW fulvic acids are aggregates held together by electrostatic interactions between carboxylic groups through combined or singular action of hydrogen bonds, polyvalent cations, hydrophobic interactions of carbon backbone structure or LMW fulvic acids covalently bound to each other or to aliphatic alcohols (Reemtsma *et al*., 2008). Reemtsma *et al*. (2008) identified the empirical formula for HMW fractions and LMW fractions from different regions in the Suwannee river delta to confirm the variances in fulvic acid molecular structures:

HMW	LMW
1. $C_{10}H_{14}O_5$	1. $C_{12}H_{16}O_3$
2. $C_{14}H_{10}O_6$	2. $C_{14}H_{18}O_6$
3. $C_{15}H_{12}O_{10}$	3. $C_{18}H_{14}O_7$
4. $C_{18}H_{16}O_{12}$	4. $C_{22}H_{28}O_8$
5. $C_{21}H_{18}O_{14}$	5. $C_{26}H_{34}O_9$
6. $C_{24}H_{20}O_{16}$	6. $C_{28}H_{36}O_{12}$
7. $C_{27}H_{22}O_{18}$	

Supramolecular structures are clusters of relatively small heterogeneous functional groups (masses around 500 Da) and the functional groups are self-assembled through hydrogen bonding held together by weak forces such as Van der Waals and CH-π and $\pi - \pi$ interactions to form large assemblies of apparently high molecular masses (Alvarez-Puebla *et al*., 2006). The overall stability of this molecular structure is related to the specific position of the different weak bonds within the molecule. The weak bonds allow for dynamic bond breaking and bond building to form new clusters characteristic of the rich diversity and complexity of the supramolecular structures (Alvarez-Puebla *et al*., 2006).

Intermolecular bonding to form a supramolecular structure is illustrated in Figure 2-4.

The intermolecular bonding to form a supramolecular structure is demonstrated (Figure 2-4) and explain the great stabilisation effect that the presence of water molecules has on the electrostatic energy in the structure.

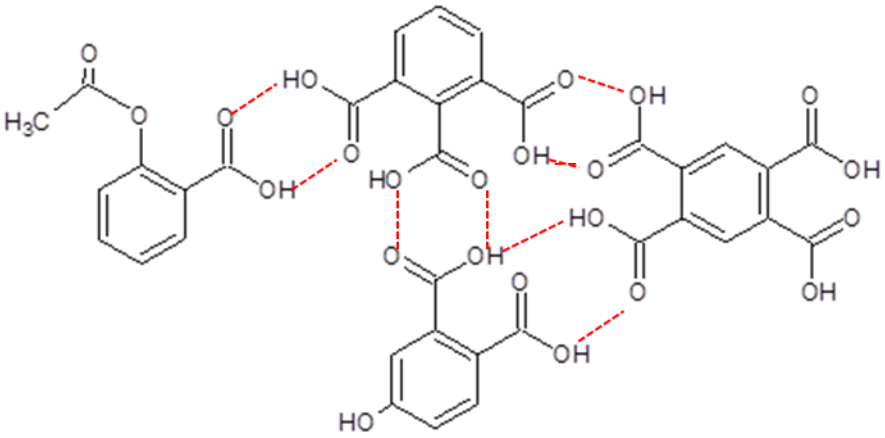


Figure 2-4. An illustration of intermolecular bonding

Alvarez-Puebla *et al.* (2006) have proposed a similar theoretical model for fulvic acid as illustrated by Figure 2-5.

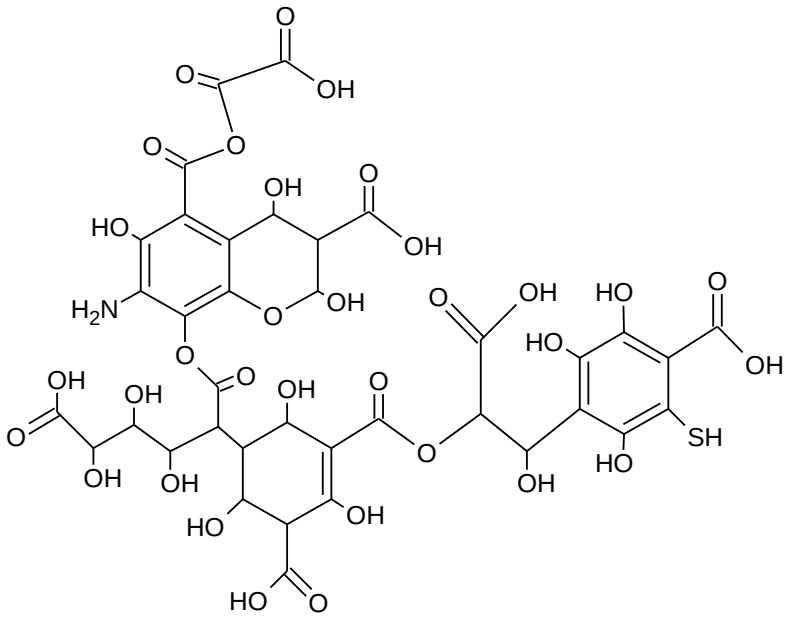


Figure 2-5. A theoretical model illustrating the complexity of chemical structure of fulvic acid.

Alvarez-Puebla *et al*. (2006) discovered that the conformation of protonated fulvic acid folds itself over to maximise Van der Waals electrostatic and H-bonds. Fulvic acid molecules have a tendency to expand because of the electrostatic repulsion generated by the charge increment solubility (dipolar moment) and electronic and vibrational energy when carboxylic and phenolic groups are ionised. The ionised fulvic acid has a higher negative charge and increased the energetic barriers and inhibited the approximation of fulvic acid caused by Brownian movements (Alvarez-Puebla *et al*., 2006). It explains the affinity of fulvic acid to form strong complexes with Fe^{3+}, Al^{3+} and especially Cu^{2+} associated with an increased solubility in natural waters (Bhutia, 2017).

The empirical formula of the structure of fulvic acid is $C_{14}H_{12}O_8$ as presented by PubChem (Pubchem CID 5359407), illustrated by Figure 2-6. However, this chemical structure is representative of a secondary metabolite from Penicillium. It is a simple, crystalline molecule with the same name as fulvic acids obtained from humus, but with different chemical and physiochemical properties.

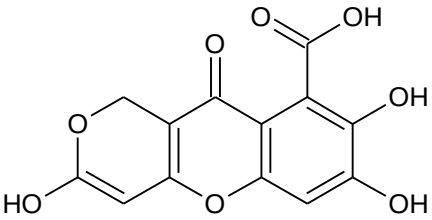


Figure 2-6. Chemical structure of fulvic acid isolated from Penicillium.

Yakimenko (2001), based on a comprehensive literature review of the structural properties of fulvic acid extracted from environmental sources, suggested that scientific evidence on the structure of fulvic acid is contradictory. The only agreement is that acid-soluble humus fractions, including fulvic acids, are the low molecular mass sub fractions characterised by a carbon skeleton composition (Yakimenko, 2001).

2.1.10 MOLECULAR CHARACTERISTICS

2.1.10.1 Chelator

Fulvic acids are highly effective chelators of heavy metals (Lubal *et al*., 2000; Pacheco & Havel, 2001), inorganic anions (Leita *et al*., 2001; Leit *et al*., 2009), halogens (Lee *et al*., 2001; Myneni, 2002), organic acids (Cozzolino *et al*., 2001), aromatic compounds (Schulten *et al*., 2001; Narri & Kim, 2002) and pesticides and herbicides (Chien & Bleam, 1997; De Paolis & Kukkonen, 1997; Schmitt *et al*., 1997; Fang *et al*., 1998; Gevao *et al*., 2000; Klaus *et al*., 2000).

The adsorption of fulvic acid by metal hydroxide surfaces was demonstrated by Filius *et al*. (2003) using a heterogeneous surface complexation model referred to as “the ligand and charge distribution model”. Filius *et al*. (2003) suggested that the surface speciation calculations of fulvic acid demonstrated that nearly all the hydroxyl groups in the adsorbed fulvic acid molecule were involved in the outer sphere complexation reactions. It included the carboxylic groups from both inner and outer sphere complexes (Filius *et al*., 2003). Schnitzer and Ghosh (1982) supported this observation from their electron spin resonance data which showed that a substantial portion of the metals in the fulvic acid complex had formed inner sphere complexes with copper and iron. Fulvic acid chelated copper complexes are divalent and iron complexes are trivalent (Schnitzer & Ghosh, 1982). Schnitzer and Ghosh (1982) suggested that the complexing of copper and iron exerted strains on the fulvic acid structure, lowering its resistance to thermal decomposition. This observation provided evidence that copper and iron are strongly complexed by fulvic acid (Schnitzer & Ghosh, 1982). The major fulvic acid functional groups involved in metal complexing are COOH and phenolic OH groups (Schnitzer and Ghosh, 1982). Metals bridging adjacent fulvic acid molecules increase the merger of the fulvic acid molecules reducing its thermal stability Saar and Weber, (1982) and Sposito *et al.* (1981) found that fulvic acids are especially reactive to iron, aluminium and copper in fresh waters (Gamble & Schnitzer, 1973) and soils (Prakash, 1971).

Linder and Murray (1987) used a modular randomised positioning of aromatic rings and functional groups to identify the predominant chelated metal-binding sites for calcium, copper, iron, magnesium, manganese and zinc ions and to estimate their concentrations within the fulvic acid molecular structure demonstrated that the most important site for zinc, manganese,

magnesium and calcium is phthalate (Linder & Murray, 1987). It was also found to be the second most important chelating site for copper. Linder and Murray (1987) concluded that the affinity for metal ions decreased in succession from copper to zinc to manganese and from manganese to calcium with the least affinity for magnesium.

Chelation therapy is used mainly in clinical toxicology (Soloway, 2011) and involves the application of chelating complexing agents in medical procedures aimed at the removal of heavy metals from the body (Soloway, 2011). The importance of fulvic acid as metal ion complexing agent was emphasised by Gamble and Schnitzer (1973). They found that fulvic acid has the ability to actively dissolve minerals and metals when in solution with water and the dissolved metallic minerals became biochemically reactive in the presence of fulvic acid. Williams (1963), Prakash (1971) and Buffle and Leppard, (1995) suggested that the chelating characteristics of fulvic acid in soil contributes significantly to enhancing the absorption of minerals and metals by plants. Prakash (1971) reported that fulvic acid had a high affinity for iron and Williams (1963) discovered that fulvic acid enhanced the absorption of vitamins and “natural” antibiotics. Buffle and Leppard (1995) reported that the mineral nutrient fulvate-complexes (organic fulvic acid molecule and nutrient ion) were rapidly absorbed by plant roots and suggested that the chelating characteristics of fulvic acid may have clinical uses. Aguilar *et al*. (2009) suggested that the metal chelating properties of fulvic acid is determined by the composition of the reactive carboxylic groups embedded in the molecular structure of fulvic acid.

Zhang *et al*. (2018) discovered that the chelated hydrogen bonding between fulvic acid and transferrin was nearly 1000-times stronger than the bonding of transferrin with other drug molecules. This finding holds promise for the use of fulvic acid in cancer therapy as transferrin is a carrier for the delivery of anticancer drugs in proliferating cancer cells (Zhang, *et al*., 2018).

2.1.10.2 Electron transport

Electron transport is the complex sequence of electron transfer from electron donors to electron acceptors via redox (both reduction and oxidation occurring simultaneously) reactions (Jayasooriya *et al*., 2016).

Humic substances are negatively charged metal complexing ligands and a number of chemical binding sites for metal ions to bind to aromatic and aliphatic carboxyl and phenolic hydroxyl groups are available in their structures. This allows fulvic acid to act as an ion exchanger by releasing metal ions of low atomic mass and chelating heavier metal ions (Aiken *et al.,* 1985; Norden & Dabek-Zlotorynska, 1997; Wershaw, 1989; Gramss *et al.,* 1999).

Fulvic acid possesses many biomedical functions related to its ability to promote the electrochemical balance in cells. They accumulate in tissue as semiquinone radicals and behave as electron donors or electron acceptors, depending on the redox state of the system (Jayasooriya *et al*., 2016; Schepetkin, *et al*., 2002).

The biomedical function of fulvic acid is confirmed by its ability to interact with xenobiotics to form stable biochemical complexes (Pacheco *et al*., 2003). Pacheco *et al*. (2003) proposed that, because of their poly-functionality, fulvic acids represents a strongly pH dependent reservoir of electron donors and electron acceptors which could hypothetically contribute to reduction-oxidation of several inorganic and organic agents. These properties of fulvic acid are abolished at the reactive sites by methylation and acetylation (Frimmel, 1998).

2.1.10.3 Bioavailability

Bioavailability is the rate and degree from the time of consumption to accessibility at cellular level of a specific compound (Rita & Akhilesh, 2015). The importance of bioavailability in medicinal applications was discussed by Rita and Akhilesh (2015), explaining that when the bioavailability of medicine diminishes, its impact on restoring normal cellular function and health is restricted.

Mirza *et al*. (2011) suggested that shilajit functions as a complexing agent for carbamazepine (CBZ) to enhance the pharmacokinetic profile of CBZ and its accessibility to the brain. Mirza *et al*. (2011) has postulated that fulvic acid could decrease the dose amount of CBZ by improving its bioavailability.

Wang *et al*. (1996b) found that fulvic acid is highly bioavailable by showing that fulvic acid was effectively absorbed and distributed in all the organs of a molecular uptake study in rats. Bioavailability for organ distribution was most prominent in the kidneys, followed in succession of distribution by the liver, spleen, bone, cartilage, skin and hair, muscles, lungs, thymus, heart and least in the brain.

2.1.11 CLINICAL PROPERTIES

Humic substances have emerged as potential sources for the development of human and animal health products (Chien *et al*., 2015; Ogner & Schnitzer, 1971; Peña-Méndez *et al*., 2004; Levine, 1989; McKnight *et al.*, 1991; Peschel & Wildt, 1988). The increased use of humic substances as antimicrobial, anti-inflammatory profibrinolytic and antitumor agents (Peña-Méndez *et al*., 2004; Rizon, 2016; Schnitzer & Khan, 1972; Van Rensburg, 2015; Yamada *et al*., 2007) have created a growing awareness among the scientific and medical communities of their potential as pharmaceutical agents (Ogner & Schnitzer, 1971; Levine, 1989; McKnight *et al.*, 1991; Peschel &

Wildt, 1988). Numerous scientific papers (Aydin *et al*., 2017; Lorentzen, 2006; Botes *et al*., 2002; Cornejo *et al*., 2011; Dekker & Medlen, 1999; Van Rensburg *et al.,* 2000; Van Rensburg, 2015; Yamada *et al*., 2007) have provided evidence that fulvic acid has clinical properties which include the inhibition of a number of inflammatory markers (Chien *et al.*, 2015; Klöcking *et al*., 2008; Malfeld, 2005), bactericidal (Kotb El-Sayed *et al*., 2012; Van Rensburg *et al*., 2000; Zhu *et al*., 2014), fungicidal (Van Rensburg *et al*., 2000; Wu *et al*., 2016) and antiviral efficacy (Joone *et al*., 2003; Peña-Méndez *et al*., 2004; Schneider *et al*., 1996; Van Rensburg *et al*., 2000) and antioxidant characteristics (Rodriquez *et al*., 2011; Shikalgar & Naikwade, 2018).

The use of fulvic acid in modern medicine was prompted by hospital studies reporting that fulvic acid dietary supplementation was effective in treating difficult viral respiratory illnesses common in children (Peña-Méndez *et al*., 2004). Schepetkin *et al*. (2009) reported that fulvic acid taken orally is effective therapy for gastritis, diarrhoea, stomach ulcers, dysentery, colitis and diabetes mellitus. The medicinal use of fulvic acid was also advocated by Rodriguez *et al*. (2011) who reported that fulvic acid applied as a tropical treatment is effective for haematoma, phlebitis, desmorrhexis, myogelosis, arthrosis, polyarthritis, osteoarthritis and osteochondritis.

2.1.11.1 Anti-inflammatory activity

The potential application of fulvic acid in the treatment of numerous chronic low-level inflammation conditions such as certain cancers, rheumatoid arthritis, type 2 diabetes, heart disease, Alzheimer's disease and depression was demonstrated by Acharya *et al*. (1988), Malfeld (2005), Van Rensburg (2015) and Van Rensburg *et al*. (2001b).

Van Rensburg *et al*. (2001b) assessed the anti-inflammatory activity of a topically applied coal-derived fulvic acid, oxifulvic acid, at concentrations of 4.5 % and 9 % respectively and compared it with 1 % diclofenac sodium and 0.1 % betamethasone for hypersensitivity in a murine contact model. Van Rensburg *et al*. (2001b) demonstrated that oxifulvic acid at concentrations of 4.5 % and 9 % respectively compared favourably with diclofenac sodium and betamethasone in suppressing cutaneous inflammatory responses. Malfeld (2005) reported that both 4.5 % and 9 % oxifulvic acid creams inhibited an elicited inflammatory reaction after 24 hours by scavenging oxidants as demonstrated by the inhibition of inflammatory markers on leucocytes at concentrations achievable for topical creams. Malfeld (2005) concluded that the anti-inflammatory effect of oxifulvic *in vivo* have demonstrated that fulvic acid is useful for the treatment of inflammatory skin diseases.

Shilajit is a potent scavenger of superoxide radicals (Schepetkin *et al*., 2002; Malfeld, 2005). Superoxide radicals induce and maintain chronic inflammation disorders and because fulvic acid

is the active biological component in shilajit, the study by Schepetkin *et al*. (2002) is confirmation of the potential use of fulvic acid as an active pharmaceutical ingredient in medicinal formulations for the treatment of chronic inflammatory disorders. This statement is supported by Chien *et al*. (2015) who have provided a molecular mechanism for the inhibition of homocysteine-induced COX-2 expression by fulvic acid in monocytes as a basis for the application of fulvic acid in manufacturing pharmaceutical therapy agents against inflammation.

2.1.11.2 Antimicrobial activity

Antimicrobial activity is the process of inhibiting and eliminating disease causing microbes (pathogens). The term “antimicrobial” is used in this study inclusively for antibacterial, antifungal and antiviral compounds.

The study by Liu *et al*. (2012) demonstrated that natural organic benzofuran containing compounds from different sources apply variable modes of action to suppress microbial infections. Liu *et al*. (2012) screened seventeen synthesised benzofuran derivatives for their antibacterial properties against *Escherichia coli*, *Staphylococcus aureus*, Methicillin-resistant *Staphylococcus aureus*, *Bacillus subtilis* and *Pseudomonas aeruginosa* and discovered that seven of the synthesised benzofuran derivatives demonstrated excellent antibacterial activities when compared to cefotaxime and penicillin.

Van Rensburg *et al*. (2001a) investigated the antimicrobial efficacy of coal-derived oxifulvic acid and found that *Streptococcus faecalis, Staphylococcus aureus, Pseudomonas aeruginosa*, *Escherichia coli, Streptococcus pyogenes, Klebsiella pneumoniaea, Proteus mirabilisa* and *Candida albicans* were sensitive to oxifulvic acid at a concentration of 15 g/L and *Enterococcus faecalis* and *Klebsiella pneumoniae* susceptible to a concentration of 5 g/L (Van Rensburg *et al*., 2001a).

2.1.11.3 Antioxidant activity

Cellular oxidation, a free radical producing chemical reaction, is known to damage various human and animal cells. The reactive oxygen species (ROS) contributes largely to the development of atherosclerosis, diabetes, cancer, neurodegenerative diseases, liver cirrhosis and cellular ageing processes (Rodriguez *et al.,* 2011). Unfortunately, synthetic antioxidants have demonstrated various side effects such as carcinogenicity and their use is increasingly restricted by health authorities (El-Moneim *et al*., 2013). This has motivated scientists in recent years to find natural antioxidants for replacing synthetic antioxidants in food and medicinal substances.

EI-Moneim *et al*. (2013) and Wang *et al*. (1996a) discovered that fulvic acid possess superoxide and hydroxyl radical scavenging properties. Numerous scientists have reported that environmental fulvic acids sourced from weathered coal (Ueda *et al*., 2004), peat (Tachibana *et al*., 2004), shilajit (Bhattacharya & Sen, 1995) and composting soil (Rodriguez *et al.,* 2011) have demonstrated powerful antioxidative properties (Bhattacharya & Sen, 1995; Ghosal *et al*., 1995).

The antioxidant property of fulvic acid is dose dependent (EI-Moneim *et al*., 2013). EI-Moneim *et al*. (2013) treated rats with a single hydrogen peroxide oral dose to induce oxidative stress and liver damage. Fulvic acid was evaluated for its protective effect by activating super oxide dismutase and glutathione peroxide activities. The *in vitro* antioxidant activity in superoxide anion radical scavenging and hydrogen peroxide scavenging assays demonstrated that fulvic acid possessed strong dose dependent antioxidant activity (EI-Moneim *et al*., 2013). EI-Moneim *et al*. (2013) suggested that the phenolic hydroxyl group and metal-chelating ability of fulvic acid explained its ROS scavenging activity.

Plaza *et al*. (2005) stated that fulvic acid has significant protective efficacy against induced oxidative stress by modulating glutathione peroxide and superoxide dismutase defence enzymes (EI-Moneim *et al*., 2013). Fulvic acid works synergistically with the non-enzymatic antioxidants glutathione, uric acid, bilirubin and vitamins C and E as well as enzymatic antioxidants such as superoxide dismutase, catalase and glutathione peroxidase (Rodriguez, *et al*., 2011). Gao *et al*. (2017) reported that antioxidant enzyme activities were significantly ($p < 0.05$) higher in loach fed a fulvic acid-added diet compared to a control group.

EI-Moneim *et al*. (2013) and Rodriguez *et al.* (2011) concluded that fulvic acid is a good candidate for use in the pharmaceutical and food industries as a natural antioxidant source against oxidative stress induced by the accumulation of hydrogen peroxide.

2.1.11.4 Immune stimulating effects

Immune stimulation restores homeostasis in the body by balancing the ratio of the different immune cells when the body is threatened by foreign pathogenic microorganisms (Riede *et al*., 1991). It is also possible that the immune response to foreign invaders is too persistent causing the development of autoimmune diseases such as rheumatism (Riede *et al*., 1991).

Vucskits *et al*. (2010) determined the production parameters, immune responses and thyroid function in rats fed different fulvic acid concentrations. They recorded a time-dependent significant ($p < 0.05$) increase in ovalbumin antibody titre with a 0.4 % fulvic acid supplementation over a 26 day period. Vucskits *et al*., 2010 found that the immune response was improved by the

14th day and reported that the mean values for the control group was 685.79 and for the fulvic acid group 1131.37 respectively. By the 26th day the control was 544.31 and the fulvic acid group 1969.83. Vucskits *et al*. (2010) discovered that the 'B'-dependent lymphoid tissue diameter of the ileum and spleen was significantly ($p < 0.05$) larger in the fulvic acid treated animals and concluded that fulvic acid supplementation resulted in a strong humoral immune stimulation.

Jayasooriya *et al*. (2016) found that fulvic acid has the ability to regulate immune-stimulating functions in a dose-dependent manner by demonstrating that the DNA-binding activities of nuclear factor-κB (NF-κB) in RAW 264.7 cells were upregulated by fulvic acid. Jayasooriya *et al*. (2016) concluded that fulvic acid, by stimulating NF-κB to promote inducible nitric oxide synthesis and nitric oxide production, is an effective regulator of immune-stimulating functions.

2.1.12 OTHER CLINICAL APPLICATIONS

The biological significance of fulvic acid has been recognized for many years (Aydin *et al*., 2017) and the use of shilajit as a rejuvenator in traditional medicine for thousands of years is a good example (Agarwal *et al*., 2007). Although fulvic acid accounts for ~90 % of all humic substances in the world, scientific understanding of the claims related to its biological properties is still limited (Aydin *et al*., 2017).

2.1.12.1 Cancer

Research by Jayasooriya *et al*. (2016) demonstrated that humic substances protects against cancer and related cancer-causing viruses. Jayasooriya *et al*. (2016) suggested a 100 % success rate in preventing tumour progression towards the cancerous state with humic substance therapies in patients with tumours of the oesophagus. Jayasooriya *et al*. (2016) concluded that fulvic acid have anticancer activities when they discovered that fulvic acid-stimulated culture media enhanced cell death in Hep3B (hepatoma) LNCaP (prostatic adenocarcinoma) and HL60 (leukaemia) human cancer cells.

2.1.12.2 Diabetes

The antidiabetic properties of fulvic acid was demonstrated by Bhattacharya (1995) who administered streptozotocin (STZ, 45 mg/kg) over a period of 28 days to induce hyperglycaemia in rats. By day 14, hyperglycaemia was significant and increased progressively for the rest of the 28 day trial period (Bhattacharya, 1995). Shilajit was administered at a dose of 50 mg and a dose of 100 mg per kg bodyweight respectively. Fulvic acid attenuated the hyperglycaemic response of

STZ from day 14 onwards for 100 mg per kg bodyweight was statistically significant to prevent the onset of diabetes mellitus (Bhattacharya, 1995).

The work of Bhattacharya (1995) was supported by the study of Trivedi *et al*. (2004) who reported that shilajit fulvic acid when orally dosed at 100 mg per kg bodyweight per day for 4 consecutive weeks works synergistically with glibenclamide at 5 mg per kg bodyweight per day orally and metformin at 0.5 g per kg bodyweight per day orally to significantly enhanced glucose lowering. Trivedi *et al*. (2004) found that the synergistic effect of glibenclamide and metformin with fulvic acid improved the lipid profile to levels well above levels recorded for these drugs when administered separately.

2.1.12.3 Neurogical disorders

Fulvic acid has cognitive and memory enhancing properties (Jaiswal & Bhattacharya, 1992; Schepetkin et al., 2009) by preventing the aggregation of Aβ peptides which are directly related to Alzheimer's disease (Verma *et al.*, 2013). This phenomenon is demonstrated by the inhibiting effect of fulvic acid on the dimer formation of Aβ17–42 peptides by disrupting the precursor Aβ17–42 trimer in a very short time interval (Verma *et al.*, 2013). Dimer formation of Aβ17–42 peptides is also detected *in vivo* in the brains of individuals with Alzheimer's disease and Down's syndrome (Miller *et al*., 2009). Verma *et al.* (2013) suggested that fulvic acid be used against Aβ17–42 mediated cytotoxicity and neurodegeneration disorders.

Cornejo *et al*. (2011) reported that Alzheimer's disease is a neurodegenerative disorder involving extracellular plaques (amyloid-β) and intracellular tangles of tau protein. Tau is the major microtubule associated protein (MAP) of a mature neuron and the microtubule network assembly promoting activity of tau, a phosphoprotein, is regulated by the degree of phosphorylation (Iqbal *et al*., 2010). Yamada *et al*. (2007) found that brain tau is three- to fourfold more hyper-phosphorylated in Alzheimer’s patients than in the normal adult brain. Tau is polymerised during this hyper phosphorylated state into paired helical filaments (Iqbal *et al*., 2010) and current therapeutic strategies are aimed at inhibiting the formation of tau filaments or to disaggregate them completely (Cornejo *et al*., 2011). Cornejo *et al*. (2011) provided evidence to suggest that fulvic acid can inhibit the aggregation of tau protein and the formation of paired helical filaments *in vitro.* Cornejo *et al*. (2011) found that fulvic acid affected the length as well as the morphology of the fibrils and suggested that fulvic acid offers a new approach in the treatment of Alzheimer's disease. Cornejo *et al*. (2011) concluded that fulvic acid can prevent pathological self-association, inhibit aggregation and promote the disassembly of tau fibrils associated with Alzheimer’s disease by binding specifically to tau.

2.1.12.4 Cartilage remodelling

Kurz *et al.* (1999) has investigated the influence of fulvic acid on collagen secretion in articular chondrocyte cultures in seven day old bovine interphalangeal joints chondrocyte monolayers and found that fulvic acid, in comparison to control cells, induced a dose dependent stimulation at 10 ppm of up to 1.7-fold on collagen type II cultures. Type II collagen is found primarily in the centre portion of joint cartilage discs to add structure and strength to connective tissues. Kurz *et al.* (1999) found that fulvic acid induced tight collagen fibre nets around a subpopulation of chondrocytes. No immune-cytochemically detectable collagen type II cells were deposited in the control cells (Kurz *et al.*, 1999). The collagen secretion was nearly threefold stronger than the ascorbic acid supplemented medium (50 µg/ml) after treatment with fulvic acid at 10 ppm (Kurz *et al.*, 1999).

2.1.12.5 Effect on allergic conditions

Ghosal *et al*. (1989) assessed the effect of shilajit against degranulation and disruption of mast cells by noxious stimuli and found that different combinations of its main constituents, fulvic acids, 4′-methoxy-6-carbomethoxybiphenyl and 3,8-dihydroxy-dibenzo-α-pyrone provided statistically significant protection against antigen-induced degranulation of sensitised mast cells. Ghosal *et al*. (1989) stated that the antigen-induced spasm of sensitised guinea-pig ileum was clearly inhibited and concluded that shilajit has the potential for clinical use in the treatment of allergic disorders. The study of Ghosal *et al*. (1989) is complimented by the studies of Yamada *et al*. (2007) and Motojima *et al.* (2011).

Yamada *et al*. (2007) found that fulvic acid extracted and purified from Canadian Sphagnum peat inhibited the antigen-antibody binding and antibody-receptor stages to conclude that fulvic acid could be useful in the treatment and prevention of allergic reactions. Motojima *et al.* (2011) discovered that fulvic acid extracted from solubilised excess sludge had an inhibitory effect on β-hexosaminidase release in human leukaemia basophilic (KU812) cells. Fulvic acid affected the expression of genes involved in signal transduction pathways, cytokine–cytokine receptor interaction pathways, immune responses, cell adhesion molecules and IgE receptor β subunit response pathways to demonstrate allergy control and various other pharmacological properties (Motojima *et al.*, 2011).

2.1.12.6 Intestinal health

The effect of dietary fulvic acid supplementation on intestinal digestive activities, antioxidant and immune enzyme activities and the composition of the microflora in juvenile loach was studied by

Gao *et al*. (2017). Fulvic acid supplementation resulted in an abundance of *Firmicute* structures, a phylum of mostly Gram-positive bacteria and *Actinobacteria* which, in the microbiome is related to improved gastrointestinal health. Gao *et al*. (2017) discovered that fulvic acid supplementation induced a concomitant reduction in the abundance of Proteobacteria, the major phylum of *Escherichia*, *Salmonella*, *Vibrio*, *Helicobacter*, *Yersinia* and *Legionellales*. These are Gram-negative bacteria and they are known for their negative impact that on gastrointestinal health. Gao *et al*. (2017) found that fulvic acid supplementation caused a noticeable reduction in the relative abundance of *Serratia*, an opportunistic pathogen, pathogenic bacteria *Acinetobacter*, *Aeromonas* and *Edwardsiella*. Fulvic acid increased the abundance of the important probiotic *Lactobacillus* in the intestine and significantly increased intestinal protease activities, antioxidant activities, lysozyme activities, complement 3 content, immunoglobulin M (IgM) content, acid phosphatase activities (ACP) and alkaline phosphatase activities (AKP) (Gao *et al*., 2017). The work of Gao *et al*. (2017) have demonstrated that fulvic acid can improve growth performance by stimulating protease and lipase activities and it was concluded that fulvic acid enhances intestinal health.

2.1.13 CLINICAL CONCERNS

Scientists have raised concerns about the utilisation of humic substance extracted from environmental sources in products for human consumption and have warned that it may be harmful to human health (Whitby & Schnitzer, 1978). Humic substances forms strong chelated complexes with metals and hydrophobic organic pollutants in natural environments (De Paolis & Kukkonen, 1997). Humic substances extracted from aquatic and soil sources are known for their chelating properties with heavy metals, organic toxicants and pesticides and are excellent vehicles for pollutant transfer (Whitby & Schnitzer, 1978), rendering the use of humic substances in pharmaceutical formulations dangerous to human health (Peña-Méndez *et al*., 2004).

Horsfall and Spiff (2005) reported that copper, lead, nickel, cadmium, cobalt, zinc and iron were isolated from various environmental fulvic acids sources. Whitby and Schnitzer (1978) discovered that dialkyl phthalates, some toxic, accumulates in bottom sediment humic materials and Peña-Méndez *et al*. (2004) provided evidence that humic acid, a soil-humic substance, is toxic to a number of mammalian cells. Klöcking (2008) stated that humic substances can chelate with heavy metals such as cadmium to form harmful chemical complexes and expressed concern about the safe use of humic substances in pharmaceutical formulations.

2.1.13.1 Safety

Soils in various regions of the world are contaminated with lead and arsenic (Mishra & Roy, 2015) increasing the health risks for using humic substance in health and nutritional products (Peña-Méndez *et al*., 2004). Fakour and Lin (2014) studied the complexation of fulvic acid with arsenic in water and confirmed that arsenic formed strong complexes with fulvic acid. Significant amounts of arsenic, arsenide (0.2 - 5 mg/L) and arsenate, respectively, chelated with only 30 mg/L FA (EL-Table *et al*., 2017).

Lead and arsenic are related to serious health concerns in young children (Mishra & Roy, 2015). Lead is a potent toxin and can damage the nervous system of embryos, breastfed infants and young children (Mishra & Roy, 2015). Only a small amount of lead poisoning is needed to delay mental development, lowering intelligence, impair hearing and affect an individual's balance (Mishra & Roy, 2015). The damage to the nervous system may be irreversible. Long-term exposure to arsenic increases the risk of skin, lung, bladder, kidney and liver cancers (Mishra & Roy, 2015).

Fulvic acid is a heat stable low molecular weight water soluble cationic colloidal material with colloidal properties that renders it susceptible to contamination (Aydin *et al*., 2017, Sabi *et al*., 2011; Sherry *et al*., 2012). It is especially reactive with Fe^{3+}, Al^{3+} and Cu^{2+} (Bhutia, 2017). EL-Table *et al*. (2017) reported that fulvic acid has a high affinity for heavy metals to form strong complexes with lead, aluminium, mercury, cadmium and chromium It is also known to form strong complexes with toxins produced by pathogenic bacteria, moulds and pesticides (EL-Table *et al*., 2017). These complexes are extremely toxic (EL-Table *et al*., 2017). Sabi *et al*. (2011) have reported that the concentration levels of heavy metal present in coal derived fulvic acid are highly toxic and potentially fatal, rendering coal-derived fulvic acid unsafe for human and animal consumption.

2.1.13.2 Quality

The efficacy of fulvic acid is determined by its quality (Schepetkin *et al*., 2003). This statement is demonstrated by the work of Malfeld (2005).

Malfeld (2005) discovered that a low concentration of coal derived oxifulvic acid had little effect on anti-inflammatory markers. Malfeld (2005) had to increase the oxifulvic acid concentration level to >100 µg/ml to be effective but warned that this concentration may be potentially toxic to cells and suggested that oxifulvic acid is not unsuitable for systemic use by humans (Malfeld,

2005). Bergh *et al*. (1997) found that the quality of coal is affected by various environmental and production factors.

Chopra *et al*. (1958) stated that shilajit consumption without preliminary purification could lead to the risk of intoxication. The presence of mycotoxins, heavy metal ions, polymeric quinones (oxidant agents) and free radicals embedded in the chemical composition of shilajit determines its safety and quality (Chopra *et al*., 1958). A report by Saper *et al*. (2008) has emphasised the danger of using poor quality humic substances and warned against the danger of using humic substances that are not supplied through regulated producers but commercialised by the internet as it may contain detectable levels of heavy metals such as lead, mercury and arsenic.

One of the main reasons for not considering fulvic acid for medicinal purposes at a commercial level, is the difficulty in extracting good quality fulvic acids economically at a large scale from natural waters and soils (Malfeld, 2005).

2.1.13.3 Consistency

The work of Ghosal (1995) has demonstrated that environmental fulvic acids, because of inconsistencies in the molecular composition of humic substances extracted from different sources, are not suitable for use in the pharmaceutical industry. Ghosal (1995) found that the physiological properties of fulvic acid extracted from various shilajit samples collected in different regions of the world differed profoundly in their molecular structures. Ghosal (1995) concluded that the diversity in the molecular structures are directly related to the different origins from where the shilajit samples were sourced.

The importance of consistency in the chemical molecular structure of humic substances for application in the pharmaceutical industry was demonstrated by Kotb El-Sayed *et al*. (2012). Kotb El-Sayed *et al*. (2012) discovered that the antioxidant and antimicrobial efficacy of fulvic acid are related to the type of plants and herbs from where fulvic acid is sourced. Plants and herbs are found in various regions where they are exposed to different climatic conditions, known to play a significant role on the humification process to ultimately determine the compositional variety in the molecular structure of fulvic acids sourced from these regions.

2.2 CARBOHYDRATE-DERIVED FULVIC ACID (CHD-FA)

CHD-FA is an internationally patented (SA Patent 2001/2419) unique carbohydrate sourced fulvic acid composition (Botes *et al,* 2017; Loxton *et al*., 2012). CHD-FA is a synthetic heavy metal free fulvic acid derived from a carbohydrate source (Sherry *et al.*, 2012). Sherry *et al.* (2012)

described CHD-FA as a heat stable low molecular weight, water soluble, cationic, colloidal material. CHD-FA is produced from a wet oxidation process using sucrose, water and oxygen and regarded as a major breakthrough in the production of a unique fulvic acid molecular structure (Loxton *et al*., 2012).

CHD-FA is not affected by environmental factors and free of heavy metals and toxic contaminants (Sabi *et al*., 2011). It has various medicinal applications (Gandy *et al*., 2012; Sabi *et al.,* 2011; Sherry *et al*., 2012; Sherry *et al*., 2013; Zhao *et al.,* 2015).

2.2.1 CHARACTERISATION

Gandy (2016) conducted a comparative analysis of the molecular structure to identify a unique marker for CHD-FA. His first aim was to record the masses of the ionisable compounds in the stock solutions of CHD-FA before using LC-MS to analyse the chemical compounds in the CHD-FA structure. Gandy (2016) demonstrated that CHD-FA consist mainly of acidic organic low molecular weight (<450) compounds and discovered that the CHD-FA molecular structure was too complex for identification of the chemical compounds embedded within the CHD-FA molecular structure.

2.2.2 CLINICAL PROPERTIES OF CHD-FA

Numerous scientific publications have demonstrated the CHD-FA possesses anti-inflammatory, antibacterial, antifungal, antiviral and antioxidant properties for oral (Gandy *et al.*, 2012; Sabi *et al*., 2011; Sherry *et al*., 2012; Sherry *et al*., 2013) and dermal (Gandy *et al*., 2012; Sabi *et al*., 2011; Zhao *et al*., 2015) applications.

A phase 1 clinical trial on the acute and subacute safety and proof-of-concept efficacy of CHD-FA was conducted by Gandy *et al.* (2012). Gandy *et al.* (2012) found that CHD-FA did not have any detrimental effects on haematological and biochemistry markers and confirmed the safety of CHD-FA for use in clinical settings.

The safety, tolerability and clinical impact of CHD-FA in a formulated wellness drink (F0210) did not have any adverse effects on the general health in one hundred and sixty six Human Immunodeficiency virus (HIV-1) positive patients (Botes *et al*., 2017). CHD-FA was well tolerated in this antiretroviral therapy-naïve study population (Botes *et al*., 2017), suggesting that CHD-FA is an active pharmaceutical ingredient that should be considered for use in medicinal applications that are currently the domain of antibiotics.

2.2.2.1 Anti-inflammatory

Sabi *et al*. (2011) evaluated the safety and the anti-inflammatory and wound healing characteristics of CHD-FA in rats and found that CHD-FA (≥100 mg/kg oral dosage) was effective in reducing carrageenan-induced paw oedema. This finding was comparable to an indomethacin oral dosage of 10 mg/kg.

A proof-of-principle study to establish CHD-FA as a safe and effective agent for preventing the onset of drug-resistant bacterial and fungal infections in military and civilian personnel who have experienced traumatic bacterial and fungal wound infections was conducted by Zhao *et al*. (2015). They found that a 4.6 % CHD-FA solution significantly up-regulated pro-inflammatory cytokine interleukin 6 (IL-6) and interleukin 10 (IL-10) in two infected models, methicillin-resistant *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Zhao *et al*., 2015). CHD-FA treated wounds displayed improvement at days 3, 6 and 10 post infection and Zhao *et al*. (2015) concluded that CHD-FA is a safe anti-inflammatory compound for the treatment of infectious wounds.

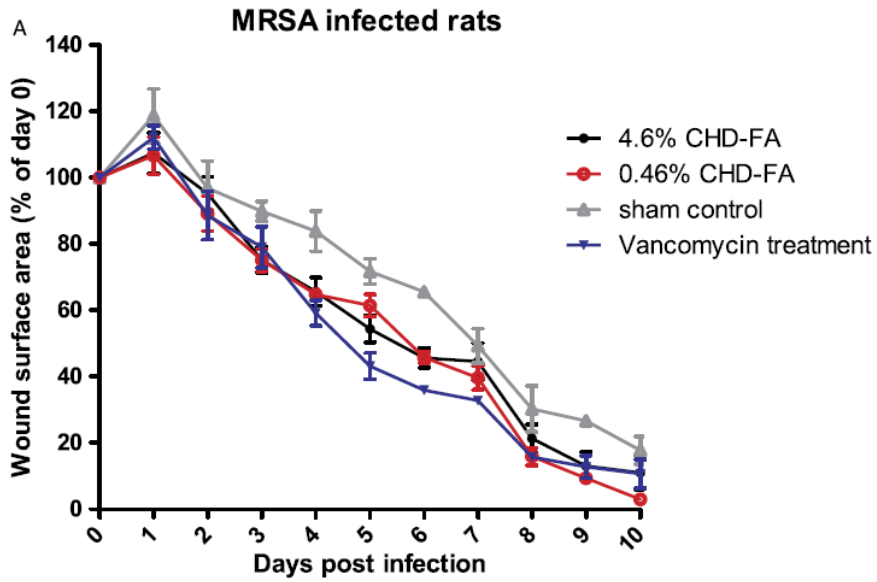
The efficacy and safety of CHD-FA in the treatment of eczema in patients 2 years and older was demonstrated by Gandy *et al*. (2012). 3.5 % CHD-FA in an emollient (buffered to pH 4.8) was compared to a placebo, Epizone A, to establish the anti-inflammatory properties of CHD-FA in patients with eczema (Gandy *et al.,* 2012). Severity, erythema, vesiculation, fissuring and scaling were examined, and significant differences were observed for severity and erythema in both placebo and CHD-FA treated groups. Gandy *et al.* (2012) reported that the CHD-FA group demonstrated a significant improvement in treating eczema and concluded that CHD-FA, when applied topically, has anti-inflammatory properties (Gandy *et al.,* 2012).

Gandy (2016) further conducted a study to assess the safety and therapeutic efficacy of CHD-FA in patients older than 2 years with in atopic dermatitis. The group treated with CHD-FA showed a significant improvement when compared to the placebo group, confirming the anti-inflammatory properties of CHD-FA, leading to a significant overall improvement of the disease. Gandy (2016) concluded that CHD-FA is safe and effective on contact with hypersensitive skin disorders. Gandy *et al.* (2012) recorded further that the flare measurement, which is an inflammatory response to an allergen, had significantly decreased with the application of CHD-FA proving that CHD-FA is an effective anti-inflammatory agent.

2.2.2.2 Antibacterial

Studies by Sabi *et al*. (2011) and Zhao *et al*. (2015) evaluated the healing efficacy of CHD-FA on wounds infected by drug resistant pathogens. Sabi *et al*. (2011) concluded that CHD-FA compared favourably with fusidic acid cream (10 mg/g) in accelerating the healing process of wounds infected with *Staphylococcus aureus*.

Zhao *et al*. (2015) analysed the healing properties of CHD-FA in multi-pathogen infected open wounds and found that CHD-FA showed strong activity against a variety of bacterial and fungal pathogens. Improved wound healing with CHD-FA treatment was demonstrated by wound surface area measurements, histopathologic examinations and expression profiling of wound healing genes (Zhao *et al*., 2015). Figure 2-7 A indicates the wound surface area measurement in rats with 1 x 108 cfu *MRSA* under different treatment. Figure 2-7 B is representative wound images taken over the 10-day experiment period from each treatment group. (Adopted from Zhao *et al.,* 2015). Approved by Prof. David Perlin for use in this thesis (Annexure B).



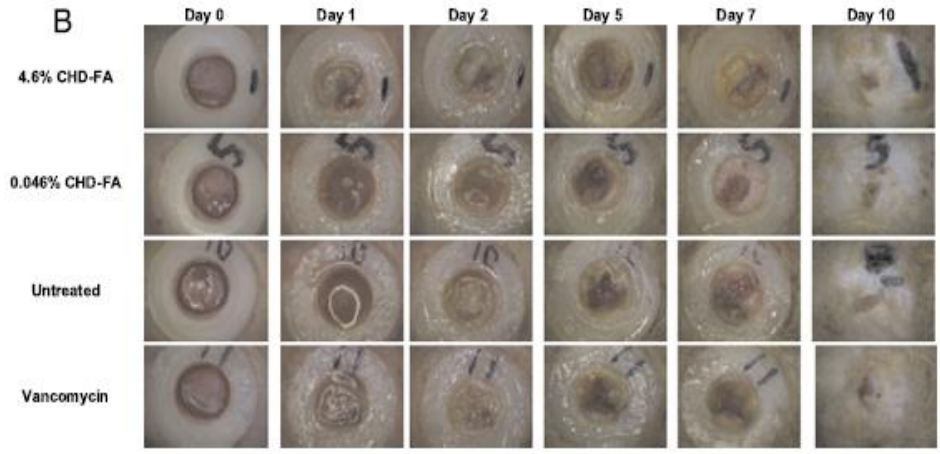
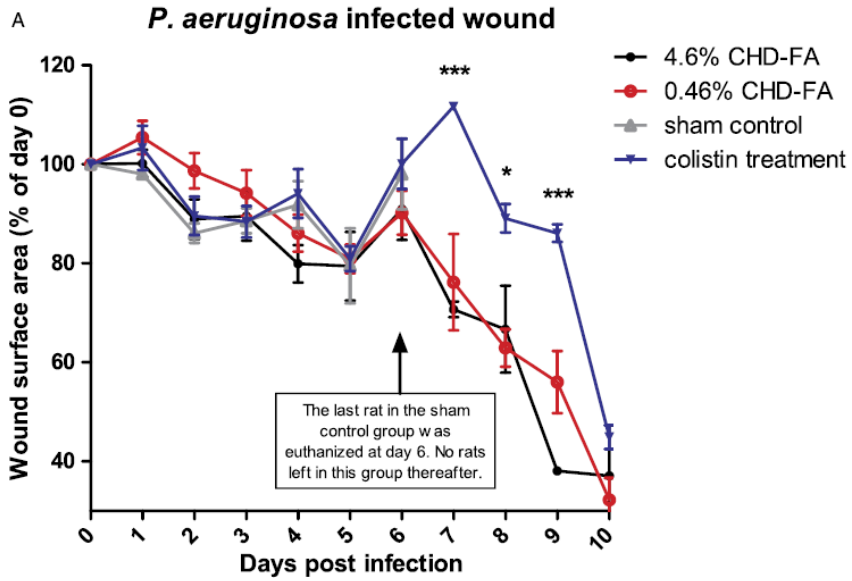


Figure 2-7. A. Wound surface area measurement in rats with *MRSA* under different treatment. B. Representative wound images from each treatment group.

Figure 2-8 A indicates the wound surface area measurement in rats infected with 1 x 107 cfu *Pseudomonas aeruginosa* under different treatment with the CHD-FA groups being compared with the Colistin control. Figure 2-8 B is representative wound images taken over the 10-day experiment period of each treatment group (Adopted from Zhao *et al.,* 2015, Approved by Prof David Perlin, see annexure B).



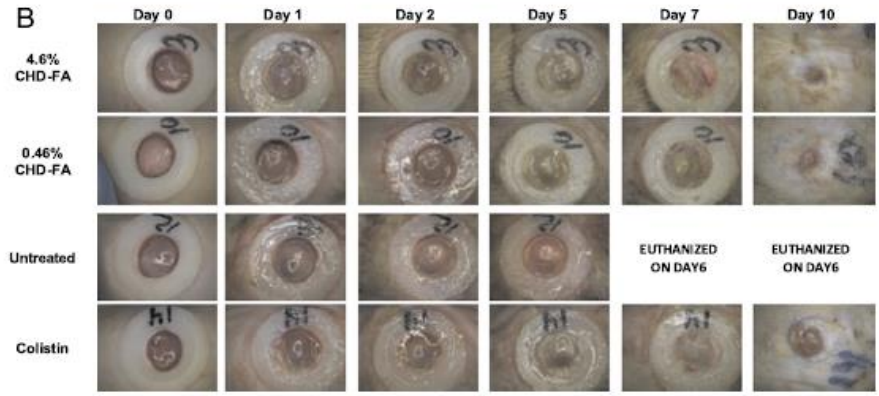


Figure 2-8. A. Wound surface area measurement in rats infected with Pseudomonas aeruginosa under different treatment. B. Representative wound images taken from each treatment group.

Zhao *et al*. (2015) concluded that CHD-FA is a promising topical remedy for drug-resistant wound infections as it accelerated the healing process of wounds infected with methicillin resistant *Staphylococcus aureus* and multidrug-resistant *Pseudomonas aeruginosa* in rats. Zhao *et al.* (2015) also established the cutaneous wound model in rats with the drug-resistant Gram-negative bacteria *Escherichia coli* and *Klebsiella pneumoniae.* Preliminary CHD-FA treatment efficacy data that included wound closure measurements and enumeration of microbial burden on wound sites were also collected on these pathogens. The conclusion of this study was that CHD-FA had excellent antimicrobial and anti-inflammatory properties and is an effective pharmaceutical agent against drug-resistant bacterial and fungal infections in military and civilian personnel suffering traumatic wound infections.

Oral infectious diseases such as periodontitis caused by microbial biofilms are associated with increased antimicrobial resistance to current medicinal therapies, suggesting an urgent need to develop safe and effective antimicrobial mouthwashes (Sherry *et al.*, 2013). Sherry *et al.* (2013) addressed this concern by investigating the antimicrobial properties of CHD-FA in the management of oral infections and discovered that CHD-FA was highly effective against planktonic and sessili bacterial biofilms with a supplementary function of down-regulating inflammation. Sherry *et al.* (2013) concluded that CHD-FA offers an attractive spectrum of clinical properties for use as an alternative treatment strategy for oral biofilm diseases.

2.2.2.3 Antifungal

Sherry *et al.* (2012) assessed the efficacy of CHD-FA as a novel antibiotic resistant modifying agent *in vitro* and found that 4 % CHD-FA (pH 2.1) combined with fluconazole was highly effective in eradicating fluconazole resistant *Candida* isolates.

Zhao *et al.* (2015) tested the efficacy of CHD-FA over a 10 day period against drug-resistant Gram-negative bacterium *Acinetobacter baumannii* and pathogenic mould *Aspergillus fumigatus.* Preliminary CHD-FA treatment efficacy data including wound closure measurements and enumeration of microbial burden on wound sites were also collected for these pathogens. CHD-FA treatment efficacy was dose dependent as demonstrated by visually observed improved wound closures for high and middle CHD-FA dose concentrations. The most prominent differences were observed on day 9 where the average wound surface areas was 21.1 % in the high dose group (4.6 %) in contrast to 38.9 % in the untreated control group. The lowest CHD-FA (0.046 %) treatment dose wound closure values were not significantly different from the untreated controls confirmed dose dependent treatment efficacy.

2.2.2.4 Antiviral

The potential application of CHD-FA in viral infections was reported by Botes *et al*. (2017) who noticed an interesting trend in patients' CD4 counts with CHD-FA exposure. CD4 counts decline was slower when compared directly to similar trends recorded in the literature for the natural progression of a viral disease.

2.2.2.5 Antioxidant

CHD-FA is an accessible source of natural antioxidants for used in the food industry (Rodriquez *et al.,* 2011). Rodriquez *et al.* (2011) alluded to possible antioxidant properties when they suggested CHD-FA has health beneficial properties.

2.2.2.6 Negative effects

The negative or adverse events reported were gastrointestinal adverse events (diarrhoea) by Botes *et al.* (2017) as well as headaches, Gandy *et al*. (2012) reported nauseaness at high dosage, however not significantly.

2.2.3 SAFETY AND TOXICOLOGY STUDIES ON CHD-FA

2.2.3.1 Observations in humans

A phase 1 clinical trial on the acute and sub-acute safety and proof of concept efficacy of CHD-FA by Gandy *et al.* (2012) provided significant evidence that 3.8 % CHD-FA is safe at oral dosages up to 80 ml per day for 7 days. On successful completion of the acute exposure safety phase which demonstrated no significant adverse effects, Gandy *et al.* (2012) performed a 3-day sub-acute toxicity test on the same volunteers for the same 3.8 % fulvic acid dose concentrations

of 20 and 40 ml respectively, administered twice daily. Vital parameters, clinical observations, ECG recordings and haematology and biochemistry full blood count, liver and kidney functions showed no abnormalities or any signs of toxicity rendering CHD-FA safe for use in humans.

2.2.3.2 Safety in immune compromised patients

The objective of a study by Botes *et al*. (2017) was to determine the safety and tolerability of CHD-FA in a pre-ART HIV-1 positive population in India. This double-blind, placebo-controlled parallel study was conducted on 332 patients. Safety, tolerability and time to the requirements of ART and/or the time to a decrease in CD4 count of 100 cells/mm^3 was recorded for subjects in each treatment group by Botes *et al*. (2017). Change in immune status was regarded as an important clinical endpoint in this study (Botes *et al*., 2017). CHD-FA did not have any negative effect on the CD4 count, HIV-1 viral load changes or to any quality of life disease-specific parameters. Botes *et al*. (2017) did no not find any significant adverse effects on the natural progression of the HIV-1 infection or on the general health of the patients, rendering CHD-FA safe for use in this population.

2.3 REFERENCES

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CHAPTER 3: IDENTIFICATION OF THE MAJOR CONSTITUENT OF CHD-FA AND A THEORETICAL MODEL FOR THE MECHANISM BY WHICH MOLECULAR FULVIC ACID IS FORMED FROM SUCROSE THROUGH A NON-CATALYTIC WET OXIDATION PROCESS

"Ready for submission" article in *ChemEngineering*, entitled:

"*Identification of the major constituent of Carbohydrate-Derived Fulvic Acid (CHD-FA) and a theoretical model for the mechanism by which molecular fulvic acid is formed from sucrose through a non-catalytic wet oxidation process."*

Introduction

This chapter presents the "ready for submission" manuscript for publication in *ChemEngineering* published by MDPI. The manuscript is presented in the required format prescribed by *Instructions for Authors,* and as outlined on the journal website:

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The manuscript begins with the title, name of author and affiliations, followed by the Abstract. The main body of the manuscript consist of an Introduction; Objectives; Methodology; Results and Discussion; Conclusion and finally Acknowledgements and References.

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Article

Identification of the major constituent of Carbohydrate-Derived Fulvic Acid (CHD-FA) and a theoretical model for the mechanism by which molecular fulvic acid is formed from sucrose through a non-catalytic wet oxidation process.

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Abstract: A comprehensive description of the non-catalytic chemical reaction process in manufacturing fulvic acid from a carbohydrate rich source has not been previously reported in scientific literature. In this study an illustrative theoretical model is proposed to demonstrate the succession of processes involved during a non-catalysed controlled wet oxidation synthesis process to form fulvic acid, identified as the major constituent in Carbohydrate-Derived Fulvic Acid (CHD-FA), from sucrose extracted from *Saccharum officinarum* (sugar cane). The decomposition of sucrose resulted in the production of various carboxylic acids demonstrated by a pH reduction. A mechanistic pathway for the transformation of sucrose into glucose and fructose, followed by further oxidative thermal degradation into cluster of low molecular weight carboxylic acids was proposed based on results obtained from mass spectrometry.

The chemical and spectroscopic properties of the CHD-FA molecular structure demonstrated similar characteristics to fulvic acid reference standards as obtained from the literature. The chromatogram overlay of CHD-FA and the penicillin-derived fulvic acid demonstrated that fulvic acid was derived from sucrose. The MALDI-TOF MS was used to desorb and ionise fulvic acid without fragmentation in order to measure the absolute molecular weight. The molecular weight of the main component in CHD-FA is 308 g/mol and this is similar to the molecular weight of penicillin-derived fulvic acid. NMR and LC-MSMS demonstrated batch-to-batch consistency in the manufacturing of CHD-FA, suggesting that CHD-FA possesses unique characteristics.

Keywords: Non-catalytic wet oxidation process, oxidative thermal degradation, re-aldol reaction, carboxylic acids, fulvic acids, Carbohydrate-Derived Fulvic Acid.

1. Introduction

Fulvic acids, often referred to as 'naturally occurring' organic acids [1], are oxidised fragments of larger humic substances [2] found primarily in peat and natural waters [3,4,5,6]. Environmental levels of fulvic acids are low and varied [2,7] and characterised by complex molecular structures [2,8,9,10,11].

The first attempt to produce a humic substance from a carbohydrate source, glucose, performed by Malguti [12], followed shortly after the discovery of Braconnot's [6] "dark brown" liquid material extracted from peat. Malguti [12] published his view on the transformation of carbohydrates to synthetic humic substances. Braconnot [13] added acids to starch or sucrose, which then formed a dark precipitate that looked like humic acids from peat. The theory that humic substances were derived from polysaccharides was supported by furan-containing compounds present in humic substances. It was demonstrated that carbohydrate synthesis yield furfural, formaldehyde and benzaldehyde [14]. The first conclusive attempt to identify the chemical structures formed during the wet oxidation process of carbohydrate demonstrated that oxygen present in natural air oxidises sucrose when in a hot neutral solution [15]. It caused an inversion of sucrose and the decomposition of the resulting hexoses [15]. The primary reaction in the thermal degradation of sucrose is the splitting of glycosidic bonds at 185 °C to form sucrose derivatives characterised by pyranose rings and various hydro-sucrose bonds [16]. The formation of aromatic compounds from carbohydrates through hydrothermolytic processes was demonstrated by Theander and co-workers [17]. They discovered that aldol condensation is a primary route for the production of aromatic compounds from saccharides. A comprehensive literature review has concluded that the chemistry of wet oxidation of a single organic compound is a complex process [18]. It involves numerous chain reactions caused by radicals of organic compounds present in the reaction mixture. The exothermic reaction of carbohydrate wet oxidation is thermally initiated by various catalytic reactions leading to chemical processes involving the removal of α-hydrogen from a carboxylic acid by hydroxide and base-induced retro-aldol reactions [18]. A mild wet oxidation manufacturing process to extract a semi-synthetic potassium-rich humic acid from bituminous coal was used to yield a synthetic derived fulvic acid, identified as oxifulvic acid [19,20]. The non-catalytic oxidation of coal with oxygen was identified as fulvic acid which did not exhibit significant acute toxicity in an animal test model [21].

A recent invention has seen the development of a pure source of fulvic acid, Carbohydrate-Derived Fulvic Acid (CHD-FA), produced by a patented non-catalytic wet oxidation process to good manufacturing practice standards (GMP) [22]. CHD-FA is free of environmental contaminants [23] and has medicinal properties [22,23,24,25,26]. This article aims to illustrate and present a theoretical model for the mechanism by which molecular fulvic acid is formed from sucrose during a non-catalytic wet oxidation synthesis of CHD-FA.

The objectives are:

1.1 First Objective

To present an illustrative model for the mechanism by which sucrose, extracted from *Saccharum officinarum* (sugar cane), is converted to organic acids in the "start-up" phase (Phase 1) of a non-catalytic wet oxidation process with oxygen and purified (reverse osmosis) water during the synthesis of CHD-FA.

1.2 Second Objective

To demonstrate that a pure carbohydrate source can produce fulvic acid during the synthesis of CHD-FA and to determine the characteristics of CHD-FA.

1.3 Third Objective

To demonstrate batch-to-batch consistency in the manufacturing of CHD-FA using a non-catalytic wet oxidation process.

2. Materials and Methods

2.1 Materials

Carbohydrate source: *Saccharum officinarum* (sugar cane) is a pure carbohydrate source from which sugar (sucrose), a natural combination of fructose and glucose, is extracted. Sugar cane is shredded and compressed into sugar cane juice. The liquid content is evaporated by heated air resulting in crystallised sugar. The sugar (sucrose) used in this study is a pure carbohydrate source. Purity is > 98%.

Oxygen source: Pure oxygen (O_2), produced by air separation processes using cryogenic liquefaction and distillation with a purity > 99.5%, was used in the wet oxidation process of this study.

Water source: Purified water used during the wet oxidation process was obtained from an in-house water purification system (reverse osmosis). This system meets with the European Pharmacopoeia water purification requirements and all the specified validations were carried out prior to the start of this study.

2.2 Equipment

A stainless steel reactor equipped with a feed flow meter, feed pump, reactor vessel and heat exchanger was used to perform the non-catalytic wet oxidation process. Oxygen flow and system pressure controls were linked to a computerised control system. The different systems ensured a standardised, safe and controlled production process.

2.3 Manufacturing process

Wet oxidation process: The process started with the preparation of a fixed concentration of sucrose solution in a mixing tank. A sugar solution was transferred in succession from the mixing tank to afeed tank and from the feed tank fed continuously to a pressurised reaction vessel at a specified rate where the solution was heated to a temperature of 160 ºC. This temperature was required for the exothermic reaction to commence. The exothermic reaction was then controlled at a high temperature of above 200 ºC. Oxygen was introduced into the reaction vessel through a series of diffusers in order to ensure an even oxygen distribution into the reactor. The critical control points are the oxygen and sucrose solution flow rates and the pressure and temperature within the reactor. A dark brown solution of organic acids (CHD-FA) referred to as the "raw API" was analysed at four hour intervals to ensure that production specifications were met.

Filtration: the intermediate API (raw product) was filtered through a 5000 Da filter to deliver a dark red-to-brown solution consisting of low molecular weight fulvic acids. Alternatively, the intermediate API (raw product) was filtered through a 400 Da filter to ensure a yellow colour fulvic acid solution consisting of fulvic acids with a low molecular mass. The 5000 Da filtered dark red-to-brown and the 400 Da yellow fulvic acids are the final CHD-FA active pharmaceutical ingredients.

Concentration: the correct CHD-FA concentration level was achieved through a controlled evaporation process.

2.4 Sampling

Start-up samples: Six samples of 100 ml each were collected from the reactor at 30 °C, 70 °C, 120 °C, 160 °C, 170 °C and 190 °C. The samples were collected during the start-up process of the non-catalytic wet

oxidation process of sucrose. Physical colour changes, demonstrative of the degradation of sucrose, and pH changes were recorded during the sampling process.

Samples for spectroscopic analysis:

- Liquid chromatography-tandem mass spectrometry (LC-MSMS)
- Nuclear magnetic resonance spectroscopy (NMR)
- Gas chromatography-mass spectrometry (GCMS)
- Matrix-assisted Laser Desorption and Ionisation Time-of-Flight Mass Spectrometry (MALDI-TOF MS)

Three 100 ml samples were collected from the sucrose solution in the reactor. These samples were used for LC-MSMS and NMR. The first sample was collected at the start of the exothermic reaction at 160 °C with the second sample at 170 °C and the third sample at 190 °C.

2.5 Analyses for objective 1

LC-MSMS:

Samples were diluted in 50 % aqueous acetonitrile to meet with the standard calibration range for mixed sugars consisting of glucose, fructose and sucrose. A Waters Synapt G2 Quadrupole-Time-of-Flight (QTOF) mass spectrometer was used in this study. The source was electrospray (negative mode) and the following settings were applied: capillary voltage, 3 kV; cone voltage, 15 V; dissolving gas (nitrogen), 650 L/h; dissolving temperature, 275 °C. The samples were introduced with a Waters Acquity ultra performance liquid chromatograph (UPLC) connected with one meter 0.003 inch PEEK tubing to the mass spectrometer.

NMR:

Samples were prepared by transferring 750 µl of each sample to a new NMR tube with a D_2O filled insert included for locking purposes. An Agilent^{Unity} Inova 600 NMR spectrometer was used to record ^{1}H NMR spectra at a frequency of 600 MHz and ^{13}C NMR spectra at a frequency of 150 MHz. ^{1}H and ^{13}C NMR semi-quantitative spectra were recorded overnight at 25 °C using default VnmrJ 4.2 software parameters. ^{1}H spectra were referenced to the residual H_2O signal at 4.79 ppm and ^{13}C spectra were referenced to an external dioxane reference standard.

2.6 Analyses for objective 2

Materials required for analyses were fulvic acid derived from penicillin (assay > 98 %), obtained from Santa Cruz Biotechnology, Texas, USA (reference standard fulvic acid - CAS 479-66-3) and two CHD-FA 5000 Da solution samples with a 5 % fulvic acid concentration of 100 ml each and two CHD-FA 400 Da solution samples with a 5 % fulvic acid concentration of 100 ml each.

LC-MSMS:

Samples from both CHD-FA 400 Da and CHD-FA 5000 Da were diluted with 50 % aqueous acetonitrile to fall within the calibration range for a mixed sugar set standard containing glucose, fructose and sucrose. A Waters Synapt G2 Quadrupole-Time-of-Flight (QTOF) mass spectrometer was used in this study. The source was electrospray (negative mode) and the following settings were applied: capillary voltage, 3 kV; cone voltage, 15 V; dissolving gas (nitrogen), 650 L/h; dissolving temperature, was 275 °C. The samples were introduced with a Waters Acquity ultra performance liquid chromatograph (UPLC) connected with one meter 0.003 inch PEEK tubing to the mass spectrometer (MS) with Solvent A: 1 % aqueous acetic acid and Solvent B: methanol with 1 % acetic acid. A short 1 minute gradient was applied starting at 100 % Solvent A to 50 % solvent B after 0.5 min and finally 100 % Solvent B after 1 min. Data

were acquired in resolution mode from m/z 50-1000. The instrument was calibrated with sodium formate and leucine encephalin was used as reference (lock mass) for accurate mass determinations. The molecular mass of main peaks identified by LC-MSMS spectra were transferred for elemental composition analysis for an empirical formula assessment (fit configuration > 70 %).

NMR:

Two samples from both CHD-FA 400 Da and CHD-FA 5000 Da were prepared by transferring 750 µl of each sample to a new NMR tube with a D_2O filled insert included for transmitter frequency regulation purposes. An Agilent^{Unity} Inova 600 NMR spectrometer was used to record ^{1}H NMR at a frequency of 600 MHz and ^{13}C NMR at a frequency of 150 MHz. The ^{1}H and ^{13}C NMR spectra were recorded overnight at 25 °C using the default VnmrJ 4.2 software parameters. The ^{1}H spectra were referenced to the residual H_2O signal at 4.79 ppm while the ^{13}C spectra were referenced to an external dioxane reference standard.

GCMS:

The fulvic acid (assay > 98 %) obtained from Santa Cruz Biotechnology, Texas, USA (reference standard fulvic acid - CAS 479-66-3) and 200 µl of the CHD-FA 5000 Da solution were transferred into a clean micro centrifuge tube and dried overnight in a speed vac at lower settings. The samples were reconstituted with 100 µl pyridine and 50 µl of BSTFA [N,O-Bis(trimethylsilyl)trifluoroacetamide] with 1 % TMCS (trimethylchlorosilane). The mixture derivatised at 60 °C for 30 min. On completion of the incubation period, samples were allowed to cool to room temperature and were then vortexed for a few seconds before being transferred into vials for GC analysis. The derivatised samples were analysed with an Agilent 6890 N gas chromatograph (Agilent, Palo Alto, CA) coupled to an Agilent 5975 MS mass spectrometer, using a polar (95 % dimethylpolysiloxane) ZB-Semivolatiles Guardian (30 m, 0.25 mm ID, 0.25 µm film thickness) GC column. The oven temperature was set to maintain 80 °C for 1 min and finally ramped at 7 °C/min to 300 °C and held for 2 min. The carrier gas was helium with a flow rate of 1 ml/min and the injector temperature was set at 280 °C in the split less mode. Mass spectra were recorded in full scan mode (40-650 m/z) with the ion source and quadrupole temperatures being maintained at 240 °C and 150 °C respectively. The transfer line temperature was maintained at 280 °C.

MALDI-TOF MS:

The MALDI-TOF MS has the ability to desorb and ionise the molecule without fragmentation and therefore measure the absolute molecular weight of the molecule. The MALDI-TOF MS instrument used had the following features and settings: a 3rd pulsed nitrogen laser (339 nm) with a maximum intensity of 2 x 10^5; negative and positive ion detection; reflectron and linear mode operation; ion path length of 3.0 m in reflectron mode and 2.0 m in linear mode. In the linear mode the mass range is 200 kDa and in the reflectron mode the range was 6000 Da with a resolution of 1000 and 10 000 respectively. The analyte / matrix samples were ablated and ionised from the sample holder with the nitrogen laser and ions accelerated into the flight tube. The best result was obtained using 1 mg/mL CHD-FA and 10 mg/mL α-cyano-4-hydroxycinnamic acid (CHCA). The obtained spectrum is in the positive ion mode.

2.7 Analyses for objective 3

Six samples of 18 batch runs over a period of four years were collected. One 100 ml sample for both CHD-FA 400 Da and CHD-FA 5000 Da from every third manufactured batch was collected for FTIR and LC-MSMS analyses. 16 Samples were collected from batches manufactured over the same period for the NMR analyses.

Fourier transform infrared spectroscopy (FTIR): A spectrum BX Fourier transform infrared spectrometer with an internal $LiTaO_3$ detector was used with the resolution set at 2 cm^{-1}, strong apodisation function, a static gain of 1,OPD velocity at 7.5 kHz, with a bi-directional interferogram direction and

number of scans set at 32. The spectrum over the conventional range at a start frequency of 4000 to an end frequency of 400 cm^{-1}.

NMR refer to 2.6.1

LC-MSMS refer to 2.6.1

3. Results and discussion

3.1 An illustrative model for the mechanism by which sucrose is synthesised to form molecular fulvic acid

The proposed theoretical pathway for the non-catalytic wet oxidation of sucrose to yield carboxylic acids is initiated by the thermal degradation of sucrose during the start-up phase. This is characterised by the splitting of glucosidic bonds. Thermal degradation of sucrose at temperatures of 110 °C, 120 °C, 130 °C and 140 °C investigated previously, reported that fructose degraded nine to tenfold faster than glucose [27,28].

The pathway is illustrated in Figure 3-1.

Sucrose $C_{12}H_{22}O_{11}$ [α-D-glucopyranoside; β-D-fructofuranosyl]

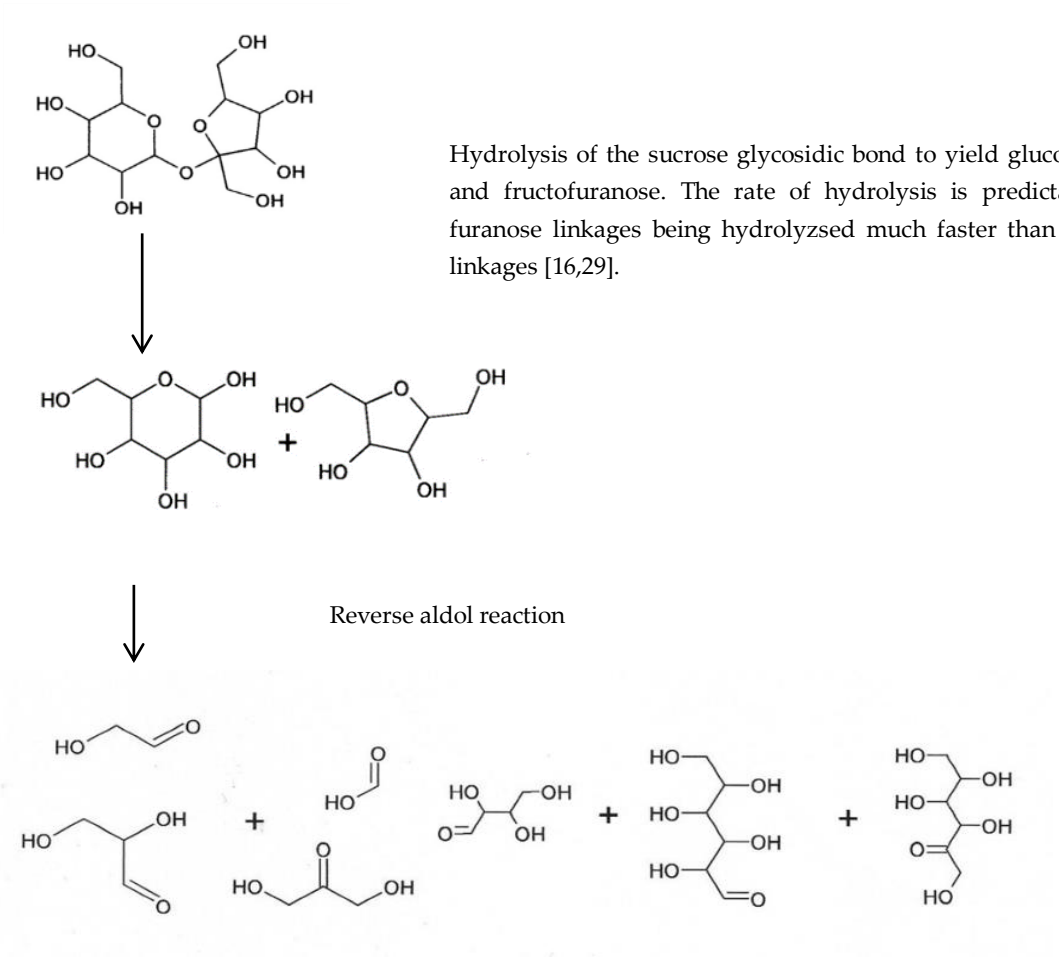


Figure 3-1. The thermal oxidative degradation synthetic pathway of sucrose.

Thermal oxidative degradation processes [16,17,28,30,31] derives oxidising power from the high solubility of oxygen at elevated pressures. Pressure and temperature increases are rectilinear and the production of carboxylic acids increases at high temperatures. The physical characteristics of the primary reaction during the thermal oxidative degradation process are demonstrated by colour changes related to the temperature increases during the start-up of the wet oxidation process. The observation of colour changing from light to dark brown as the temperature increases (Photo 1-6). At the start-up phase of the wet oxidation process, six samples at 30 °C, 70 °C, 120 °C, 160 °C, 170 °C and 190 °C were collected**.** The physical characteristics of these samples are indicated by the photos. Photo 1 is the sample collected at 30 °C, the second photo is the sample collected at 70 °C, photo 3 depicts the sample collected at 120 °C while photos four to six show the samples collected at 160 °C, 170 °C and 190 °C respectively.

Photos 1-6: Colour changes at different temperatures for samples during the start-up of the wet oxidation process.

1 2 3 4 5 6

The colour changes observed in this study, ranging from light-to-dark brown with an increase in temperature was reported previously [30] and it was suggested that the colour changes are related to the formation of certain dehydrated aromatic compounds during increasing temperatures such as hydroxymethyl-2-furfural (5-HMF) or 2-furfural. Levulinic acid and other smaller carboxylic acids formed during the oxidation process may also contribute to the changes. Golon and Kuhnert [32] suggested that the heating of disaccharides yield thousands of compounds formed by a small number of unselective and chemo-selective reactions, predominantly oligomerisation and dehydration and hydration reactions. These reactions are responsible for the colour changes. Darkening of heated glucose and maltose solutions was also reported recently [29].

It was proposed that thermal degradation products of glucose and maltose such as furfural, 5-methylfurfural and 5-HMF are responsible for the development of the dark colour observed during the thermal oxidative degradation process. The increase in yields of organic acid yields presented in the present study is confirmed by previous findings [33]. Colour and pH changes at different temperatures reported in Table 3-1 are commonly observed during thermal oxidative degradation processes. It is clear from Table 3-1 that the increase in temperatures and pressures during thermal oxidative degradation processes are directly correlated to a decrease in pH demonstrative of an increase in organic acids yields.

Table 3-1. Colour and pH changes at different temperatures in thermal oxidative degradation.

Sample	Temperature	Colour	pH
1	30 ºC	Transparent natural water colour	5.62
2	70 ºC	Off-white	5.25
3	120ºC	Light yellow	4.15
4	160 ºC	Dark yellow	3.62
5	170 ºC	Light Reddish brown	2.61
6	190 ºC	Dark brown	2.16

Table 3-1 provides evidence that carboxylic acids are formed during the non-catalytic wet oxidation of sucrose demonstrated by the reduction in the pH of the solution. This study has provided evidence that the formation of carboxylic acids is directly correlated to the combination of high temperatures, high pressures and heating time. This statement is confirmed by the finding that sucrose is transformed into glucose and fructose during the oxidative thermal degradation process as reported in Table 3-2. It supports the findings of a previous study [16] which found that there was no sucrose present in the reaction mixture after 30 min at 185 °C and that chromatography showed that the presence of longer retention times is associated with the of new products.

Table 3-2. Changes in concentrations of sucrose, glucose and fructose at different temperatures in the thermal oxidative degradation process.

	Concentration mg/L		
Samples	**Glucose**	**Fructose**	**Sucrose**
Sample 1 at 160 °C	83298.2	84173	1417
Sample 2 at 170 °C	92671.5	49476.0	45
Sample 3 at 190 °C	24648	1452	0

The thermal degradation of sucrose was analysed with LC-MSMS. The first step was to obtain a chromatogram from a reference sucrose solution reported in Figure 3-2 to serve as a reference standard in the present study. The retention time is 18.5 minutes.

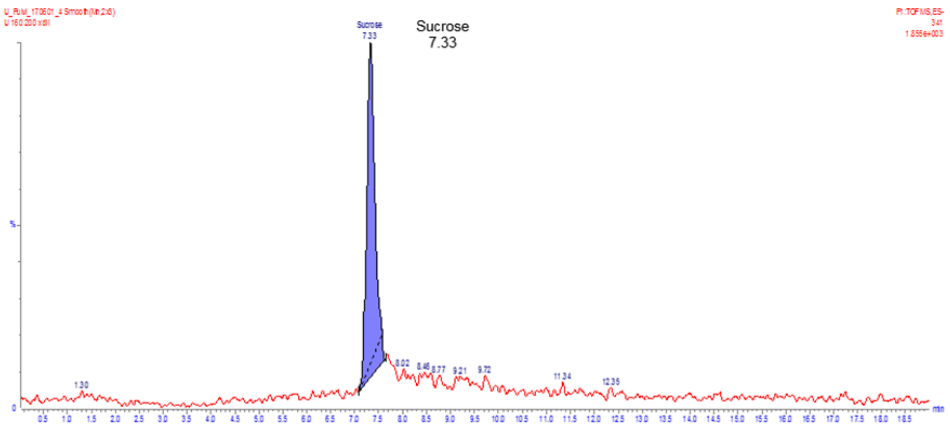


Figure 3-2. Reference standard: Sucrose solution (12 %) at 30 ºC, retention time 18.5 min.

Figures 3-3, 3-4 and 3-5 are chromatograms of samples obtained from the reactor at the start of the exothermic reaction in the present study. It demonstrates the step-by-step changes that occur in both glucose and fructose concentrations and supports the splitting of the glycosidic bonds during thermal oxidative degradation. Figure 3-3 is the chromatograph showing the presence of glucose and fructose concentration at the start of the exothermic reaction. No carboxylic acids are present.

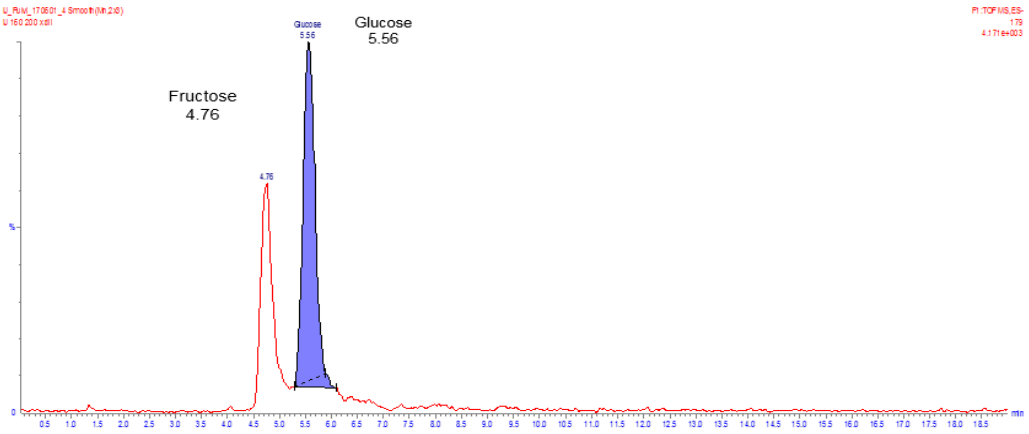


Figure 3-3. Sample 1: Solution from reactor at 160 °C.

Figure 3-4 shows a decrease in fructose concentration at 170 °C and the possible presence of carboxylic acids/other degradation products suggesting that fructose is degraded into carboxylic acids.

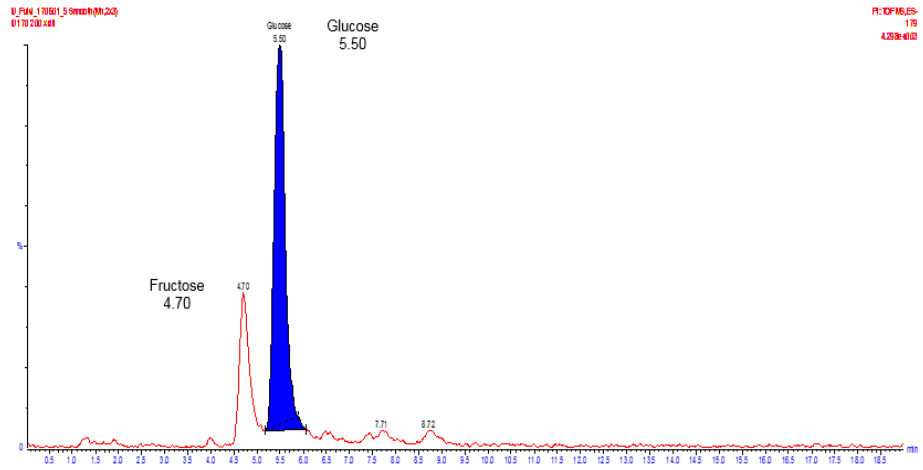


Figure 3-4. Sample 2: Solution from reactor at 170 °C.

The splitting of glycosidic bonds during thermal oxidative degradation is confirmed by the absence of sucrose at 190 °C (Figure 3-5). The likely presence of carboxylic acids from the degradation of fructose at 170 °C (Figure 3-4) is shown in Figure 3-5. A decrease in glucose concentration with the formation of carboxylic acids is demonstrated by an increase in the number of peaks with a concurrent drop in pH, indicated by Figure 3-5. (also see Table 3-2)

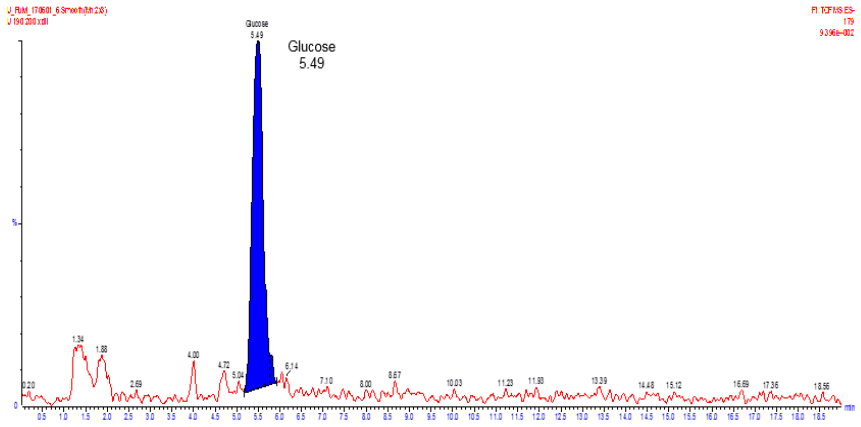


Figure 3-5. Sample 3: Solution from reactor at 190 °C.

LC-MSMS in Figures 3-2, 3-3, 3-4 and 3-5 respectively support previous research work stating that wet oxidation splits the glycosidic bond in sucrose to yield glucose and fructose [16,29,32]. It is then degraded further into carboxylic acids as shown by ^{1}H NMR in Figure 3-6. The main feature of the degraded products is the additional proton signals appearing in the region between 1.8 and 5.5 ppm. The increase in upfield (right side of spectrum) proton signals indicates that different types of degradation compounds have formed concomitantly with the increase in temperature.

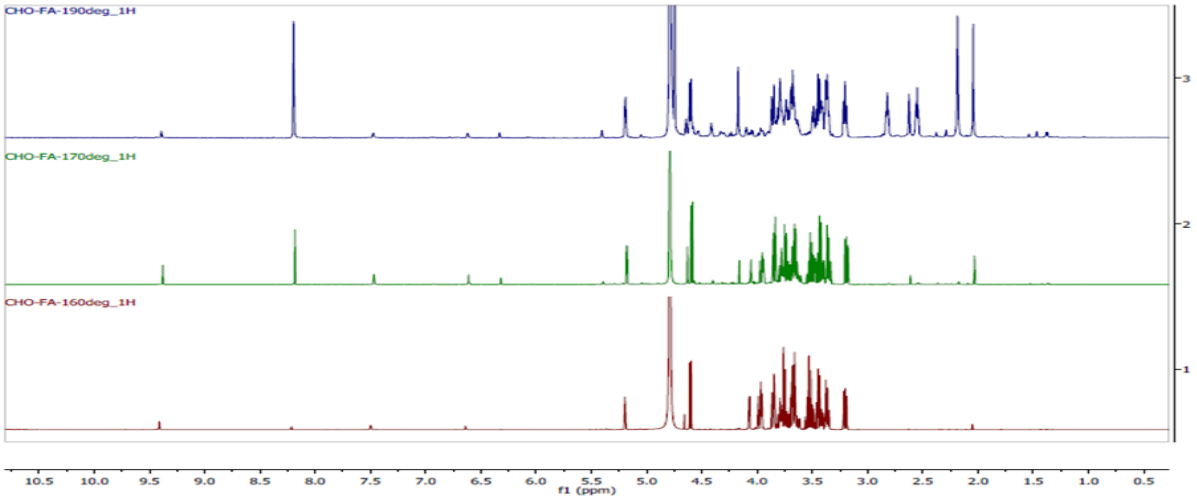


Figure 3-6. ^{1}H NMR spectra of degraded sucrose obtained from the reactor at 160 °C, 170 °C, 190 °C.

The NMR data at 160 °C is representative of a mixture of mainly glucose and fructose. Similarly, the sample at 170 °C shows predominantly glucose, a decrease in fructose and an increase in degradation products. Finally, glucose is still present at 190 °C but fructose is no longer present and an increase in the number of peaks associated with the degradation products is shown. A number of new and increased proton signal intensities demonstrate an increased concentration of these species in the spectra. The stronger signals (1.9 ppm and 4.2 ppm) at 190 °C have possibly originated from protons belonging to chemical fragments formed during oxidation. The signals at 3.2 ppm, originally from protons on CH_3-groups bonded to aliphatic chains and the signals at 4.0 to 5.5 ppm are partially due to alcoholic OH-group protons [34]. It is argued, based on the following two observations, that under the present experimental conditions, ^{1}H NMR spectra revealed loose structures that are not cleaved by oxidative degradation:

- The permanence of sugar proton signals between 2.8 and 5.5 ppm.
- The spectral pattern 1.9 to 4.2 ppm showing a high degree of oxidation.

The ^{13}C NMR spectra of oxidative thermal degradation of sucrose into glucose and fructose and then into carboxylic acids are illustrated in Figure 3-7. The ^{13}C NMR spectra demonstrated that different carbon types appear at different temperatures during the start-up of the wet oxidation process as measured at 160 °C, 170 °C and 190 °C. The transformation of the carbohydrate carbons (60 – 90 ppm) to carbonyl carbons (160 – 190 ppm) and aliphatic carbons (0 – 60 ppm) is proposed [35].

The exothermic reaction commenced at 160 °C and thereafter temperature, oxygen supply and sugar solution flow rates were controlled. It is suggested that, at this point of elevated temperatures and high pressure conditions, a myriad of reactions are possible. The different types of chemical reactions during typical wet oxidation of organic compounds include auto-oxidation, oxidative thermal degradation, hydrolysis, decarboxylation and polymerization [18] can be divided into three stages: chain initiation, propagation and termination.

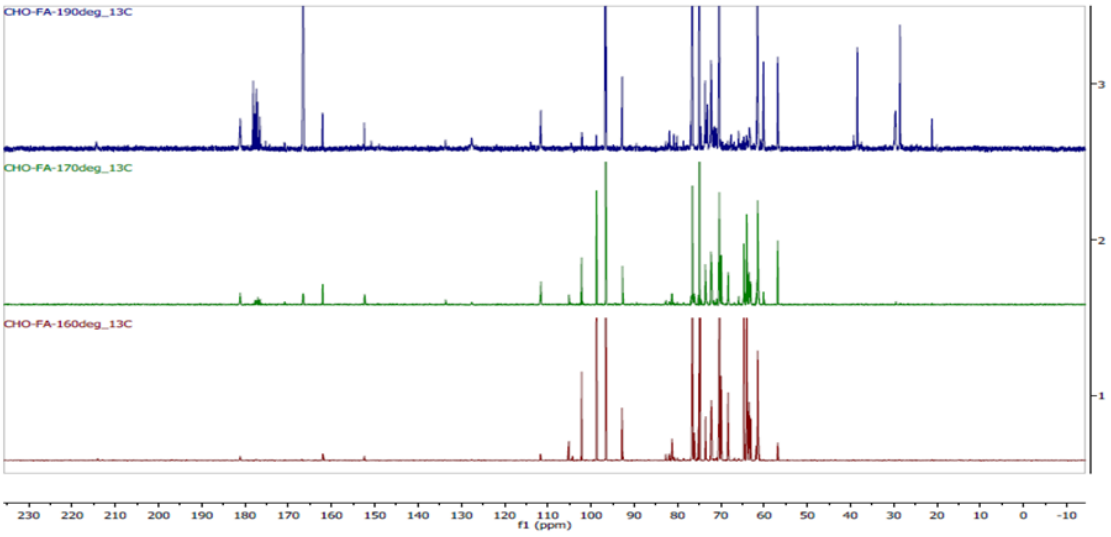


Figure 3-7. ^{13}C NMR spectra of degraded sucrose obtained from the reactor at 160 °C, 170 °C, 190 °C.

Auto-oxidation is the oxidation of sucrose isomers via free-radical reactions involving oxygen. Hydrolysis rates, which enforces sucrose glycosidic bond splitting to yield glucopyranose and fructofuranose are predictable with furanose linkages being hydrolysed much faster than pyranose linkages. Aldose sugars will undergo dehydration to form deoxy sugars and furfurals through 2-3-enolisation to form 3-deoxyglucosone. Further dehydration occurring down the chain with subsequent ring closures producing hydroxymethylfurfural and furfural from pentoses. Decarboxylation, where carboxyl groups are replaced with protons, is an additional occurring chemical reaction during the wet oxidation process [16,17,18,28,30].

Oxidative thermal degradation occurs when the glucosidic hydroxyl group reacts with the alcoholic hydroxyl group of a second sucrose isomer. The structural difference between each fraction is not only quantitative as per the presence of the same structure, however, different in size and concentrations of functional groups but also qualitative. Each fraction corresponds to a different and distinctive structural pattern as indicated by the isolation of Levoglucosan by a thermal polymerisation mechanism [36]. Co-oxidation involves the oxidation of an organic compound by free radical intermediates during wet oxidation.

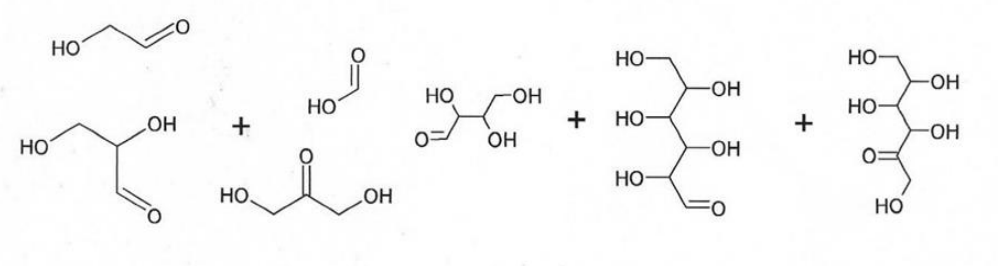
It was proposed that the free-radical reaction mechanism for the formation of propionic acid involves sixteen steps, confirming the suggestion that a myriad of reactions is involved during exothermic reactions [37]. Studies have confirmed that a range of different chemical reactions occur during wet oxidation processes, including Diels-Alder condensation, domino reaction, oxidation, SN1 and SN2 reactions (nucleophilic substitution), retro-aldol condensations, re-aldol reactions, keto-enol tautomerism, benzyl rearrangement, transformation (van Ecken reaction), thermal polymerisation, enolisation and mutarotation [17,18,28,30]. Aromatic rings are common end-products of these reactions due to their thermodynamic stability.

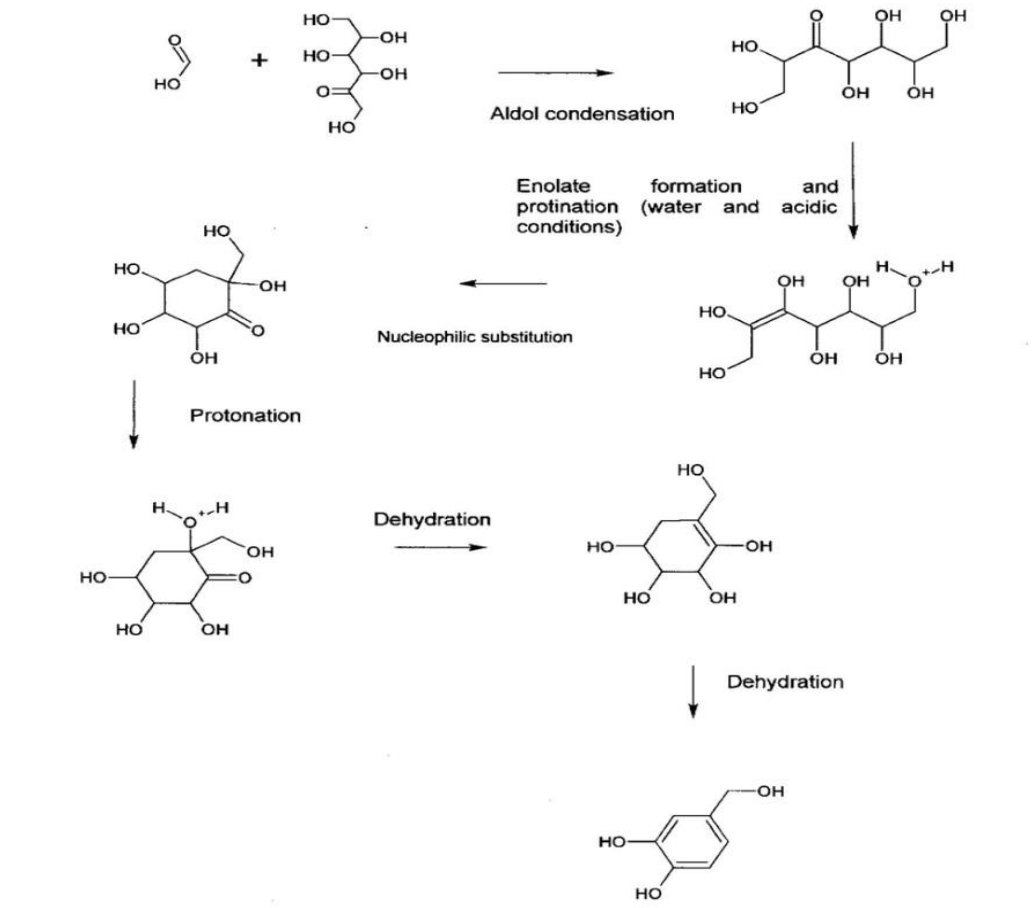
Low molecular mass carboxylic acids undergo a minimum of four reactions: (i) reactions involving cleavage of the OH bond, (ii) attack by a nucleophile on the C-O bond, (iii) decarboxylation and (iv) attack on the α-carbon. In monocarboxylic acids, the attack of O_2 and O free-radicals on the C–O bond and on the α-carbon is considered a major reaction in breaking the parent acid into compounds with lower molecular mass [31]. Different products derived from glucose and fructose, such as 1,6-anhydroglucose, formic acid, 5-HMF, erythrose and glyceraldehyde, are explained by isomerisation and bond cleavage due to the mechanism of Lobry de Bruyn-Alberda van Ekenstein (LBEA) transformation [38]. The formation of gluconic acid, formic acid and glucaric acid by an uncatalysed wet oxidation process was also reported [30].

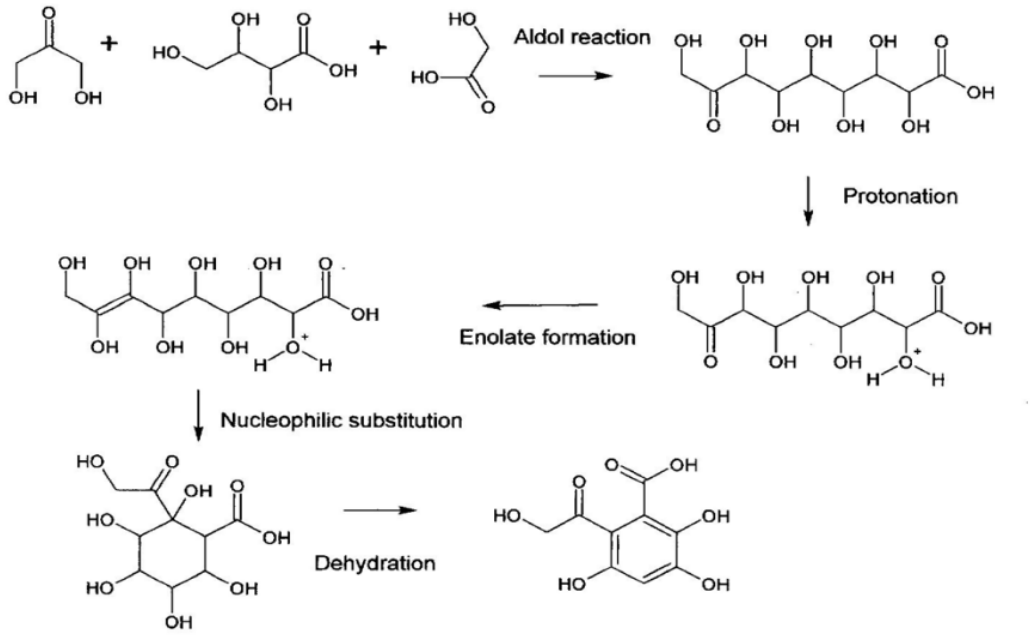
Oxidative thermal degradation causes the formation of C_2, C_3, C_4, C_5 and C_6 acids, indicating that the aldolisation of lower carbonyl intermediates, the Cannizarro reaction of low molecular carbonyl intermediates and/or the direct cleavage of 1-deoxydiuloses derived from the parent sugars, may occur [28]. C_6 acid was also detected and provides evidence that it is obtained from retro-aldol and re-aldol reactions [17,39]. It is evident from previous work that the formation of phenolic products such as 5-hydroxymethyl-2-furaldehyde, 2-furaldehyde, 1,2-dihydroxybenzene, 4-hydroxybenzoic acid and 3,8-dihydroxy-2-methylchromone from carbohydrates is possible [17].

The proposed theoretical pathway presented for the formation of fulvic acid by the present study is illustrated in Figure 3-8. The myriad of reactions during the exothermic reaction of low molecular weight carboxylic acids as "building blocks" are well illustrated here. Figure 3-8 clearly shows that this chemical pathway is a one-pot synthesis process and it must be stated that all the reactions are taking place within the reactor. It should be stated that a one-pot synthesis has a more comprehensive meaning than simply a "cascade or a domino or a tandem reaction". The concept of a one-pot synthesis encompasses all the different reaction processes in a single reactor. One-pot synthesis reactions are reactions where the fragments and intermediates generated during the processes are converted into various desired intermediate compounds and eventually into final products. The one-pot synthesis is well documented [40].

Figure 3-8 is an illustrative demonstration of the theoretical pathway during the one-pot synthesis process of sucrose to form CHD-FA. It should be noted that the last path demonstrated in Figure 3-8 is probably due to sample preparation and that the molecular structure, the second last structure in the pathway, is actually the main component of CHD-FA.




Aldol condensation
Enolate formation and protination (water and acidic conditions)
Nucleophilic substitution
Protonation
Dehydration
Dehydration


Aldol reaction
Protonation
Enolate formation
Nucleophilic substitution
Dehydration

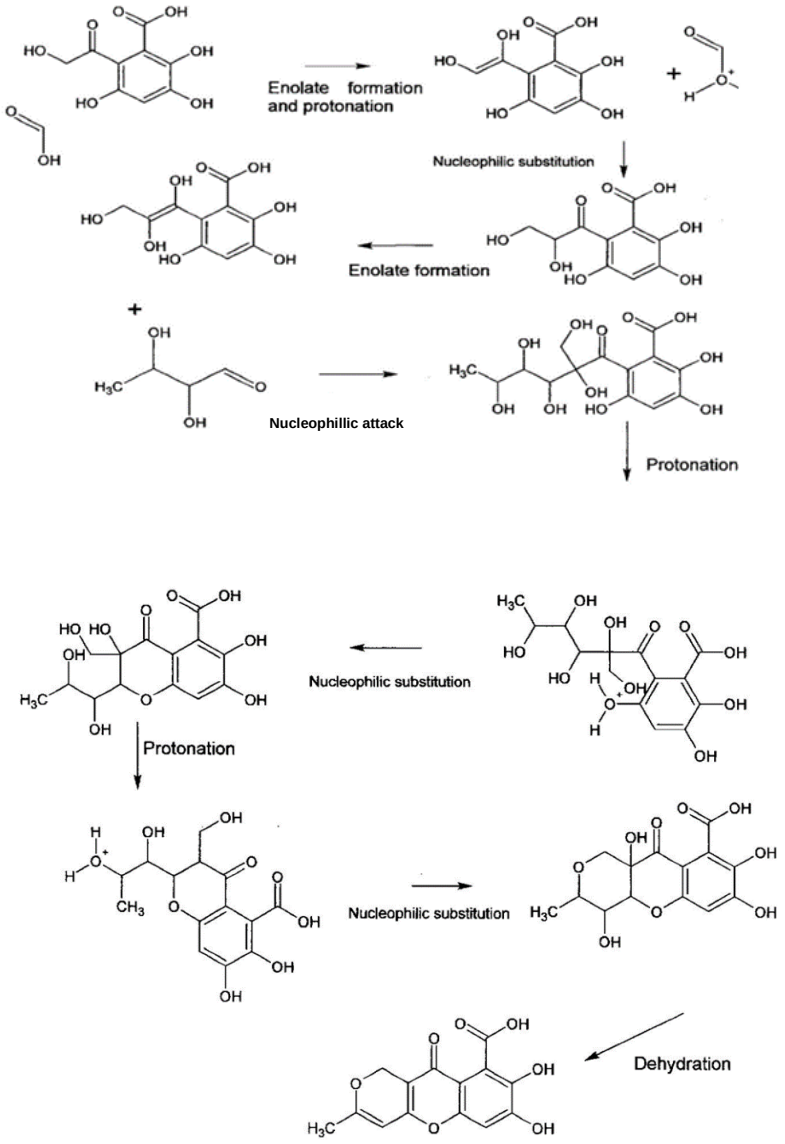


Figure 3-8. Theoretical pathway for the formation of molecular fulvic acid in CHD-FA.

3.2. Chromatographic and spectroscopic properties of CHD-FA

CHD-FA has unique chromatographic and spectroscopic properties which correspond with the penicillin-derived fulvic acid reference standard used in this study. The chemical and spectroscopic properties of CHD-FA are identified by GC-MS, NMR and LC-MSMS.

GC-MS:

In order to confirm that molecular fulvic acid was derived from sucrose, GC-MS of CHD-FA was compared to the penicillin-derived fulvic acid reference standard as illustrated in Figure 3-9. GC-MS data shows a clear overlay of fulvic acid present in CHD-FA (black) and fulvic acid from the reference standard (blue) after derivatisation with BSTFA. Both spectra exhibit retention times of approximately 21.2 min. The

chromatogram overlay of CHD-FA and the penicillin-derived fulvic acid reference standard confirms that fulvic acid was derived from sucrose through a non-catalytic wet oxidation process. Other smaller peaks were ignored as the objective of this study was to establish the presence of fulvic acid within CHD-FA solution.

These chromatograms are presented in Figure 3-9.

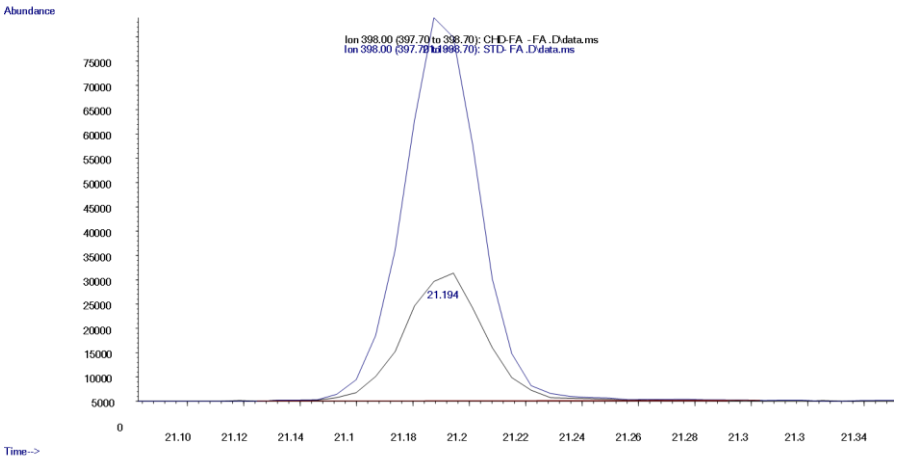
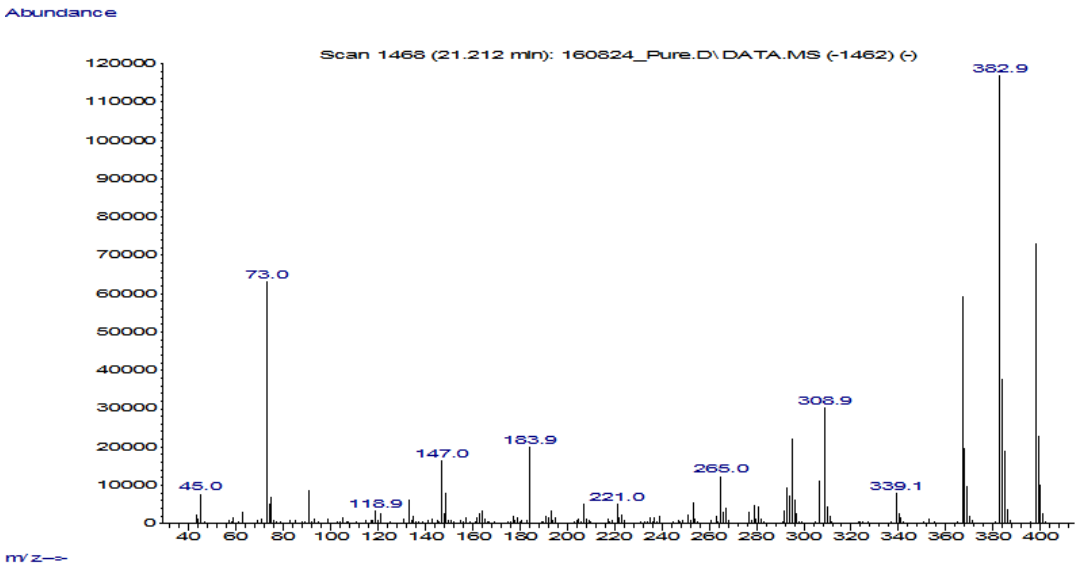


Figure 3-9. Overlay of GC-MS chromatograms of CHD-FA (black) and the fulvic acid reference standard (blue).

The GC-MS data represented in Figure 3-10 and Figure 3-11 provides specific information on the defragmentation pattern of fulvic acid. The typical fragmentation is the loss of $(CH_2)_n$, leading to the molecular weight loosing 28 Da (411 Da – 383 Da; 367 Da – 339 Da). The expected loss in CO_2 (44 Da) and reduction in spacing of 28 Da (147 Da – 118.9 Da) may also be related to CO unit losses [48]. GC-MS retention time of CHD-FA is 21.2 min. The GC-MS fragmentation patterns (Figure 3-11) show a degree of consistency in space loss for 16 Da, 28 Da and 44 Da respectively. The fragmentation of the derivatised molecule produced at m/z 307 and losing the m/z 76 fragment (losing 44 Da, 16 Da and another 16 Da) demonstrates some consistency in fragmentation and is conclusive evidence for the existence of an ordered CHD-FA structure with a polymeric character. Similar defragmentation patterns are observed in the penicillin-derived fulvic acid (Figure 10). These results lead to the idea of the existence of an ordered structure with polymeric character.



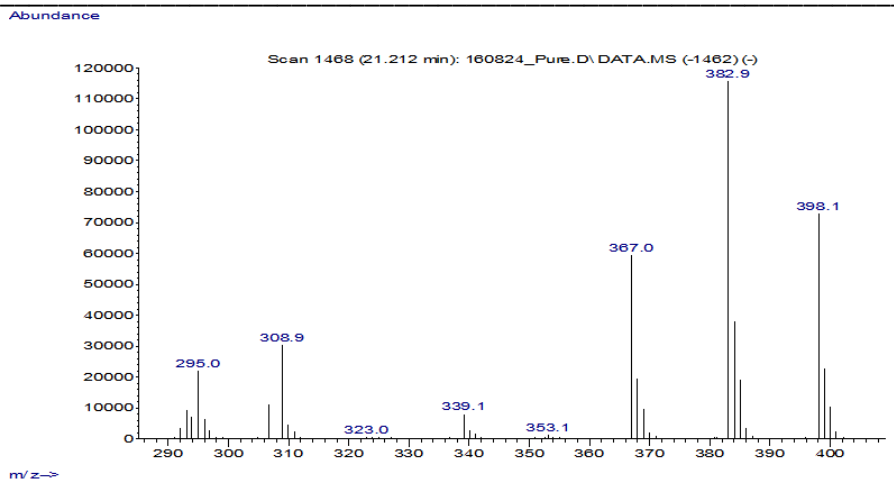


Figure 3-10. GC-MS spectra of penicillin-derived fulvic acid.

A similar defragmentation pattern was noted in the penicillin-derived fulvic acid, represented in Figure 3-10.

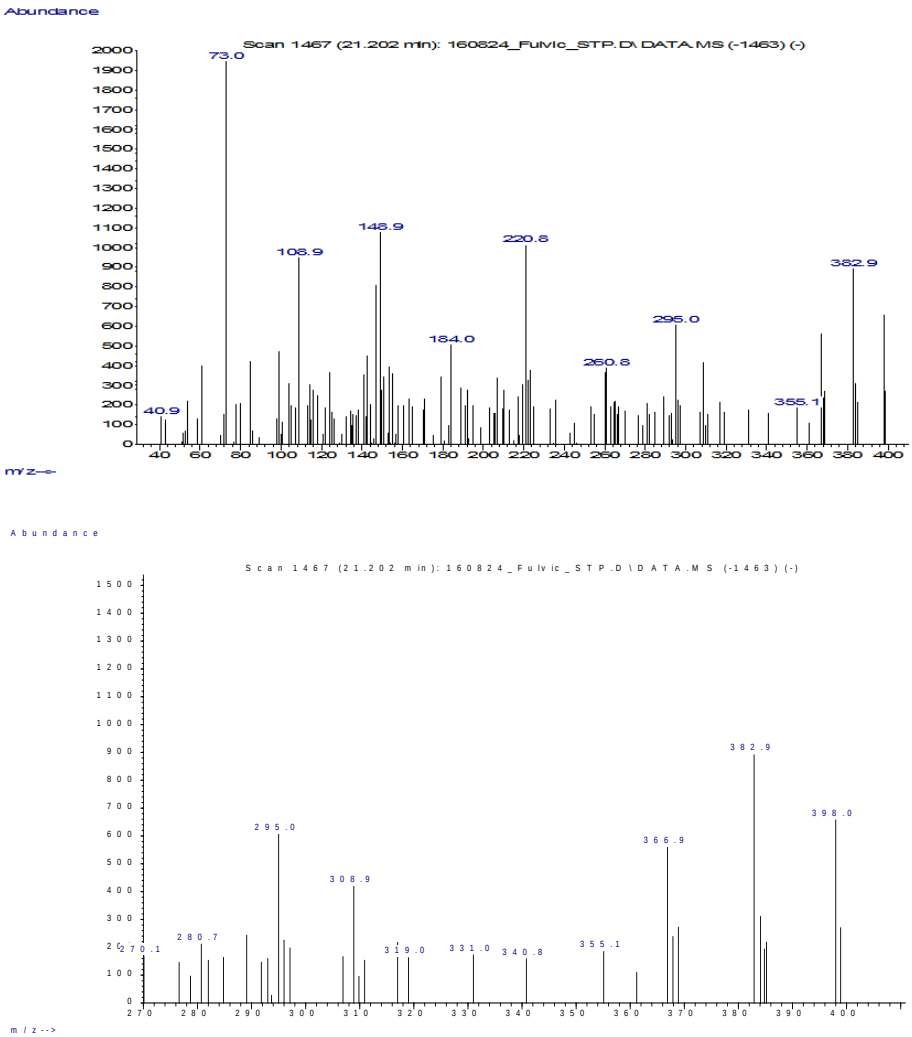


Figure 3-11. GC-MS spectra of molecular fulvic acid in CHD-FA.

GC-MS of molecular fulvic acid present in CHD-FA, reported in Figure 3-11, compares favourably to the penicillin-derived fulvic acid shown in Figure 3-10 and demonstrates directly compatible similarities in the fragmentation patterns of both fulvic acids.

NMR:

Two samples were collected from the two final 400 Da and 5000 Da CHD-FA products produced during the sucrose wet oxidation process to establish the carbon distribution. CHD-FA carbon distribution was compared to established carbon distribution profiles of humic and fulvic acids characterised for the fulvic acid reference standards from IHSS [35]. Carbon functional group distribution profiles are usually established by using solution state ^{13}C NMR [35]. The carbon NMR spectra for fulvic acids are divided into six major bands. These bands represent characteristic carbon functionalities commonly found in humic substances [35] and are:

- aliphatic carbons from 0 to 60 ppm
- carbohydrate carbons from 60 to 90 ppm
- olefinic carbons from 90 to 110 ppm
- aromatic carbons from 110 to 165 ppm
- carboxyl carbons from 165 to 190 ppm
- ketonic and aldehydic carbons from 190 to 220 ppm.

The quantitative distributions of ^{13}C nuclei in fulvic and humic acids were determined by recording ^{13}C NMR spectra using inverse gated decoupling and pulse delays of sufficient duration to ensure quantitative results. The NOE factors can provide some insight into the relaxation mechanisms of the ^{13}C nuclei in the fulvic and humic acid molecules and it is therefore convenient to measure these concurrently with the quantitative carbon distributions. The ^{13}C NMR for CHD-FA and environmental fulvic acids are compared in Table 3-3. It shows that CHD-FA and environmental fulvic acids from various sources have similar relative carbon functionality distributions in the six regions integrated in each spectrum. Table 3-3 is a demonstration of the notion that fulvic acid is synthesised from sucrose through a wet oxidation process [35].

The spectra were divided into three areas based on the chemical shift: saturated aliphatic-C area (10-110 ppm), aromatic- and olefinic-C area (110-165 ppm) and carbonyl-C area (165-216 ppm). Furthermore, the range of 60 – 110 ppm was differentiated as carbohydrate-C area from the aliphatic-C area. Saturated aliphatic-C and aromatic-C is generally separated at 105 ppm on the spectrum. The presented study found that two kinds of carbons were separated at 110 ppm, related to the broadening band for anomeric carbons to 110 ppm. The relative carbon composition was obtained by calculating the percentage of each assigned area within the total area [53].

Table 3-3. Tabulated percentage carbon distribution of fulvic acids from different sources.

PERCENTAGE CARBON DISTRIBUTION OF CHD-FA COMPARED WITH PERCENTAGE CARBON DISTRIBUTION OF OTHER FULVIC ACID DETERMINED FROM SEMI-QUANTITATIVE CARBON-13 NUCLEAR MAGNETIC RESONANCE SPECTRA						
	Ketonic, Aldehydic carbons 190-220 ppm	Carboxyl, Ester, Amide carbons 165-190 ppm	Aromatic carbons 110 - 165 ppm	Olefinic carbons 90-110 ppm	Carbohydrate carbons 60 - 90 ppm	Aliphatic carbons 0-60 ppm
CHD-FA 400 DA	1.9	42.3	4.3	0.1	5.4	46
CHD-FA 400 DA	1.4	46.3	1.3	-0.1	2.8	48.3

CHD-FA 5000 DA	2.48	39.1	5.76	0.6	6.99	46.34
CHD-FA 5000 DA	0.58	38.38	7.86	1	9.6	42.58
SUWANNEE FULVIC [43]	7	48	24	5	11	33
NORDIC FULVIC [43]	10	61	31	7	12	18
SOIL FULVIC [43]	12	56	30	1	9	22
PEAT FULVIC [43]	7	64	34	3	9	20

The principal differences between CHD-FA and environmental fulvic acids are the high percentage of aliphatic carbons and the low percentage in aromatic carbons in CHD-FA when compared to environmental fulvic acids (Figure 3-12).

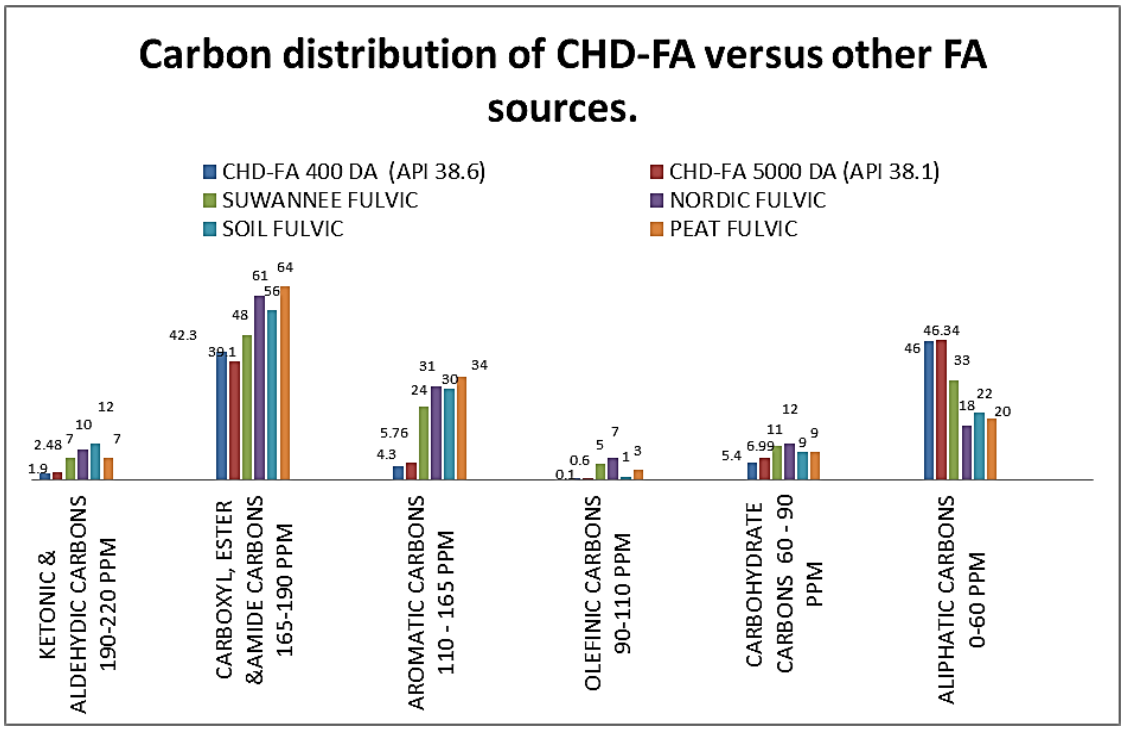


Figure 3-12. Graphic layout of carbon functionality intensity distribution of fulvic acid from different sources.

Previous studies have reported significant variations in resolution patterns for similar fulvic acid fractions obtained from different environmental sources [41,42].

LC-MSMS:

The LC-MSMS spectrum of CHD-FA was compared to the LC-MSMS spectrum of a penicillin-derived fulvic acid. Figure 3-13 is the recorded LC-MSMS spectrum of CHD-FA showing a characteristic mass-to-charge ratio (m/z) distribution value representative of a CHD-FA "fingerprint".

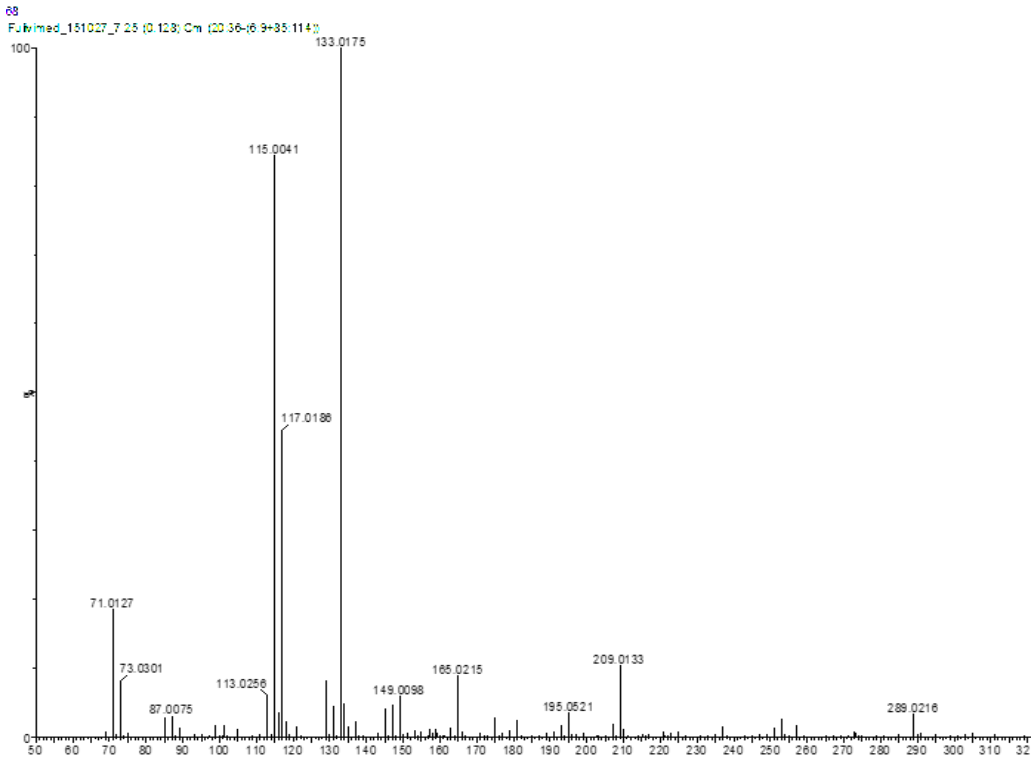


Figure 3-13. LC-MSMS spectrum of CHD-FA

The LC-MSMS data of CHD-FA (Figure 3-13) shows a main constituent with a molecular weight of 290.02 g/mol. This molecular weight is referenced to an anhydrofulvic acid, possibly caused by dehydration that could have occured during the sampling process. This was compared to a penicillin-derived fulvic acid with a molecular weight of 308.242 g/mol and molecular formula $C_{14}H_{12}O_8$ [43] shown in Figure 3-14.

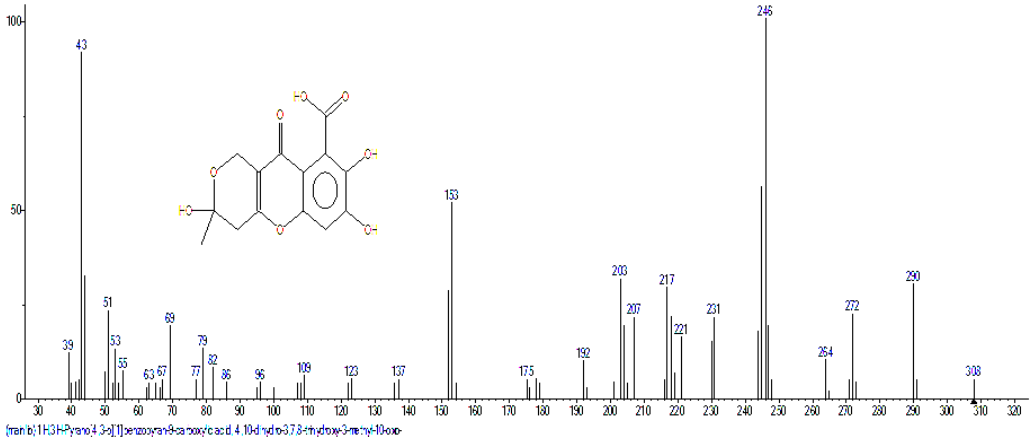


Figure 3-14. LC-MS spectrum of penicillin-derived fulvic acid

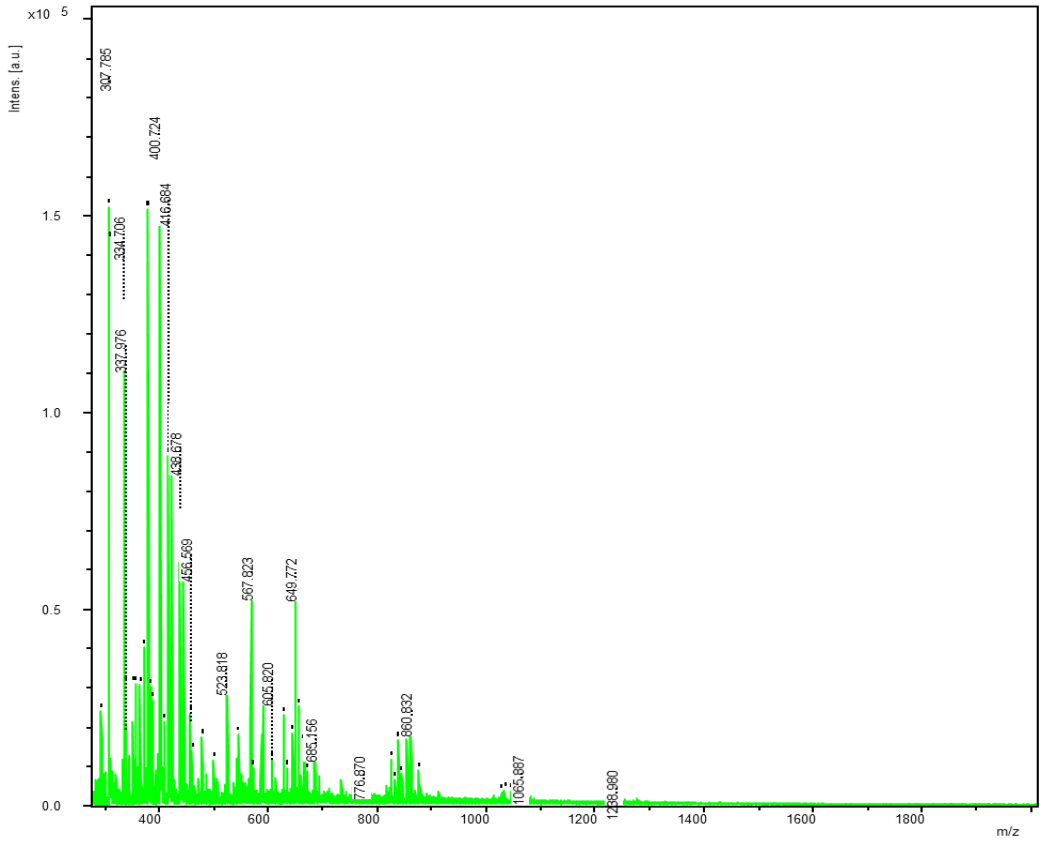
LC-MSMS spectrum (Figure 3-14) shows that CHD-FA consists of multiple compounds with molecular weights ranging between 72 – 290 g/mol. The number of different peaks and the complexity of the spectra illustrated in Figure 3-14 provide evidence that CHD-FA has a supramolecular nature.

Fulvic acids consist of C, H and O in very similar ratios across the molecular weight range. Each nominal mass comprises of multiple components characterised by varyations in the empirical formula. Components with the same empirical formula are most likely consisting of multiple isomers, resulting in a complex mixture of compounds with similar chemical properties, at least in the case of polycarboxylic fulvic acids and model compounds of CHD-FA of lower molecular mass [44].

3.5 MALDI-TOF MS

MALDI-TOF MS spectra (Figure 3-15) shows the mass data generated for single charged intact structures in CHD-FA and provides no evidence of fragmentation. The mass spectrum (Figure 3-15) provides evidence of 64 molecular ion signals present in CHD-FA. The MALDI-TOF-MS data presented in Figure 3-15 provided conclusive evidence that the main constituent present in CHD-FA has a molecular weight of 307.785 g/mol. The area under each peak represents the natural abundance of the components [45] and is well demonstrated in Figure 3-15, reporting a CHD-FA MALDI mass spectrum for a m/z range of 200–2000 Da.

1.



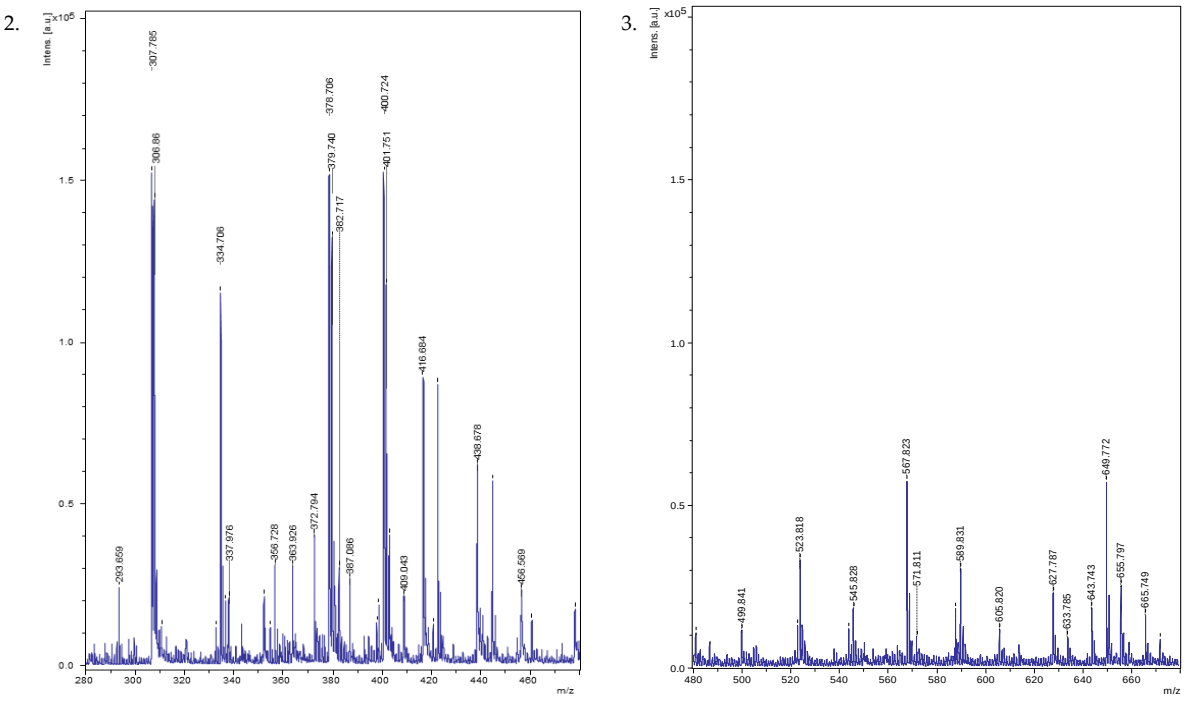


Figure 3-15. 1) MALTI-TOF MS spectra for CHD-FA; 2) Magnified in mass range 280–480 Da; 3) Magnified in mass range 480–680 Da.

Molecular mass (M_i, m/z) and the integration of each peak area (N_i) are summarised in Table 3-4. The 24 structures of the 64 molecular ion signals representative of components in CHD-FA identified by MALDI-TOF-MS present a natural abundance (% area) of 80%. The major constituent in the CHD-FA structure has a 20.8 % natural abundance and the balance is unidentified clusters of carboxylic acids. The number average molecular weight (Mn) and weight average molecular weight (Mw) of CHD-FA with standard deviations are 437.7 ± 264.7 and 487.1 ± 244.5 Da respectively. Polydispersity (Mw/Mn) is calculated as 1.1. The calculations of the number average molecular weight (M_n) and the weight average molecular weight (M_w) are based on the following two equations [45,46]:

$$M_n = \sum N_i M_i / \sum N_i \qquad M_w = \sum N_i M_i^2 / \sum N_i M_i$$

Table 3-4. Calculation of molecular weight and natural abundance of CHD-FA.

m/z (M_i)	Area (N_i)	N_i*M_i	$N_i*M_i^2$	Natural Abundance (% Area)	M_n	M_w	M_w/M_n
293.7	669.0	196447.8	57688706.2	0.84%	**437.7**	**487.1**	**1.1**
307.8	**13062.4**	**4020691.9**	**1237593803.1**	**20.8%**			
356.7	748.8	267131.8	95293288.2	0.94%			
363.9	938.8	341636.5	124330486.3	1.18%			
372.8	1097.9	409273.0	152574544.0	1.38%			
378.7	**8305.3**	**3145254.3**	**1191125881.4**	**10.40%**			
379.7	**2895.4**	**1099482.9**	**417517897.8**	**3.63%**			
382.7	774.7	296504.5	113477273.7	0.97%			
387.1	550.7	213148.8	82506882.6	0.69%			
400.7	**8163.9**	**3271458.0**	**1310952597.3**	**10.22%**			
401.8	**2183.6**	**877271.6**	**352444730.3**	**2.73%**			

403.0	1286.0	518293.1	208880351.6	1.61%
416.7	3941.6	1642399.5	684361380.0	4.94%
422.7	2491.6	1053308.1	445281358.9	3.12%
438.7	2723.3	1194638.5	524061596.7	3.41%
444.7	1607.5	714916.7	317941609.8	2.01%
523.8	944.1	494521.3	259039383.6	1.18%
567.8	1805.7	1025346.3	582215289.4	2.26%
589.8	845.7	498792.7	294203631.6	1.06%
627.8	930.8	584317.9	366826891.7	1.17%
649.8	2075.9	1348852.0	876446062.9	2.60%
655.8	740.7	485775.2	318570046.9	0.93%
860.8	887.2	763729.9	657443439.3	1.11%

MALDI-TOF MS was employed to determine molecular weights and molecular weight distributions of CHD-FA. This study shows similarities to previous work which has demonstrated that fulvic acid have 9–40 components in the molecular weight (m/z) range of 200 to 900 Da. Average molecular weights of 360–410 Da and weight average from 370–420 Da were reported for fulvic acid [47,48]. The number average molecular weight (M_n) and the weight average molecular weight (M_w) with standard deviations presented in the present study are respectively 437.7 ± 264.7 Da and 487.1 ± 244.5 Da for CHD-FA. Polydispersity (M_w/M_n) calculated as 1.1 provides conclusive evidence that CHD-FA is a polydisperse character with a narrow molecular weight distribution.

Fulvic acids are complex mixtures of molecular species with different molecular weight and functional groups, which contributes to deviations reported in the results of this study, suggesting that CHD-FA is a complex supramolecular structure. MALDI-TOF MS identified 308 Da as the highest intensity peak in the spectrum with a natural abundance of 20.8 %. It is the most prominent component in CHD-FA. The empirical formula is $C_{14}H_{12}O_8$. It is similar to the empirical formula of penicillin-derived fulvic acid. The molecular structure is illustrated by Figure 3-16. The next two prominent peaks in the spectrum have a natural abundance of 14.03 % and 12.95 % respectively. This is conclusive evidence that the most prominent structure in CHD-FA, identified as the main component, and form combinations with the smaller carboxylic acids to form structures illustrated in Figure 3-17.

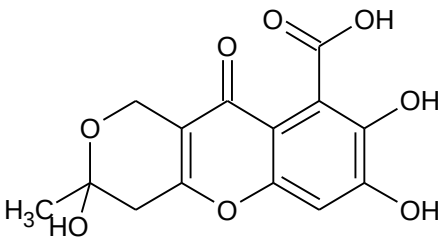


Figure 3-16. Illustration of the main component in CHD-FA.

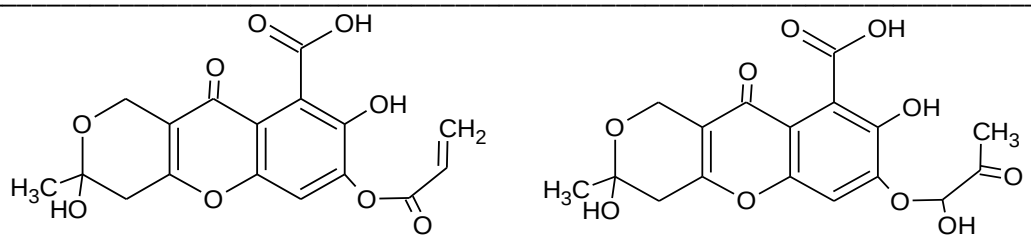


Figure 3-17. Illustration of molecular CHD-FA structures with natural abundance of 14.03 % and 12.95 % respectively.

3.3 Batch-to-batch consistency was demonstrated by various well-known methods such as the FTIR, NMR and LC-MSMS.

FTIR: The FTIR spectra of CHD-FA (Figure 3-15 and Figure 3-16) are similar to FTIR for fulvic acids extracted from different environmental sources [34,41,49,50,51,52]. The main absorption bands for environmental fulvic acids are characteristic of hydrogen–bonded OH stretching for both phenolic and carboxylic groups and ranges between 3450 – 3050 cm^{-1}. Fulvic acid is characterised by a broad and strong absorption band between 3400 cm^{-1} and 3200 cm^{-1} and indicative of υ(O-H) stretching for carboxylic, alcohol and phenol groups. The strong absorption band at 1636 cm^{-1} is a function of C=O and aromatic C=C stretching. C-O absorption bands are between 1500 and 1100 cm^{-1}. FTIR on six CHD-FA 400 Da samples and six CHD-FA 5000 Da samples from six different batches manufactured over a period of four years, reported in Figure 3-18 and Figure 3-19 respectively, demonstrate batch-to-batch consistency for CHD-FA 400 Da and CHD-FA 5000 Da.

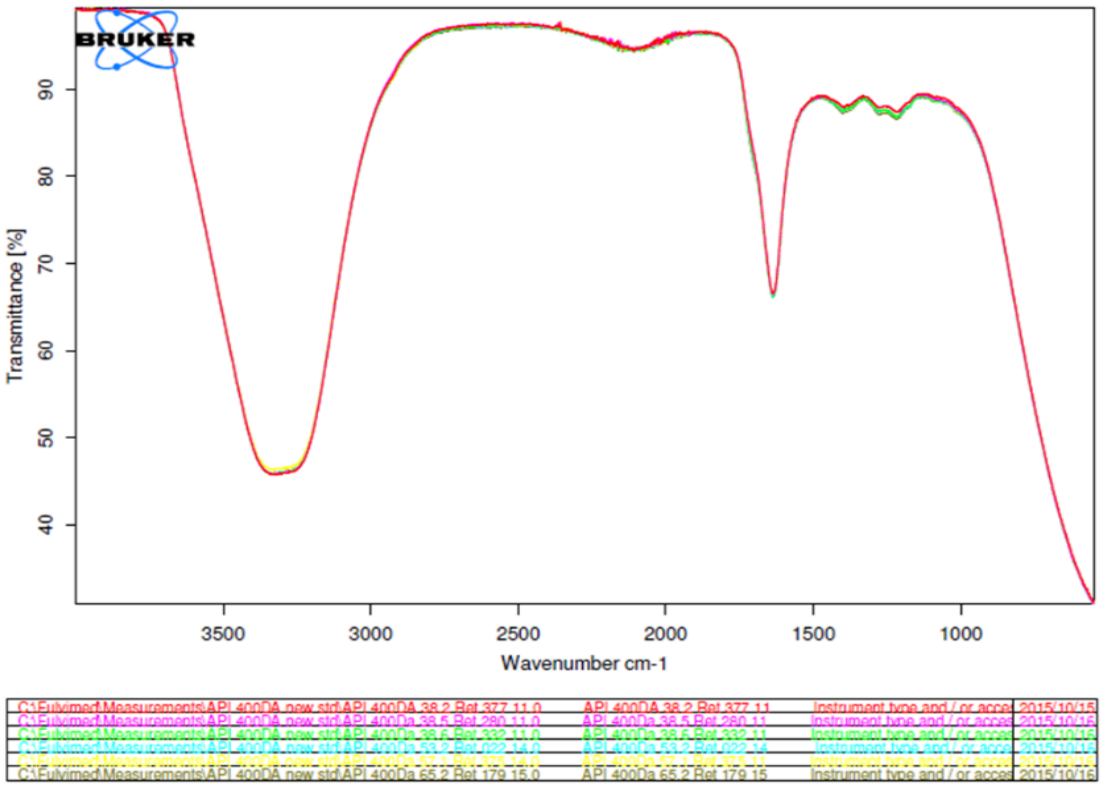


Figure 3-18. FTIR spectra of six samples from six different batches of CHD-FA 400 Da.

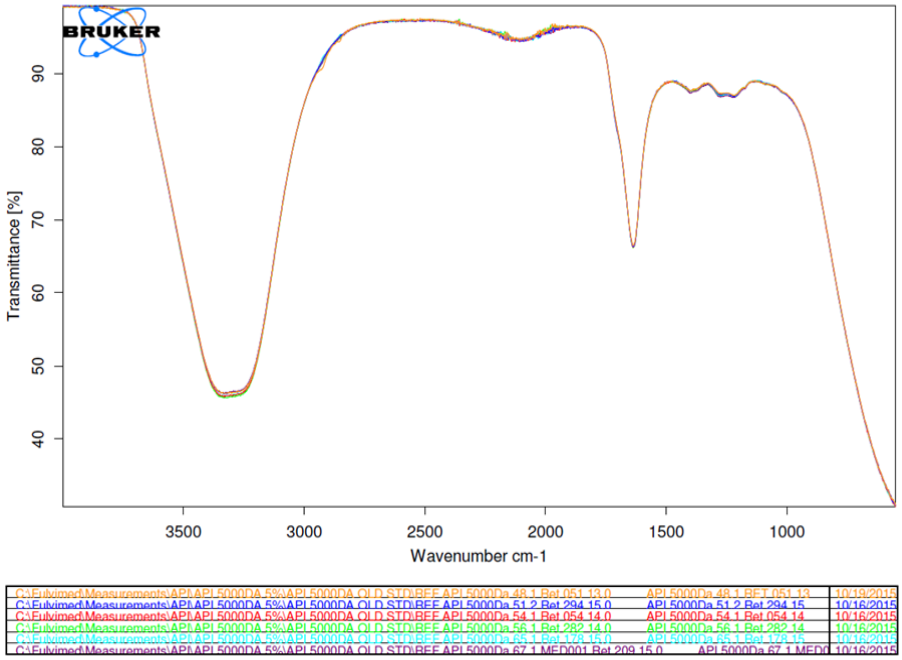


Figure 3-19. FTIR spectra of six samples from six different batches of CHD-FA 5000 Da.

NMR: Seventeen samples to establish the carbon functionality distribution in the solution produced during a wet oxidation process were collected from the two final CHD-FA products, 400 Da and 5000 Da respectively. The percentage carbon distribution was compared to percentage carbon distribution profiles for humic and fulvic acids characterised according to the fulvic acid reference standards from the IHSS, established by using solution state ^{13}C and ^{1}H NMR [35]. ^{13}C NMR for CHD-FA 400 Da and CHD-FA 5000 Da are reported in Table 3-5.

Table 3-5. Percentage carbon distribution of fulvic acid in CHD-FA 400 Da and CHD-FA 5000 Da

PERCENTAGE CARBON DISTRIBUTION OF CHD-FA COMPARED WITH CARBON DISTRIBUTION OF OTHER FULVIC ACID DETERMINED FROM QUANTITATIVE CARBON-13 NUCLEAR MAGNETIC RESONANCE SPECTRA						
CHD-FA Batch runs	Ketonic, Aldehydic carbons 190-220 ppm	Carboxyl, Ester, Amide carbons 165-190 ppm	Aromatic carbons 110 - 165 ppm	Olefinic carbons 90-110 ppm	Carbohydrate carbons 60 - 90 ppm	Aliphatic carbons 0-60 ppm
CHD-FA 400 Da (run 38.2)	1.67	40.16	4.95	0.26	6.17	46.79
CHD-FA 400 Da (run 38.5)	-0.01	49.83	2.66	0.36	5.9	41.25
CHD-FA 400 Da (run 38.6)	0.36	42.3	3.54	-0.12	6.29	47.37
CHD-FA 400 Da (run 53.2)	0.46	40.85	5.09	0.17	6.4	47.02
CHD-FA 400 Da (run 57.1)	0.67	43.84	6.66	0.01	5.68	49.14
CHD-FA 400 Da (run 65.2)	0.3	43.42	4.24	0.05	5.54	46.46

CHD-FA 5000 Da (run 38.1)	2.48	39.1	5.76	0.6	6.99	46.34
CHD-FA 5000 Da (run 45.2)	0.58	38.38	7.86	1	9.6	42.58
CHD-FA 5000 Da (run 46.1)	2.2	42.42	3.75	1.05	4.08	46.5
CHD-FA 5000 Da (run 48.1)	0.68	40.38	4.15	0.91	8.06	45.81
CHD-FA 5000 DA (run 48.2)	1.28	38.96	4.42	0.78	6.58	47.97
CHD-FA 5000 Da (run 65.1)	0.66	40.94	3.85	0.35	7.14	47.07
CHD-FA 5000 Da (run 54.1)	1.31	37.84	5.88	0.93	9.54	44.5
CHD-FA 5000 Da (run 67.1)	0.66	36	6.45	1.09	10.98	44.82
CHD-FA 5000 Da (run 56.1)	0.57	39.06	4.87	0.86	8.52	46.12
CHD-FA 5000 Da (run 51.2)	0.35	38.06	4.87	1.18	10.37	45.18

The ^{13}C NMR data for CHD-FA from 16 different batch runs presented in Table 3-5 demonstrated no major differences in the relative carbon functionality distribution between CHD-FA 400 Da and 5000 Da. It is confirmation of batch-to-batch consistency in the manufacturing of CHD-FA from sucrose. ^{13}C NMR has provided conclusive evidence (Figure 3-20) that the different size filters used in CHD-FA manufacturing, 400 Da and 5000 Da respectively, have no major influences on carbon distributions in both fulvic acids. Additional NMR data is required from more CHD-FA batch runs to provide statistical evidence to support these observations in molecular consistancy.

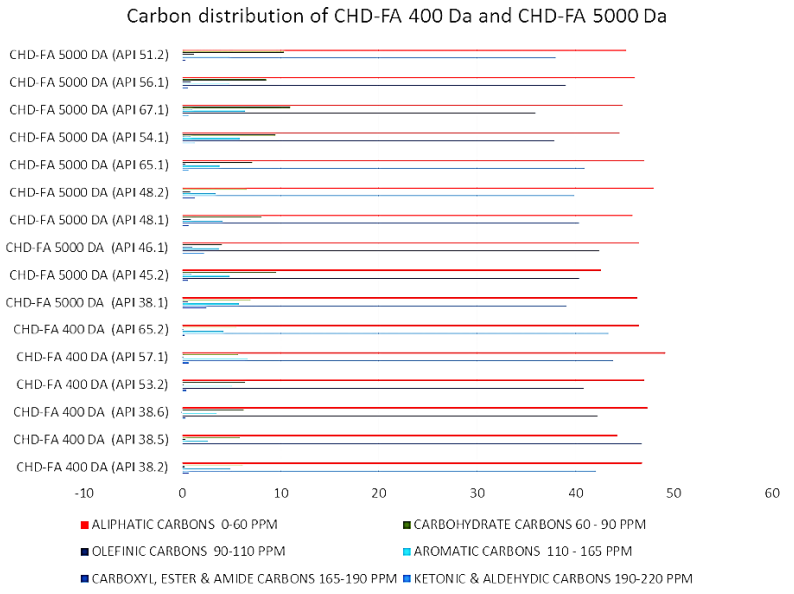


Figure 3-20. Carbon functional group distribution from CHD-FA 400 Da and CHD-FA 5000 Da.

LC-MSMS: Previous work has suggested that LC-MSMS quickly and easily, on a qualitative basis, differentiate between fulvic acid fractions from various geographical origins [44]. The difference in chromatographic behaviour is a measure of the relative polarity of compounds that make up the fulvic acid fraction from different environmental sources. Fulvic acids consist mainly of C, H and O with corresponding ratios across the molecular weight range. Each nominal mass is a composition of multiple components within the molecular structure of fulvic acids extracted from different sources and within the same source, suggesting a specific empirical formula for each fulvic acid [44]. In contrast to this diversity in the molecular structures of environmental fulvic acids, LC-MSMS data for the present study did not show any variances in chromatographic mass spectra for CHD-FA 5000 Da (Figure 3-21) and CHD-FA 400 Da (Figure 3-22) respectively. The specific structural features for CHD-FA 400 Da and 5000 Da were unchanged over a period of 4 years, providing conclusive evidence that CHD-FA manufactured by sucrose synthesis during controlled non-catalytic wet oxidation has a unique "fingerprint", demonstrated by its batch-to-batch molecular consistency over 32 batch runs.

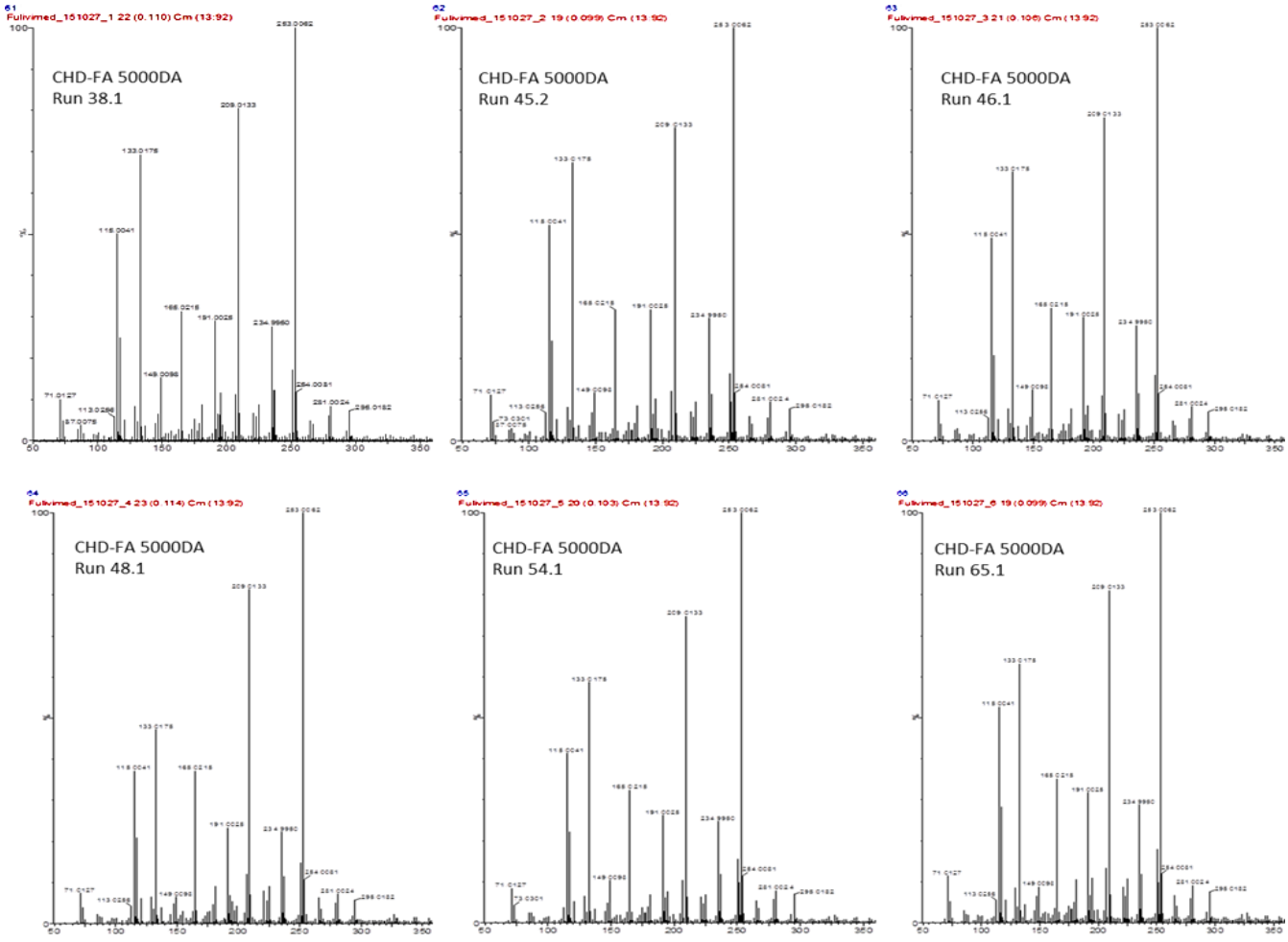


Figure 3-21. LC-MSMS spectra of six batches from CHD-FA 5000 Da.

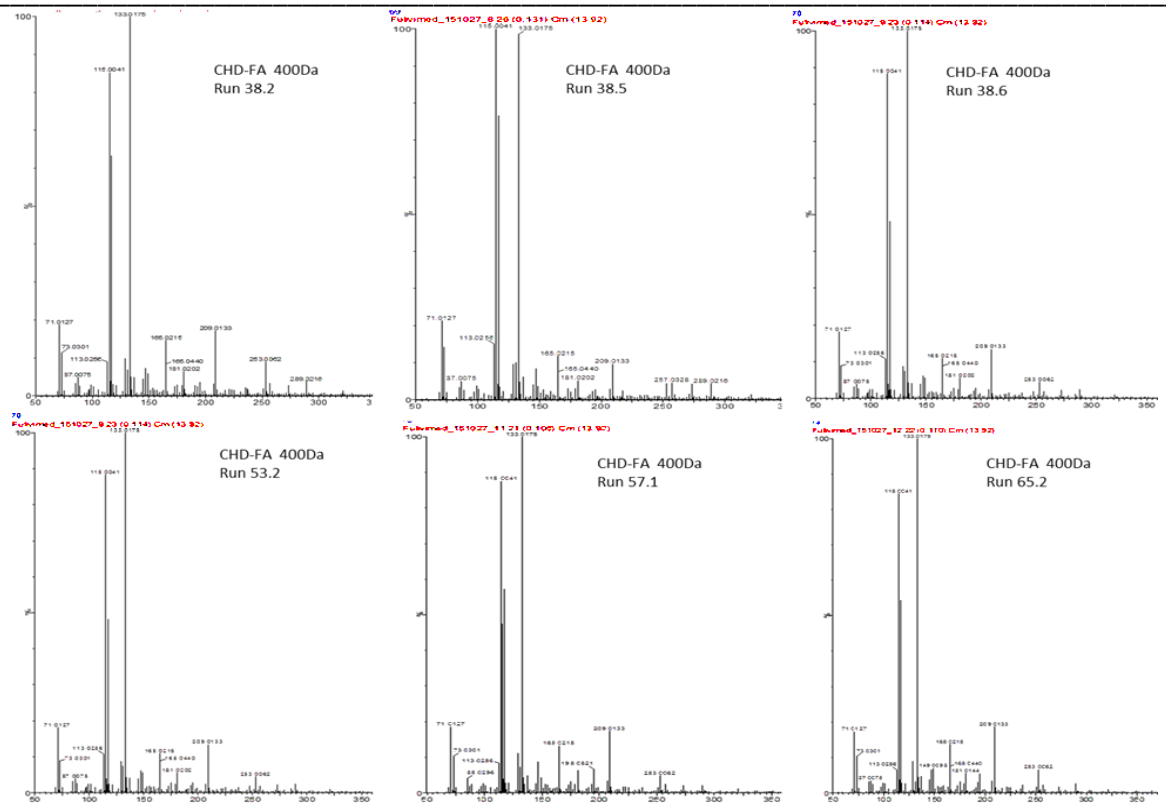


Figure 3-22. LC-MSMS spectra of six batches from CHD-FA 400 Da.

4. Conclusion

Conclusive evidence is provided to state that fulvic acid was synthesised from sucrose during a one-pot-non-catalytic wet oxidation process of sucrose. A detailed description of the theoretical pathway for the synthesis of CHD-FA, a unique fulvic acid from a carbohydrate source, consists of a myriad of chemical reactions. This takes place during the degradation of sucrose inside a reactor. The detailed step-by-step analysis of the degradation process of sucrose in the presence of oxygen during Phase 1 of the controlled exothermic reaction at high temperatures, high pressure and controlled sucrose flow rates presented in this paper has not been reported previously. It is also confirmed that a gradual increase in temperature during the exothermal degradation of sucrose into fructose and glucose is associated with a corresponding reduction in pH. This reduction in pH correlates with changes in the chemical composition of the sucrose molecules from carbohydrate to carboxylic acids. Progressive colour changes in the reactor solution from light yellow to dark brown demonstrated this finding. LC-MSMS spectra showed that CHD-FA consists of multiple compounds with molecular weights ranging between 72 – 290 g/mol. The number of different peaks and the complexity of the spectra provide evidence that CHD-FA has a supramolecular nature. MALDI-TOF MS measured 64 molecular ion signals in the mass spectrum of CHD-FA and identified 308 g/mol as the highest intensity peak with a natural abundance of 20.8 % in the spectrum and confirmed it as the most prominent component of the CHD-FA structure ($C_{14}H_{12}O_8$). This structure is similar to penicillin-derived fulvic acid.

Batch-to-batch consistency over four years is evidence that synthetic fulvic acid derived from sucrose consistently present the same chromatographic and spectroscopic properties for the molecular structure of CHD-FA. This is conclusive of a unique fingerprint nature for CHD-FA.

5. Patents: The manufacturing process is patented by PfeinsmitH Ltd, a subsidiary of Fulhold Pharma Ltd.

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Conflicts of Interest: The authors declare no conflict of interest

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CHAPTER 4: CHARACTERISATION OF CARBOHYDRATE-DERIVED FULVIC ACID AND IDENTIFICATION OF THE BACKBONE STRUCTURES EMBEDDED IN THE FULVIC ACID STRUCTURE

"Ready for submission" article in *Molecules*, entitled:

"*Characterisation of Carbohydrate-Derived Fulvic Acid and identification of the backbone structures embedded in fulvic acid structure."*

Introduction

This chapter presents the "ready for submission" manuscript for publication in *Molecules* published by MDPI. The manuscript is presented in the required format prescribed by *Instructions for Authors,* and as outlined on the journal website:

https://www.mdpi.com/journal/Molecules/instructions

The manuscript begins with the title, name of author and affiliations followed by the Abstract. The main body of the manuscript consist of an Introduction; Objectives; Methodology; Results and Discussion; Conclusion and finally the Acknowledgements and References.

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Characterisation of Carbohydrate-Derived Fulvic Acid and identification of the backbone structures embedded in the fulvic acid structure

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Abstract: Fulvic acid is a complex, heterogeneous supramolecular structure and it has been documented that, despite numerous analytical techniques available in modern science, fulvic acids from various environmental sources are extremely difficult to isolate for structural identification. Fulvic acid plays a very important role in sustaining equilibrium in the environment and the identification of its molecular composition will assist in formulating products to assist in human health. The present study aimed to identify the backbone structures of Carbohydrate-Derived Fulvic Acid (CHD-FA), a synthetic fulvic acid derived from sucrose. Fourier transform infrared (FTIR) spectroscopy, nuclear magnetic resonance (NMR), gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MSMS) were used. CHD-FA has broad and strong FTIR absorption bands for carboxylic-, alcohol- and phenolic group υ (O-H) stretching between 3400 and 3200 cm^{-1}. Absorption bands at 1560 cm^{-1} are characteristic of C=O and aromatic C=C conjugated with C=O. FTIR recorded O-H deformation and C-O stretching of phenolic OH between 1440 and 1200 cm^{-1}. ^{13}C NMR revealed a number of signals in the regions of aliphatic carbons and carbohydrate carbons and less in aromatic carbons and carboxyl regions and a reduced number in ester and amide carbons. GC-MS provided detailed information on the defragmentation pattern of molecular fulvic acid of CHD-FA. LC-MSMS was first used to resolve complex fulvic acid structures into smaller groups of identical-mass product ions. Follow-up LC-MSMS stages of product ion resolutions were used to trace fragmentation patterns to more specific backbone structures. This study identified 24 prominent peaks as the backbone structures embedded in the CHD-FA molecular structure and it is concluded that CHD-FA has a supramolecular nature.

Keywords: Carbohydrate-Derived Fulvic Acid, FTIR, NMR, GC-MS, LC-MSMS, backbone structures, carboxylic acids.

1. Introduction

The heterogeneity of molecular structures in the humic substance "family", humin, humic and fulvic acids, is a result of the different ecosystems (vegetation, climate and topography) from where they are

sourced [1,2,3,4]. They are self-assembled hydrogen-bond supramolecular compositions of relatively small heterogeneous molecules (masses around 500 Da) [5]. A humic substance supramolecular structure is stabilised by weak forces, predominantly hydrophobic (Van der Waals, CH–π and π–π) and the hydrogen bonds are responsible for the molecular mass of the substances. Chemical structures are varied [6] and related to the specific location and vertical soil layer depth within a geographical area [7] from which it is sourced. Chemical analytical techniques provide bulk information on humus structures [8] and a number of singular and/or combinational technique investigations have demonstrated that humic compounds are complex mixtures [9]. Numerous attempts to characterise it as a single chemical structure were unsuccessful [10,11,12]. Fulvic acid, the smallest group of the humic substances family, has a low molecular weight [13,14] ranging from a few hundred to a few thousand g/mol [15] and are sourced from humus rich soils [13,16], sediment [17,18] and aquatic environments [19]. FTIR, used to identify the specific functional groups within a molecular structure [20], has also been used to characterise fulvic acids extracted from aquatic [2,21], soil [22,23], waste [24] and coal [25] sources. In addition, GC-MS analysis on fulvic acids extracted from different environmental sources has identified carbohydrates, phenols, benzenes and lignin phenol structures [1,22,26,27,28,29,30,31]. LC-MSMS studies have quantified fulvic acid fractions from different sources [32,33,34,35]. Fulvic acid is therefore described as a cluster of acidic organic polymers [36] with a highly complex *supramolecular* nature related to its origin [10,37,38,39] and characterised by carboxylic acids, phenols and hydroxyl functional groups [40].

Carbohydrate-Derived Fulvic Acid (CHD-FA), produced by a wet oxidation process from sucrose, water and oxygen, is a unique patented fulvic acid [41]. CHD-FA has a broad molecular weight distribution and a complex structure which is free of environmental contaminants and known to have various medicinal applications [29,42,43,44,45]. The present study employed special analytical methods to characterise CHD-FA and to identify its backbone structures.

2. Methodology

2.1 Fourier Transform Infrared (FTIR) spectroscopy

A spectrum BX Fourier transform infrared spectrometer with an internal $LiTaO_3$ detector was used in this study. The resolution was set at 2 cm^{-1}, strong apodization function, a static gain of 1, OPD velocity at 7.5 kHz, with a bi-directional interferogram direction and number of scans set at 32. The spectrum over the conventional range was started at a wavenumber of 4000 cm^{-1} and ended at a wavenumber of 400 cm^{-1}. A 100 litre CHD-FA 5 % solution sample was evaporated to a fulvic acid concentration of 33 %. Only 0.1 ml of the CHD-FA sample (33 %) was used for the FTIR analysis.

2.2 Nuclear Magnetic Resonance spectroscopy (NMR)

Four samples were collected from four different 5000 Da batches of CHD-FA and one sample from a 400 Da batch. A volume of 750 µl of each sample was transferred to a new NMR tube with a D_2O filled insert included for locking of the instrument. An Agilent^{Unity} Inova 600 NMR spectrometer was used to record ^{1}H NMR at a frequency of 600 MHz and ^{13}C NMR at a frequency of 150 MHz, respectively. The ^{1}H and ^{13}C NMR spectra were recorded overnight at 25 °C using default VnmrJ 4.2 software parameters. The ^{1}H spectra were referenced to the residual H_2O signal at 4.79 ppm while the ^{13}C spectra were referenced with an external dioxane reference standard.

2.3 Gas Chromatography-mass Spectrometry (GC-MS)

200 µl of the final reactor product (CHD-FA solution) was transferred into a clean micro centrifuge tube and dried overnight in a speed vacuum at low settings. The dried CHD-FA was reconstituted with 100 µl pyridine and 50 µl BSTFA [N,O-Bis(trimethylsilyl)trifluoroacetamide] containing 1 % TMCS (trimethylchlorosilane) was added to this mixture. The mixture derivatised at 60 °C for 30 min. On

completion of the incubation period, samples were allowed to cool to room temperature and were subsequently vortexed for a few seconds before being transferred into vials for GC analysis. The derivatised samples were analysed by an Agilent 6890 N gas chromatograph (Agilent, Palo Alto, CA) coupled to a Agilent 5975 mass spectrometer, using a polar (95 % dimethylpolysiloxane) ZB-Semivolatiles Guardian (30 m, 0.25 mm ID, 0.25 μm film thickness) GC column. The oven temperature was set to maintain 80 °C for 1 min and finally ramped at 7 °C/min to 300 °C and held for 2 min. The carrier gas was helium with a flow rate of 1 ml/min and the injector temperature was set at 280 °C in the split-less mode. Mass spectra were recorded in full scan mode (40–650 m/z) with the ion source and quadrupole temperatures being maintained at 240 °C and 150 °C, respectively. The transfer line temperature was maintained at 280 °C.

2.4 Liquid Chromatography-mass Spectrometry LC-MSMS

A Waters Synapt G2 Quadrupole-Time-of-Flight (QTOF) mass spectrometer was used in this study. The source was electrospray (negative ionisation mode), the capillary voltage was set to 3 kV, the cone voltage was set to 15 V, the dissolving gas (nitrogen) was set at 650 L/h and the dissolving temperature was 275 °C. The samples were introduced with a Waters Acquity ultra performance liquid chromatograph (UPLC) connected with PEEK tubing (1 m, 0.003“) to the mass spectrometer (MS). The solvent system consisted of solvent A (1 % acetic acid) and solvent B (methanol with 1 % acetic acid). A short 1 min gradient was applied over the tubing starting at 100 % solvent A and adjusting the solvent to 50 % solvent B after 0.5 min, and finally 100 % solvent B after 1 min. Data was acquired in resolution mode from 50–1000 m/z. The instrument was calibrated with sodium formate and leucine encephalin was used as reference (lock mass) for accurate mass determinations.

3. Results

3.1 FTIR

CHD-FA is characterised by a broad and strong absorption band between 3400 and 3200 cm^{-1}, indicative of υ(O-H) stretch intermolecular bonding of carboxylic, alcohol and phenolic groups. The FTIR spectrum of CHD-FA is reported in Figure 4-1.

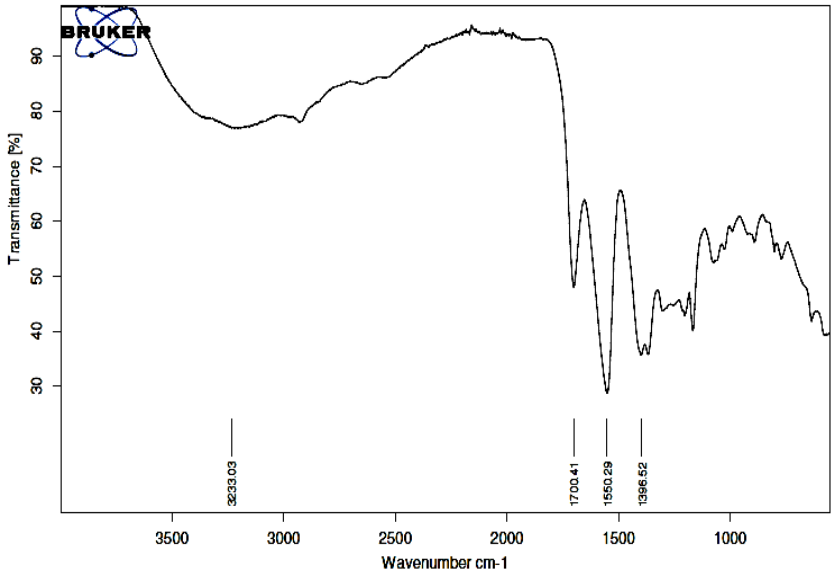


Figure 4-1. FTIR spectrum of CHD-FA with the FA concentration at 33 %.

Strong absorption bands were recorded for CHD-FA in the aromatic ring stretch (1630–1620 cm^{-1}), carboxylic and hydroxyl regions. Strong bands between 1590 cm^{-1} and 1520 cm^{-1} (Figure 4-1) are characteristic of C=O and aromatic C=C conjugating with C=O functions. Absorption bands between 1400 and 1200 cm^{-1} (Table 4-1) are characteristic of O-H deformation and C-O stretching of phenolic OH groups, indicative of O-H and C-O deformation of COOH functional groups in CHD-FA. FTIR phenolic O-H bonds, carboxylic acids and aromatic rings were present in the spectra of all the CHD-FA fractions. FTIR spectra are summarised in Table 4-1.

Table 4-1. FTIR absorption bands of CHD-FA and possible functional group assignments.

Frequency (cm^{-1})	Assignment
3400-3200	O-H stretching of carboxylic, alcohol and phenolic groups
2940-2840	Aliphatic C-H stretching
1725-1718	C=O stretching of COOH and ketones
1620-1600	Aromatic C=C, strongly H-bonded C=O of conjugated ketones
1600-1585	C=C stretch within the ring
1590-1520	COO^- symmetric stretching
1500-1400	C=C stretch within the ring
1400-1200	C-O stretching and OH deformation of COOH
1170-950	C-O stretching of polysaccharide-like substances

3.2 NMR

A FTIR spectrum (Figure 4-1) combined with ^1H NMR spectra (Figure 4-2) provided a sensitive and unbiased overview of all the functional groups in CHD-FA. ^1H NMR spectra (Figure 4-2) was recorded from -2 ppm to 14 ppm in order to cover all the sample protons that appear between 0 ppm and 10 ppm. ^1H NMR spectra for CHD-FA 5000 Da batches were compared to one batch from 400 Da and shown at the bottom of Figure 4-2. All spectra were recorded using the same parameters.

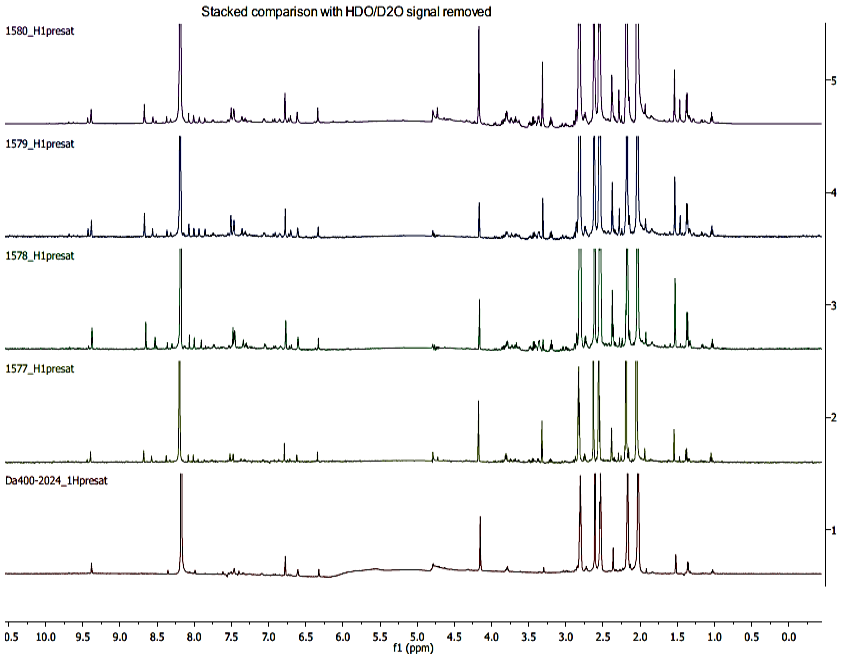


Figure 4-2. [1]H NMR spectra comparing CHD-FA 5000 Da to 400 Da.

A marked reduction in the aromatic (6 – 8 ppm), aldehyde and organic acid protons in the 9 – 10 ppm region of the 400 Da spectra is demonstrated.

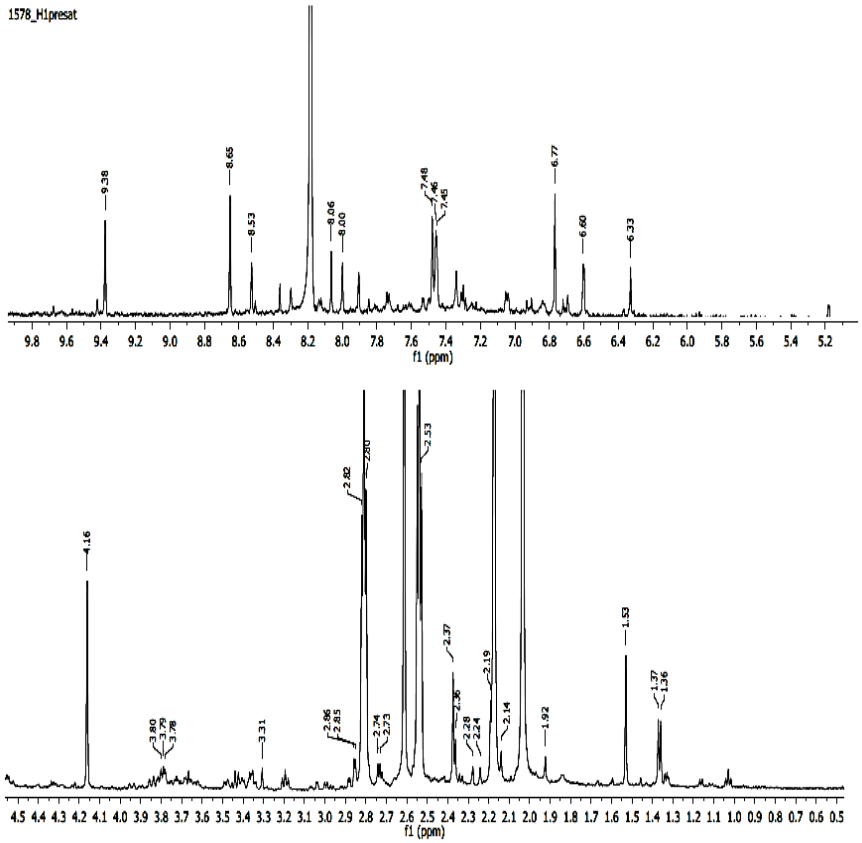


Figure 4-3. [1]H NMR spectra of CHD-FA showing expanded regions of the spectrum.

^{1}H NMR signals ranging between 1.8–2.9 ppm (Figure 4-3) are associated with the CH_3 and CH_2 protons that appeared in this region, also shown by FTIR (Figure 4-1). ^{1}H NMR 4.0–5.5 ppm is protons on the sugar alcoholic CH-OH groups. Signal 1.9 ppm is derived from protons on ester CH_3–CO groups and corresponds to the sharp FTIR 1392 cm^{-1} band (Figure 4-1). Small proton signals (Figure 4-3) between 6.0–6.8 ppm may represent protons in maleic, muconic and fumaric acid derivatives [46].

^{13}C NMR spectra (Figure 4-4) showed aliphatic CH_2, CH_3 carbon signals at ± 30 ppm and unprotonated carboxyl carbon signals at ± 170 ppm. The carbohydrate CH-carbons appeared at 60–82 ppm and signals between 111 ppm and 136 ppm may be assigned to aromatic carbons. Relative NMR signal intensities within a spectra is representative of a semi-quantitative fingerprint determined by the concentration within a sample. The overall carbon signal profile of the various CHD-FA spectra were very similar showing notably low or few aromatic carbons. ^{13}C NMR for the different CHD-FA batches were all recorded according to the same sample preparation and acquisition parameters.

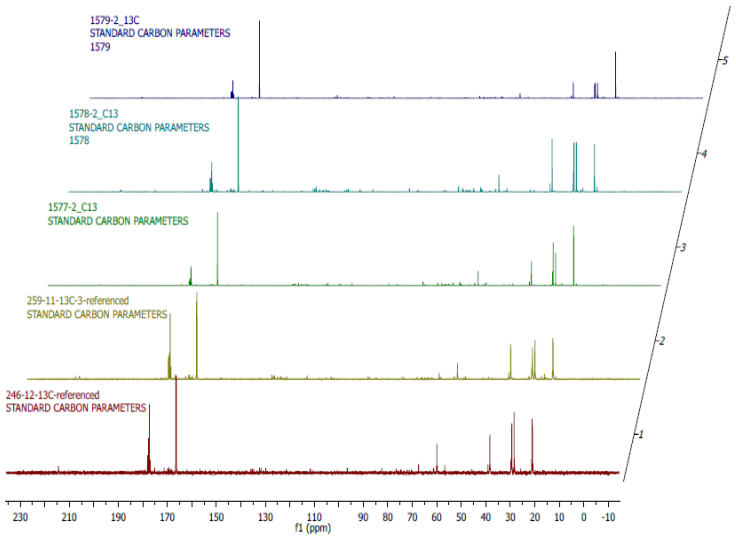


Figure 4-4. ^{13}C NMR spectra represent 5 different batches of CHD-FA.

The ^{13}C NMR spectra (Figure 4-4) showed that the carbon profile of CHD-FA is characterised by a number of the fulvic acid "characteristic" aliphatic, hetero-aliphatic, aromatic and carbonyl carbon signal intensities ranging from 0 ppm to 220 ppm. Table 4-2 is a comparative semi-quantitative summary of the six major integral-regions for ^{13}C NMR spectra relativant to humic substances. The ^{13}C NMR spectra regions were established by the International Humic Substances Society (IHSS) [47].

Table 4-2. A summary of the ^{13}C NMR spectrum of CHD-FA

PERCENTAGE CARBON DISTRIBUTION OF CHD-FA	
KETONIC & ALDEHYDIC CARBONS 190–220 ppm	0.575
CARBONYL CARBONS 165–190 ppm	43.42
AROMATIC CARBONS 110–165 ppm	4.52
OLEFINIC CARBONS 90–110 ppm	0.12

CARBOHYDRATE CARBONS 60–90 ppm	6.00
ALIPHATIC CARBONS 0–60 ppm	46.34

3.3 GC-MS

The GC-MS data presented in Figure 4-5 provides specific information on the defragmentation pattern of fulvic acid in CHD-FA. The typical fragmentation is the loss of $(CH_2)_n$, leading to the molecular weight loosing 28 Da (411 Da – 383 Da; 367 Da – 339 Da). The loss in CO_2 (44 Da) was expected and reduction in spacing of 28 Da (147 Da – 118.9 Da) may also be related to CO unit losses. GC retention time of CHD-FA is 21.2 min. The GC-MS fragmentation patterns (Figure 4-5) show a degree of consistency in spacing losing 16 Da, 28 Da and 44 Da. respectively. The fragmentation of the derivatised molecule produced at m/z 307 and losing the m/z 76 fragment (losing 44 Da, 16 Da and another 16 Da) demonstrates some consistency in fragmentation and is conclusive evidence for the existence of an ordered CHD-FA structure with a polymeric character.

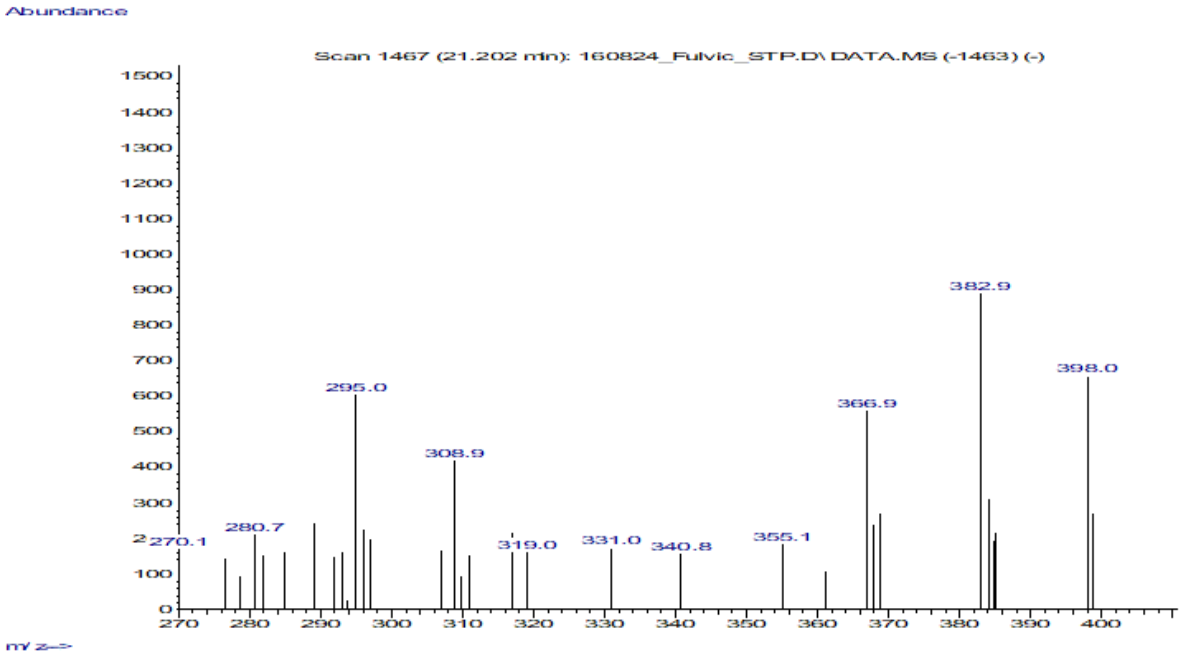


Figure 4-5. The GC-MS spectrum of the CHD-FA

3.4 LC-MSMS

LC-MSMS functioning in a negative ion electrospray mode was used to provide a more in-depth analysis of CHD-FA. The LC-MSMS spectra illustrated in Figure 4-6 shows that CHD-FA consists of multiple structures with molecular weights ranging between 72 to 290 g/mol. The number of different peaks and complexity of spectra provide evidence of a supramolecular nature. The LC-MSMS chromatogram (Figure 4-6) shows the presence of 24 major peaks in CHD-FA. The peaks can be used to identify the potential principle backbone structures embedded in CHD-FA. The empirical formula was derived from the National Institute of Standards and Technology (NIST) mass spectral search program with a most probable match of > 80 % and ion molecular weight ranges of m/z 70 to 290 m/z (Figure 4-6).

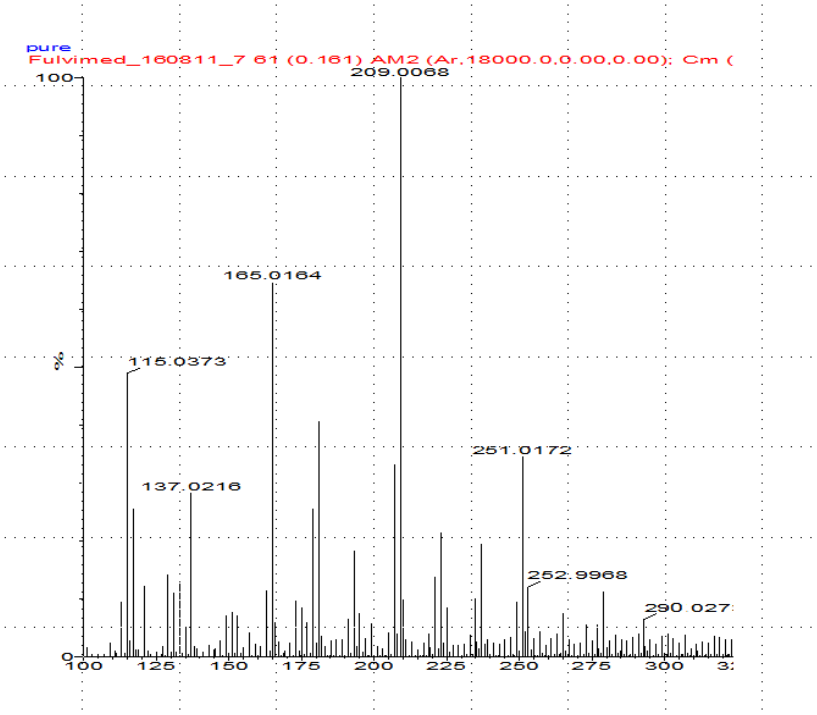


Figure 4-6. LC-MSMS spectrum of CHD-FA.

The 24 backbone structures of CHD-FA (Figure 4-6) are presented in Table 4-3. In order to give and indication of the accuracy of the database search, 10 components were randomly selected to determine similarities. The percentage similarity for Succinic acid was 100 %; Malic acid 99.8 %; 1,2,3-Benzenetricarboxylic acid 88.8 %; Phthalic acid 99.1 %; Maleic Acid 98.1%; 1,2,4,5-Benzenetetracarboxylic acid 97 %; 7,8-Dihydroxy-3-methyl-10-oxo-1H,10H-pyrano[4,3-b]benzopyran-9-carboxylic acid 92.5 %; Butanoic acid / butyric acid 91.8 %; 2,5-Bis(dihydroxymethyl)terephthalic acid 92.5 %; Acetylsalicylic acid 84.3 %. It must be noted that CHD-FA is also characterised by numerous smaller components (Figure 4-6) not reported in this study.

Table 4-3. The potential constituents of CHD-FA as determined by LC-MSMS.

Empiric formula	Index Name
$C_3H_4O_2$	2-Propenoic acid or Acrylic acid
$C_3H_4O_3$	Pyruvic acid
$C_5H_6O_3$	6-Hydroxy-2*H*-pyran-3(6*H*)-one
$C_4H_4O_4$	Maleic Acid
$C_5H_8O_3$	Levulinic acid
$C_4H_6O_4$	Succinic acid
$C_5H_6O_4$	Itaconic Acid
$C_5H_8O_4$	Glutaric Acid
$C_4H_6O_5$	Malic Acid
$C_4H_8O_5$	Butanoic acid / butyric acid
$C_7H_6O_3$	4-Hydroxybenzoic acid or Salicyclic acid
$C_5H_6O_5$	α-Ketoglutaric acid

$C_5H_8O_5$	2-Hydroxyglutaric acid
$C_4H_6O_6$	2,3-Dihydroxysuccinic acid / Tartaric acid
$C_8H_6O_4$	Phthalic acid
$C_5H_{10}O_6$	2,3,4,5-Tetrahydroxypentanoic acid
$C_9H_8O_4$	2-Acetoxybenzenecarboxylic acid or Acetylsalicylic acid / Aspirin
$C_8H_6O_5$	4-Hydroxy-1,2-benzenedicarboxylic acid
$C_6H_{10}O_7$	d-Glucuronic acid
$C_6H_{12}O_7$	d-Gluconic acid
$C_9H_6O_6$	1,2,3-Benzenetricarboxylic acid
$C_{10}H_6O_8$	1,2,4,5-Benzenetetracarboxylic acid
$C_{10}H_{10}O_8$	2,5-Bis(dihydroxymethyl)terephthalic acid
$C_{14}H_{10}O_7$	7,8-Dihydroxy-3-methyl-10-oxo-1H,10H-pyrano[4,3-b]benzopyran-9-carboxylic acid

It is possible that one or more of these components are held together with intermolecular bonds to form supramolecular structures [5,10,37,38,39].

4. Discussion

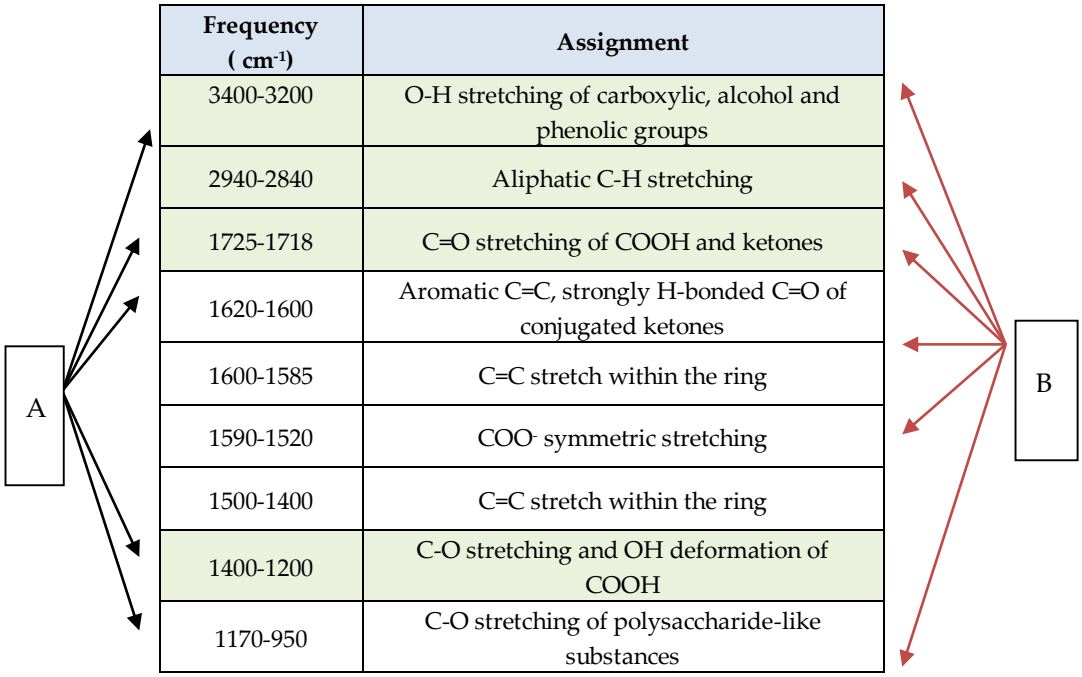
S*upramolecular* structures are formed by numerous molecules held together by a number of weak intermolecular bonds that, at any instant, contributes to the overall stability of the molecular structure [5,10,37,38,39]. Intermolecular dynamics of bond breaking and bond making is an important aspect in supra- molecules that allows for the rich diversity and complexity of their molecular structures [48]. This study has added a new perspective on the structural composition of CHD-FA, identified by spectroscopical and chromatographic techniques as a supramolecular structure.

4.1 FTIR

Research concluded that the overlapping FTIR absorption bands in fulvic acids obtained from marine, estuarine and lacustrine sediment sources range between 3400 cm^{-1} and 1040 cm^{-1} [1,21,49,50]. The major bands are between 3400 cm^{-1} and 1040 cm^{-1} [49]. Absorption bands at 2940 cm^{-1} are characteristic of the asymmetrical C-H stretching of aliphatic groups in the fulvic acid structure [21]. Fulvic acids are characterised by H-bonded OH groups (3400–3300 cm^{-1}), aliphatic C–H stretching (2940–2840 cm^{-1}), C=O stretching of COOH (1725–1718 cm^{-1}), aromatic C=C, strongly H-bonded C=O (1550 cm^{-1}), C-O stretching and OH deformation of COOH (1280–1230 cm^{-1}) and C–O stretching of polysaccharide (1040 cm^{-1}). The present study identified similar absorption bands for CHD-FA reported in Figure 4-1. The broad band between 3400 and 3200 cm^{-1} (Figure 4.1) is characteristic of υ(O-H) stretch for carboxylic, alcohol and phenolic groups. A strong absorption band at 1550 cm^{-1} for CHD-FA (Figure 4-1) is demonstrated for C=O and aromatic C=C conjugated with C=O. This finding supports previous studies [21,25,26,46] reporting aromatic C=C, COO^- and hydrogen-bonded C=O FTIR absorption bands near 1640 cm^{-1} in fulvic acids. Phenolic and carboxylic functional groups are characteristic of synthesised fulvic acids [24,25]. Fulvic acids extracted from different sources exhibit similar FTIR bands suggestive of "core" structures distinctive of the chemical composition of fulvic acid not influenced by origin [25].

This study concluded that the FTIR spectra (Figure 4-1) of CHD-FA summarised in Table 1 are similar to the spectra published for fulvic acids extracted from different environmental sources [21,24,25,26,46,49]. The same aliphatic peaks (1130, 1450, 2930 cm^{-1}) and OH and COOH peaks (1224, 1420, 1720 cm^{-1}) are

characteristic for CHD-FA. Similarities and differences in FTIR absorption bands are illustrated by Figure 4-7.



Frequency (cm^{-1})	Assignment
3400-3200	O-H stretching of carboxylic, alcohol and phenolic groups
2940-2840	Aliphatic C-H stretching
1725-1718	C=O stretching of COOH and ketones
1620-1600	Aromatic C=C, strongly H-bonded C=O of conjugated ketones
1600-1585	C=C stretch within the ring
1590-1520	COO^- symmetric stretching
1500-1400	C=C stretch within the ring
1400-1200	C-O stretching and OH deformation of COOH
1170-950	C-O stretching of polysaccharide-like substances

Figure 4-7. FTIR absorption bands of CHD-FA (A) and the environmental FA (B).

4.2 NMR

^{1}H NMR for fulvic acids from various sources [22,35,47,49,51,52,53,54] are characterised by signals at 1.2 ppm (highly shielded aliphatic CH) and 3.1 to 3.5 ppm for aliphatic side chains attached to an aromatic ring. Prominent signals near 3.7 and 3.9 ppm may represent OCH and CO-CH_3 groups respectively [22]. Signals at 3.2 ppm originate from protons on CH_3-O groups bonded to aliphatic chains [46,55]. Signals recorded in the 4.0–5.5 ppm are protons on sugar alcoholic OH groups. These signals also suggest the presence of C-C and C-O chemical bonds in the primary structures of humic materials [46]. Methoxyl carbons occur in the 50 to 62 ppm region and acetal and hemiacetal carbons overlap with aromatic carbons from 90 to 110 ppm; olefinic carbons overlap with aromatic carbons in the 110 to 150 ppm region and lactone, ester and amide carbons overlap with the carboxyl carbons in the 165 to 190 ppm region [47]. Aliphatic methyl esters (signals at 171 ppm) and aromatic and α,β-unsaturated methyl esters (signals at 165 ppm) indicate that formic, acetic and succinic acids are present and are postulated to be bound by ester linkages to various hydroxyl groups in fulvic acid [53]. Fulvic acid is therefore characterised by aliphatic, aromatic and carboxylic acid structures and demonstrate signals for the presence of polysaccharides in the aliphatic region between 65 and 105 ppm [55]. The strong signals for COOH groups and weak signals in the aromatic region suggest high contents of carboxylic groups [55]. The phenolic signals in fulvic acid are origin specific [49] as demonstrated by their prominence in aquatically derived fulvic acid when compared to terrestrial samples [54]. Fulvic acid is therefore more aliphatic, less aromatic and substantially more carboxylic in nature than humic acid [55] as indicated by the differences in the NMR spectra of fulvic acid extracted from various environmental sources.

^{1}H NMR: A comparison between the CHD-FA FTIR (Figure 4-1) and ^{1}H NMR (Figure 4-2) demonstrated that FTIR (Figure 4-1) does not provide sufficient information on the presence of aliphatic chains and had to be complimented by ^{1}H NMR (Figure 4-2). CHD-FA has many proton signals in the region between 1.8 and 4.0 ppm, ranging between 2 ppm and 2.7 ppm for sinals and with maximum

intensity. This finding is similar to environmental fulvic acids [22,46,49,51,52,53]. Noteworthy is the signals recorded between 6.5–8.8 ppm (Figure 4-3). These signals are representative of aromatic protons in CHD-FA. Small signals caused by protons from maleic, muconic and fumaric acid derivatives [46] were recorded for the olefinic region of 6.0–6.8 ppm (Figure 4-3). CHD-FA ^{1}H NMR (Figure 4-3) demonstrated similarities between CHD-FA and environmental fulvic acids.

^{13}C NMR: The carbon spectra of CHD-FA were integrated according to the same six bands established by the IHSS standard reference for fulvic and humic acids (Table 4-2). Fulvic acids are more aliphatic, less aromatic and substantially more carboxylic in nature than humic acids [55]. The carbon distribution of CHD-FA is also more aliphatic and carboxylic and less aromatic in nature. Up to 43 % of the carboxylic acid groups characterised in fulvic acid (Table 4-2) is attributed to malonic acid, phthalic acid and salicyclic acid [53]. Similar proton signal profiles were recorded in the sample of CHD-FA (Table 4-3). ^{13}C NMR (Figure 4-4) identified carboxylic groups in CHD-FA as demonstrated by signals at 165–190 ppm for carboxylic acid and ester carbons. High intensity signals recorded at 60 ppm (Figure 4-4) may arise from carboxylic acid aliphatic CH2-carbons C_6 and CH-carbons C_2 – C_5 [56]. Strong COOH group signals for FTIR spectra (Figure 4-1) together with weak signals in ^{13}C NMR aromatic region (Figure 4-4) demonstrates a high content of carboxylic groups in the chemical structure of CHD-FA. This is also a principal character of environmental fulvic acid molecular structures.

4.3 GC-MS

Early GC-MS studies on environmental fulvic acid have identified fragments of carboxylic acids and polycyclic aromatic compounds indicative of hemipic acid, dimethoxy-benzenedicarboxylic acid, pyromellitic acid and mellitic acid [13]. GC-MS identified differences in the chemical structures of environmental fulvic acids. Variations in furan-derived compounds and simple aromatic- and cyclic polycarboxylic acids were identified in fulvic acids isolated from Elliott soil, Suwannee River and Waskish peat, providing evidence of a *supramolecular* structure [1]. Thirty-seven compounds identified in fulvic acid grouped together as: (1) benzene carboxylic, phenolic and methyl benzene carboxylic acids; (2) naphthalene carboxylic acids; (3) pyridine carboxylic acids; and (4) aliphatic acids support the *supramolecular* structure theory [31]. The complexity of a supramolecular structure is supported by a fulvic acid structure for which 47 compounds accounting for 95 % of the total fulvic acid composition was identified [40]. The highest mass fractions were diethyl succinate and diethyl malonate accounting for 29 % and 17 % of the total respectively. Low molecular weight components such as aliphatic and benzene carboxylic acids are the "building blocks" of fulvic acid extracted from soil and are held together by weak linkages of hydrogen bonds [22]. CHD-FA is characterised by a high percentage of aliphatic carbons characterised by extensive fragmentation demonstrated by weak parent ion peaks. This finding has confirmed previous reports that furan-derived compounds and simple aromatic- and cyclic polycarboxylic acids are characteristic of the molecular structure of fulvic acids [1]. GC-MS provided information on the defragmentation pattern of molecular CHD-FA structure. The typical fragmentation is the loss of $(CH_2)_n$, leading to a decrease in molecular weight. The analysis of the defragmentation pattern can be used to determine the connectivity of the ions in the parent structure. Fragmentation patterns (Figure 4-5) show consistency in spacing losses of 16 Da, 28 Da and 44 Da. The differences of 14 Da and 28 Da between adjacent peaks is associated with the presence of aliphatic carboxylic groups in fulvic acid. This is consistent with the presence of carboxylic, alkyl, carbonyl and aromatic functional groups [5,6]. Fragmentation of the derivatised molecules produced m/z 307 caused by the loss of a m/z 76 constituent. GC-MS offered conclusive evidence of the complexity of the CHD-FA molecular structure.

4.4 LC-MSMS spectra of CHD-FA

LC-MSMS is an effective technique to differentiate between different fulvic acid structural compositions [33,57]. LC-MSMS was used in the present study to provide information on the molecular weights of the backbone structures of CHD-FA presented in Figure 4-6. Fulvic acids resolves into groups of

identical mass parent ions and the fragmentation patterns can be traced to more specific carboxylic acids including coumalic acid, hydroxyphenylacetic acid, phenyl malonic acid, pyromellitic acid, coumarin-3-carboxylic acid and chelidonic acid. [57]. The highest relative fulvic acid fragment content reported previously was succinic and malonic acids accounting for 29 % and 17 % of the total respectively [58]. Fulvic acid molecular fragments are similar for different environmental sources [32,57,33]. Figure 4-8 illustrates some of the identified fragments [33]. Several CHD-FA components have now been identified as backbone structures embedded in CHD-FA (Table 4-3).

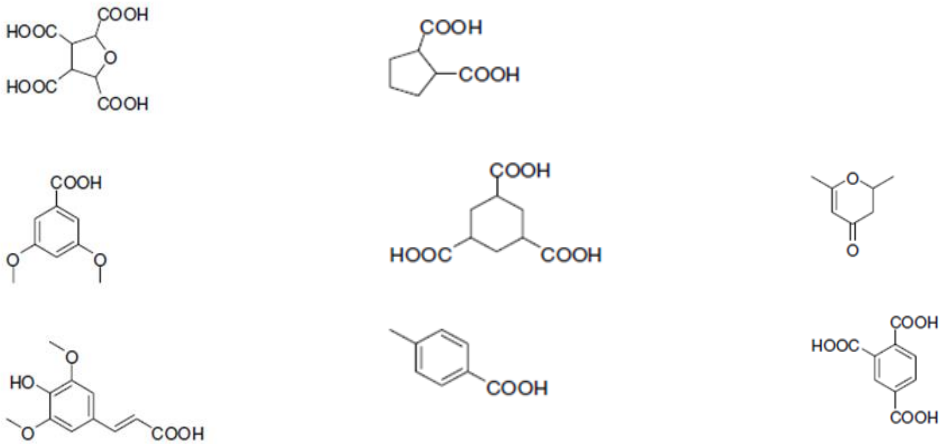


Figure 4-8. Backbone structures of fulvic acid as identified.

5. Conclusion

The present study has provided conclusive evidence for the use of a multi mass spectrometric approach in the characterisation of CHD-FA. CHD-FA has strong FTIR absorption bands at 3400 cm^{-1} and 1200 cm^{-1}. The six major humic substances characteristic for ^{13}C NMR signals established by the IHSS are also characteristic of CHD-FA. The presence of more carboxyl and aliphatic carbons in CHD-FA when compared to the fulvic acid reference standards from IHSS, suggest unique characteristics for CHD-FA. GC-MS provided specific information on the defragmentation pattern of its molecular structure. The differences of 14 Da and 28 Da space losses between adjacent peaks confirm the presence of aliphatic carboxylic groups in CHD-FA. This is consistent with the functional carboxylic, alkyl, carbonyl and aromatic groups and is expected to be present in CHD-FA. There is some consistency in the CHD-FA fragments, suggesting the existence of an ordered structure with a polymeric character. This study identified 24 prominent peaks as the backbone structures embedded in the CHD-FA molecular structure, providing evidence to support the supramolecular nature for CHD-FA. This information provides a basic understanding of the complexity of CHD-FA and forms a platform for investigating the biological properties of CHD-FA. The focuses of future research on CHD-FA should aim to quantify and identify the different characteristics for each of the components, inclusive of the synergistic effect of the different components and the molecular mechanism of the biological activities of these components.

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CHAPTER 5: CARBOHYDRATE-DERIVED FULVIC ACID (CHD-FA) IS A UNIQUE SUPRAMOLECULAR FULVIC ACID WITH BIOLOGICAL PROPERTIES

"Ready for submission" article in *International Journal of Molecular Sciences*, entitled:

"Carbohydrate-Derived Fulvic Acid (CHD-FA) is a unique supramolecular fulvic acid with biological properties"

Introduction

This chapter presents the "ready for submission" manuscript for publication in the *International Journal of Molecular Sciences* published by MDPI. The manuscript is presented in the required format prescribed by *Instructions for Authors,* and as outlined on the journal website:

https://www.mdpi.com/journal/ijms/instructions

The manuscript begins with the title, name of author and affiliations followed by the Abstract. The main body of the manuscript consist of an Introduction; Objectives; Methodology; Results and Discussion; Conclusion and finally the Acknowledgements and References.

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Article

Carbohydrate-Derived Fulvic Acid (CHD-FA) is a unique supramolecular fulvic acid with biological properties

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Abstract: The medicinal uses of humic substances and fulvic acid have been known for many years. Medicinal products containing humic and fulvic acids with therapeutic indications of inflammation, bacterial infections, allergies, arthritis, rheumatism and various other conditions are freely available. The safety and clinical efficacy of drugs are determined by their physicochemical properties and fulvic acids extracted from environmental sources showed variation in their molecular composition. Antioxidant and antimicrobial efficacy of fulvic acid is affected by differences in climatic conditions. Carbohydrate-Derived Fulvic Acid (CHD-FA) is free of heavy metals and environmental pollutants and safe for pharmaceutical interventions. CHD-FA has anti-inflammatory, antibacterial, antifungal, antiviral and antioxidant properties. The supramolecular structure of CHD-FA is characterised by twenty-four principal backbone structures embedded in CHD-FA. The backbone structures are associated with each other and with the parent structure through intermolecular bonding to form a supramolecular structure. This study presents a literature review on the pharmaceutical properties of fulvic acid. This review provides evidence pertaining to the complexity of CHD-FA and the biological properties of the backbone structures embedded in CHD-FA. It offers insight into the anti-inflammatory, antibacterial, antifungal, antiviral and antioxidant properties of CHD-FA and forms a platform for further investigating into the biological properties of CHD-FA.

Keywords: carboxylic acids, anti-inflammatory, antibacterial, antifungal, antiviral, antioxidant

1. Introduction

The medicinal use of humic substances dates back to Ayurvedic traditions and Eastern medicines [13,89]. New humic substance based pharmacological drugs e.g. Shilagen, Diabecon 400, Geriforte, Pilex, Rumalava, Salhumin® and Humex® are commercially available [135,137,138] and their therapeutic properties include anti-inflammatory, antibacterial, antitoxic, antiulcerogenic, antiarthritic and antiallergic applications [84,135,137,183]. Mechanistic studies conducted during the early 2000's on potassium humate derived from bituminous coal found that potassium humate stimulated lymphocyte proliferation by

increasing the production of cytokine interleukin-2 (IL-2) [71,167], especially in human immunodeficiency virus (HIV) infected patients [71]. Fulvic acid, a humic substance common in rich organic humus soil and form by the decomposition of ancient plant deposits, is described as an acidic metabolite [33] characterised by highly complex chemical structures [1,19,40,136,157,170,187]. Fulvic acid is extracted from various environmental sources for its pharmaceutical properties [2,54,56,102,117,118,121,122,186]. It is also synthetically produced by wet oxidation processes [17,30,31,48,49,146,156] e.g. oxifulvic acid derived from coal [17,20,121,165]. Oxifulvic acid have clinical properties [11,20,36,79,91,108,165,167,186] demonstrated by effectively inhibiting inflammatory markers [25,75]. Fulvic acid is highly bactericidal [83,165,195], fungicidal [165,184], antiviral [71,121,140,166] and is an effective antioxidant [129,144]. The antioxidant properties are related to the reactive oxygen species (ROS) scavenging activities of fulvic acid [144].

The safety and pharmacological efficacy of drugs are determined by their physicochemical nature [97] and fulvic acids extracted from shilajit samples collected in different regions had markedly different physiological properties showing significant variances in chemical structures [55]. Antioxidant and antimicrobial efficacy was determined by climatic conditions impacting on the composition of specific plants and herbs from which fulvic acid is sourced [83]. The most prominent complement-fixing system in fulvic acid is related to the relatively low molecular weight fraction of the carboxylic groups in the molecular structure [139]. The complement-fixing system plays an essential role in innate immunity, determining anti-inflammatory responses and the destruction and removal of pathogens [139]. This is proved by the Mantel test and Redundancy Analysis (RDA) recordings of COOH and OH peaks which correlated significantly with the inhibition rates of fulvic acid against phytopathogenic fungi [184].

A pure fulvic acid composition derived from carbohydrate source by wet oxidation, Carbohydrate-Derived Fulvic Acid (CHD-FA), is free of heavy metals and environmental pollutants [11,49,50,142]. It is safe for pharmaceutical inventions, non-toxic and safe for oral [51,142,143,193], dermal and wound healing applications [143,144,194]. CHD-FA possesses anti-inflammatory, antibacterial, antifungal, antiviral and antioxidant properties [51,142,143,193]

2. Clinical properties of CHD-FA

2.1 CHD-FA demonstrates anti-inflammatory properties

The anti-inflammatory properties of CHD-FA are well documented and reports by various studies are summarised in Table 5-1:

Table 5-1. Anti-inflammatory properties of CHD-FA.

AIM	INVESTIGATOR	OUTCOMES
Phase 1 clinical study of the acute and sub-acute safety and proof-of-concept efficacy wheal measurements (an inflammatory response to an allergen).	Gandy [51]	CHD-FA significantly decreased the flare measurement, which is an inflammatory response to an allergen. CHD-FA showed a significant decrease in wheal formation in the skin prick test, and thus proved to act as an anti-inflammatory agent.
Medicinal properties and applications of a Carbohydrate-Derived Fulvic Acid.	Gandy [49]	The investigator evaluated five characteristics of atopic dermatitis: severity, erythema, vesiculation, fissuring and scaling. CHD-FA showed a significant improvement when compared to the placebo, thus demonstrating the anti-inflammatory properties of CHD-FA.
CHD-FA inhibits carrageenan-induced inflammation and enhances wound healing: efficacy and toxicity study in rats.	Sabi [130]	CHD-FA at dosages of 100 and 135 mg/kg reduced the inflammation significantly over a 7 h trial period. Indomethacin at dosages of 10 mg/kg reduced the inflammation significantly over a 7 h trial period similar to CHD-FA. CHD-FA, at a dosage of 50 mg/kg, had no effect on the development of oedema.
Eczema topical application *In vivo:* Single-centre, double-blind, placebo (emollient) controlled, parallel-group comparative study, drug or placebo applied twice daily for 4 weeks Visual Analogue Scale (VAS) Investigators global assessment	Gandy [50]	3.5 % CHD-FA in an emollient (buffered to pH 4.8). Significant differences were observed for both severity and erythema. 3.5 % CHD-FA in an emollient (buffered to pH 4.8). A significant improvement was observed compared to the placebo group for investigator assessment of global response to treatment 97 point scale: 0 = completely clear; 1 = almost clear (about 90 %); 2 = marked improvement (75 %); 3 = moderate improvement (50 %); 4 = slight improvement (25 %); 5 = no change (moderate to severe disease) and 6 = worst) Epizone A® (buffered with acetic acid) placebo (pH 4.8) emollient. Significant differences were observed for both severity, erythema and scaling. CHD-FA showed a significant improvement when compared to the placebo treated group, thus demonstrating the anti-inflammatory properties of CHD-FA. Severity of itching was evaluated via a visual analogue scale. VAS was defined on a 10 cm line in where point 0 refers to clear allergic dermatitis and point 10 refers to the most severe allergic dermatitis. A significant decrease was observed for both groups, thus indicating that both treatments alleviated the patients' perception of their itching which is an indication of disease severity. Severity of itching - Greater and more significant alleviation of symptoms experienced was observed by the CHD-FA group, thus indicating this to be a more effective treatment.
The anti-inflammatory properties of	Malfeld [95]	Concentrations of 750 and 1000 μg/ml significantly ($p < 0.05$) decreased proliferation in

CHD-FA scavenging of oxidants inhibition of the expression of inflammatory markers on leucocytes at concentrations achievable in topical cream formulations.	phytohaemagglutinin (PHA)-stimulated lymphocytes. Significantly ($p < 0.05$) decreased the expression of CD25 by PHA-stimulated lymphocytes at concentrations of 250 μg/ml and higher but had no effect on the resting control cultures. Significantly ($p < 0.05$) decreased the expression of CD38 by PHA-stimulated lymphocytes treated with concentrations of 250 μg/ml and higher, compared to the untreated control culture. Significantly ($p < 0.001$) decreased the expression of human leukocyte antigen-DR isotype (HLA-DR) by PHA-stimulated lymphocytes at 100 μg/ml and higher. Concentrations of 100 and 125 μg/ml significantly ($p < 0.05$) decreased ferricytochrome C reduction in pharbol myristate acetate (PMA)-stimulated neutrophils. Concentrations of 250 μg/ml and higher showed a highy significant ($p < 0.001$) decrease in ferricytochrome C. Decreased ferricytochrome C reduction by stimulated neutrophils at concentrations of 750 and 1000 μg/ml fulvic acid ($p < 0.05$). Concentrations of 250 μg/ml and higher had a highly significant effect on the degranulation of N-formyl-methionyl-leucyl-phenylalanine / cytocalasin B (FMLP/CB) stimulated neutrophils ($p < 0.0001$). CR3 expression by resting neutrophils decreased significantly ($p < 0.05$) after treatment at concentrations of 750 and 1000 μg/ml. The decrease was highly significant ($p < 0.001$) at 100 μg/ml. The 100 μg/ml fulvic acid concentration resulted in a very significant decrease ($p < 0.001$) and the 125 μg/ml and higher concentrations resulted in a significant decrease ($p < 0.05$) of CR3 expression by PMA-stimulated neutrophils. Very significant inhibition of CR3 expression by FMLP/CB stimulated neutrophils at concentrations of 250 μg/ml and higher ($p < 0.001$) of fulvic acid. No significant differences in the Lactate Dehydrogenase (LDH) release by neutrophils and lymphocytes in either the untreated control or those treated with the fulvic acid concentrations up to 1000 μg/ml. No significant differences in the membrane stability of erythrocytes in the untreated control or those treated with fulvic acid concentrations up to 1000 μg/ml. No quenching in the Myeloperoxidase (MPO) and the ferricytochrome C experiments when concentrations up to 1000 μg/ml were added to the supernatant after the neutrophils had been stimulated. No quenching was seen in either experiment. No quenching in the CR3 experiment when concentrations of up to 1000 μg/ml were added after the neutrophils had been stimulated and just before they were analysed on the flow cytometer.

2.2 CHD-FA demonstrates antibacterial properties

CHD-FA has broad-spectrum activity against microbial biofilms. Biofilms are disaggregated by CHD-FA and display a fibrous appearance cause by cell lysis and the release of intercellular components of bacterial cells after exposure to CHD-FA [143]. CHD-FA is effective against *Porphyromonas gingivalis, Streptococcus mitis* and *Streptococcus mutans* at 0.8 % [143]. CHD-FA MIC plantonic of 0.125 % and MIC sessile of 0.5 % showed efficacy against *Enterococcus faecalis* and *Fusobacterium nucleatum* [143]. The endpoint minimum inhibitory concentration (MIC_{90}) was used to demonstrate efficacy of CHD-FA at 0.125 % against *Escherichia. coli, Enterobacter aerogenes, Enterobacter cloacae, Klebsiella pneumoniae,* Methicillin Resistant *Staphylococcus aureus* (MRSA), Methicillin susceptible *Staphylococcus aureus, Pseudomonas aeruginosa.* CHD-FA at 0.06 % is effective against *Streptococcus pyogenes* and *Enterococcus faecium* [193].

Topical applications of CHD-FA have shown effectiveness in drug-resistant wound infections with [193]:

- Potent antimicrobial activity against a broad range of drug-resistant bacteria and fungi.
- Complete inhibition of bacterial growth at 0.06 - 0.12 % CHD-FA.
- Complete inhibition of fungal growth at 0.125 - 0.5 % CHD-FA.
- A reduced microbial burden in *MRSA* and *Pseudomonas aeruginosa* infected wounds by one log or greater.
- A significant improvement of wound closure (healing).

An infected wound preliminary proof-of-principle study [193], used to confirm the wide-spectrum antimicrobial properties of CHD-FA and to evaluate the efficacy of topical applications of CHD-FA on wounds in the early stage of MRSA or *Pseudomonas aeruginosa* infections in an *in vivo* study showed the following:

- Antimicrobial efficacy (MIC50 (minimum inhibitory concentration) and MIC90) for drug-sensitive and multi-drug resistant bacteria at a concentration of ≤ 0.125 % CHD-FA.
- Antimicrobial efficacy (MIC50 and MIC90) for drug-resistant fungi at a concentration of ≤ 0.5 % CHD-FA.
- MRSA-histopathologic evaluation of CHD-FA and untreated wound samples from day 3 showed higher scores in both macrophage and fibroblast categories in 4.6 % CHD-FA treated wounds.
- Decrease in cellular inflammation and an increase in epithelialisation suggested that wound healing was significantly improved with CHD-FA treatment.
- *Pseudomonas aeruginosa* infections showed that the infection and inflammation in the wounds treated with CHD-FA were controlled relative to untreated and colistin-treated wounds, as indicated by lower neutrophils scores as early as day 3.
- IL-10 (anti-inflammatory factor), one of the most important anti-inflammatory cytokines in which its overexpression promotes wound healing was threefold more highly expressed than the untreated control at day 3, suggesting that CHD-FA treated wounds were more likely to progress into the next stage of healing by lessening the severity of inflammation.
- IL-10 and PTGS2 (signal transduction) were up-regulated by an additional threefold in CHD-FA-treated wounds compared with the sham control, indicating a more balanced inflammation response and progression toward accelerated wound healing in the CHD-FA-treated wounds.
- On day 10 when most genes were restored to baseline expression, four genes (inflammatory chemokine), CCL12 and CCL7, pro-inflammatory cytokine IL-6, and matrix metalloproteinase (MMP9) were still highly expressed in the sham control compared to the CHD-FA-treated wounds.

- The key biomarker of impaired wound healing, IL-6 was constantly overexpressed from day 3 to day 6 in the untreated wounds. In contrast, the significantly reduced (25-fold) expression of IL-6 in the CHD-FA treated wounds, manifested accelerated and better controlled wound healing.

2.3 CHD-FA demonstrates antifungal properties

The endpoint MIC_{90} efficacy of CHD-FA against *Absidia corymbifera, Aspergillus flavus, Aspergillus fumigatus, Aspergillus niger, Aspergillus terreus* and *Candida albicans* is 0.5 %. MIC_{90} of CHD-FA at 0.25 % is effective against *Penicillium chrysogenum* and *Penicillium marneffei* and it is effective against *Rhizopus oryzae* and *Fusarium solani* at 0.5 % [193]. CHD-FA is active against planktonic and sessile *Candida albicans* at concentrations of 0.125 % and 0.25 % respectively [142]. CHD-FA is fungicidal by acting through disruption of the cell membrane [142].

2.4 CHD-FA demonstrates antiviral properties

An interesting trend was noticed when CHD-FA was administered to patients infected with the HIV-1 virus. Patients' CD4 counts declined slower compared to natural progression trends of viral diseases reported in the literature [21]. CHD-FA was well tolerated as a wellness drink in a study population on antiretroviral therapy [21].

2.5 CHD-FA demonstrates antioxidant properties

The effect of CHD-FA on lucigenin enhanced chemiluminescence of neutrophils, either resting or stimulated by phorbol myristate acetate (PMA) and on the oxygen scavenging properties thereof in a cell free system using the Trolox Equivalent Antioxidant Capacity (TEAC) assay, showed strong antioxidant activity detectable at concentrations of 500 mg/ml. Scavenging of the 2,2-azinobis (3-ethylbenzothiazoline 6-sulfonate) radical cation, using the TEAC, assay confirmed that the antioxidant activity is due to chemical activity and not to the inhibition of the membrane associated NADPH oxidase system of neutrophils [116]. CHD-FA possesses antioxidant properties *in vitro* and possesses properties as an effective immunomodulator for the treatment of diseases associated with an overproduction of reactive oxidants by phagocytes. This needs to be confirmed by *in vivo* systems. The antioxidant properties of CHD-FA partially support the health beneficial properties of this compound and it is a good candidate to be used in nutritional or food industries as an accessible source of natural antioxidants [123].

3. Investigation of the clinical properties of the backbone structures embedded in CHD-FA.

The World Health Organisation has instructed that medicinal plant studies must include the identification of chemical constituents and the determination of their biological activities for use as ingredients in health products [172,182]. In view of this instruction by WHO and with the aim to provide scientific evidence for the use of CHD-FA as an active pharmaceutical ingredient in the manufacturing of natural medicines.

A previous study by the author (submitted for publication) demonstrated that 7,8-dihydroxy-3-methyl-10-oxo-1*H*-pyrano[4,3-b] chromene-9-carboxylic acid is the most prominent component of the CHD-FA structure. It, together with several carboxylic acids, forms the backbone structures of CHD-FA's supramolecular structure. The backbone structures include malic acid, maleic acid, levulinic acid, succinic acid, propenoic acid, phthalic acid, arabonic acid, itaconic acid, glucuronic acid, glutaric acid, benzene tri- and tetracarboxylic acids. The author has also reported (in the affore mentioned article submitted for publication) that the backbone structures are associated with each other as well as with the parent structure through intermolecular bonding and that it is the specific bonding sites of the different carboxylic acids which are responsible for the unique supramolecular structure of CHD-FA. Similar findings were reported previously [113,115,190,194] and assist in providing a basic understanding of the complex composition of supra molecules such as CHD-FA. The present study aimed to provide

information to substantiate the anti-inflammatory, antimicrobial, antifungal and antioxidant efficacy for CHD-FA by identifying and reviewing the clinical properties of the most prominent compounds identified as the backbone structures embedded in CHD-FA.

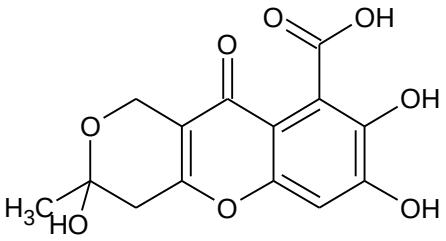

Figure 5-1. Presentation of the main component in CHD-FA.

3.1 Anti-inflammatory activities of carboxylic acids

In general, non-steroidal anti-inflammatory drugs (NSAIDs) structurally consist of an acidic moiety (carboxylic acid), which is attached to a planar, aromatic functionality. Therefore, the NSAIDs are characterised by the following properties:

- All are relatively strong organic acids with a pKa range of 3 – 5. Most are carboxylic acids. The carboxylic acid / acidic group is essential for the inhibition of cyclooxygenases (COX).
- The carboxylic acid / acidic group within these compounds serves as a major binding group (ionic binding) with proteins.
- The carboxylic acid / acidic group also serves as a major site of metabolism by conjugation. Therefore, a major pathway of clearance for many NSAIDs is glucuronidation and inactivation followed renal elimination.
- Salicyclic acid and acetylsalicylic acid (aspirin) are prominent backbone structures of CHD-FA and have potent anti-inflammatory activity with mild analgesic and antipyretic properties, and these compounds bind with high affinity to COX-1.

Propionic acid derivatives such as the "profens" are all strong organic acids, and include compounds such as fenoprofen (a), ibuprofen (b) and naproxen illustrated as a; b; and c respectivelyin Figure 5-2. These compounds are anti-inflammatory agents with antipyretic and analgesic activity. All these compounds are carboxylic acids.

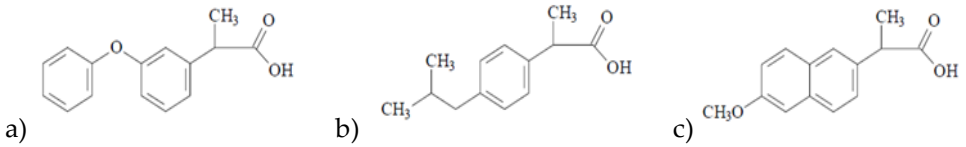

Figure 5-2. The structures of profens: a) Fenoprofen; b) Ibuprofen; c) Naproxen

3.2 Antibacterial activities of carboxylic acids

Carboxylic acids exhibit bacteriostatic and bactericidal properties depending on the physiological status of the organism and the physicochemical characteristics of the external environment. Given the weak acidic nature of most of these compounds, pH is considered a primary determinant of effectiveness related to its effects on the concentration of undissociated acids formed [128]. It has been traditionally assumed that undissociated forms of carboxylic acids can easily penetrate the lipid membrane of the bacterial cell and once internalised into the neutral pH of the cell cytoplasm dissociate into anions and protons [127]. Generation of both species potentially presents problems for bacteria that must maintain a near neutral pH cytoplasm to sustain functional macromolecules. Export of excess protons requires

consumption of cellular adenosine triphosphate (ATP) and may result in depletion of cellular energy [127].

The synergistic bactericidal effects of medium-chain fatty acids (MCFAs) and organic acids (OAs) against *Escherichia coli* and the underlying mechanism of action expose bacterial cells to combined treatments causing membrane disruption and disintegration and/or cell death (irreversible damage) [77]. Disruption of the bacterial membrane facilitates the entry of other antimicrobial compounds into the cytoplasm [77]. A major advantage of combined treatment with very low concentrations of natural antimicrobial compounds is that it is very cost-effective [77].

3.3 Antifungal activities of carboxylic acids

The effect of eight organic acids (propionic, acetic, formic, lactic, tartaric, citric, oxalic and malic acids) used as antifungal agents on the growth of four fungi *(Aspergillus flavus, Penicillium purpurogenum, Rhizopus nigricans and Fusarium oxysporum)* showed no relationship between the efficacy of organic acids and the end pH [64]. The inhibition of microbial growth increases by lowering the pH of the media, and most micro-organisms are susceptible to antimicrobial effects in the presence of organic acids [64]. The hydrophobic nature of most organic acids allows free diffusion of the protonised forms through cell membranes. This diffusion process takes place spontaneously due to pH and osmolarity gradients that exist between the inner and outer sides of the cell. The intracellular pH is higher than extracellular, and the acid undergoes dissociation as soon as it enters the cytoplasm and then decreases the intracellular pH by releasing the proton [64]. The cell allocates the main part of its energy content to eliminate the newly formed protons resulting in slower growth kinetics. A relationship exists between the chemical structure of organic acids and the fungal growth inhibition. The higher inhibitory effect is related to similarities in the chemical structures of formic acid, acetic acid and propionic acid as well as in the low pKa [64]. The antifungal activity of four weak organic acids against *Saccharomyces cerevisiae* proved that the synergistic effects of a combination of weak acids is more effective than single weak acids [82]. The inhibitory effect of the weak organic acids is caused by a sudden decrease of extracellular pH and the increased permeability of the cell membrane [82].

3.4 Antiviral activities of carboxylic acids

The efficacy of a combination of organic acids against various viral infections provided evidence that the synergistic effect of the organic acids, demonstrated by the patent (WO 2012/161558Al) is a function of the combined efficacy of lactic acid, citric acid, malic acid, citric acid, tartaric acid and orthophosphoric acid [80]. The invention described the treatment method and/or prophylaxis of *Coxsackie* virus. The antiviral effect of an organic acid was also investigated [46] and comprised of two or more polycarboxylic acids selected from the group consisting of malonic acid, succinic acid, glutaric acid, adipic acid, pimelic acid, fumaric acid, maleic acid, tartaric acid, malic acid and citric acid in a registered patent (US 8,034,844 B2) [46]. The efficacy of organic acids in the prevention of rhinovirus infections was also reported [161] and the immediate and residual antiviral activity that was demonstrated by salicylic, pyroglutamic, fumaric and benzoic acids exceeded the positive control of 2 % iodine, 4 % chlorhexidine, 1 % triclosan, and 62 % ethanol. Benzalkonium chloride (0.13 %) was also tested and showed no residual antiviral activity in this assay.

3.5 Antioxidant activities of carboxylic acids

The high antioxidant activity of short-chain organic acids and polyphenols is demonstrated for grape juice [90] and pomegranate juices [154] and the antioxidant activity of natural sterols and organic acids e.g. citric, malic, lactic, fumaric and tartaric acids is well documented [96]. The reaction mechanism involves either the inhibition of the chain reaction of pro-oxidant radicals or the sequestration of free radicals [96]. A mixture composed of oxalic, citric, malic quinic and fumaric acids exhibited a

concentration-dependent scavenging ability against diphenyl-2-picryl-hydrazyl (DPPH) [128] and provide evidence that the synergistic mechanism of different organic acids in a composition determines the efficacy of the supramolecular product.

3.6 Malic Acid

The anti-inflammatory property of malic acid [141] is demonstrated by its ability to reduce serum levels of TNF-α [151]. Antimicrobial activity was proved by a marked reduction in the total bacterial count in the faeces of Nile tilapia (*Oreochromis niloticus*) fish fed diets supplemented with malic acid [64]. Bactericidal efficacy was confirmed by the discovery that malic acid has completely inactivated *Salmonella enterica* serovar and *Listeria monocytogenes* on fruit [126] and meat [58]. Malic acid in combination with other carboxylic structures have demonstrated antibacterial efficacy against *Escherichia coli* O157:H7 and related strains of enterohemorrhagic *Escherichia. coli*, [92], *Cronobacter sakazakii* [78] and *Salmonella gaminara* [42]. Fungicidal efficacy was proved against *Candida* spp [111] and malic acid significantly inhibited the growth of *Colletotrichum gloeosporioides*, a phytopathogenic fungus [73]. Antiviral properties were reported on the antiviral effects of ethanol-based sanitizers with different concentrations of malic acid against murine norovirus and *feline calici* virus F4 [5,41]. Antioxidant properties are well known [69,75,125,180].

3.7 Maleic Acid

Maleic acid is a chelating agent when used as a root bio modifier on the cemented surface of teeth and preliminary evidence for 7 % maleic acid solution as an anti-inflammatory agent when used as a root conditioning agent was proposed [168]. Maleic acid derivative prevent liver fibrosis at an early phase for *in vitro* and *in vivo* studies through the inhibition of reactive oxygen species, inhibition of inflammation and the activation of hepatic stellate cells [188]. Antimicrobial efficacy is known for bactericidal [14,44] and fungicidal uses [14]. Maleic acid eradicates *Enterococcus faecalis* biofilms *in vitro* at ≥ 1 minute of contact [44]. The antimicrobial efficacy of 7 % maleic acid was investigated and reported to eliminate *Enterococcus faecalis* and *Staphylococcus aureus* bacteria as well as *Candida albicans* fungi at different time intervals [14]. Fungicidal efficacy was also reported [110] for an apple cider vinegar containing maleic acid against *Candida* spp., suggesting the use of substances containing maleic acid as possible therapeutic alternatives for patients with denture stomatitis. Applications in antiviral treatments were also reported [22,81]. A comprehensive explanation for the antioxidant mechanism of maleic acid was offered in a study that investigated the effects of maleic acid on chromium (a highly toxic environmental pollutant that negatively affects plant growth and development) uptake and mitigation of chromium toxicity [94].

3.8 Levulinic acid

The use of levulinic acid for anti-inflammatory applications showed that > 23% of the levulinic market volume share in 2013 was for pharmaceutical applications [59]. An antimicrobial efficacy against *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Aspergilus niger* and *Candida albicans* during the development of new cosmetic formulations to replace chemical preservatives with ingredients such as ≤ 0.3 % w/w levulinic acid that have antimicrobial properties, confirmed the microbicidal efficacy of levulinic acid [119]. Levulinic acid (4-oxo-pentanoic acid) is active against Gram-positive and Gram-negative bacteria, moulds and yeasts [119]. Antioxidant properties of levulinic acid were demonstrated by the thermal degradation characteristics of fructose to organic acids e.g. levulinic acid has an increased antioxidant activity [181]. Levulinic acid has antioxidant properties for for use in pharmaceutical chemistry [32].

3.9 Succinic acid

Succinic acid has cerebro-protective, cardio-protective, immune-tropic and stress-protective characteristics [88]. The anti-inflammatory properties of succinic acid were described by using an

exudative inflammation model of carrageenan-induced paw oedema of rats as well as reporting on the hepatic-protective efficacy of rats injured with carbon tetrachloride and the renal protection actions of succinic acid [88]. It takes the form of an anion, succinate, which has multiple biological functions in living organisms e.g. regulating the production of inflammatory cytokines [105]. Succinate can also activate the immune cells through its receptor, SUCNR1 and exacerbates disease [105]. Succinate is a metabolite in innate immune signalling which leads to enhanced IL-1β production during inflammation [152]. Antimicrobial efficacy of succinic acid was demonstrated against *Streptococcus suis* [52] and in cultures of two strains of *Escherichia coli*, three strains of *Salmonella* sp. and two strains of *Clostridium perfringens* [147] and also against chronic viral hepatitis [148]. Succinic acid has very strong antioxidant properties and may be used as cyto-protective medicines in hepatology [148]. Clinical trials on the efficacy of metabolic correctors based on succinic acid as pathogenic treatment in chronic viral hepatitis have proved to correct the insufficiency of the antioxidant defence mechanisms [148]. Succinic acid boosts cerebellar function following short-term treatment [44]. Succinic acid crosses the blood-brain barrier and penetrates the cerebellar tissue and bypasses dysfunctional complex I in cerebellar tissue without disrupting complex II or downstream oxygen consumption, restoring complex III inhibition by antimycin A and prevents damage of outer mitochondrial membranes [44]. Within Purkinje neurons, succinic acid diminishes Purkinje dendritic atrophy and averts loss of Purkinje soma. On a behavioural level, succinic acid lessens the cerebellar ataxia phenotype, it was highlighted the important health benefits of succinic acid.

3.10 Benzenetricarboxylic acid

The anti-inflammatory use of 1,2,3-benzenetricarboxylic acid was proposed by using a plant extract from *Excoecaria agallocha* (*Euphorbiaceae*), which is characterised by bioactive compounds that include derivatives of 1,2,3-benzenetricarboxylic acid [70]. This work supported the identification of the insecticidal components of *Saraca asoca* bark, which contains derivatives of 1,2,3-benzenetricarboxylic acid [57]. It was confirmed the bactericidal efficacy of 1,2,3-benzenetricarboxylic acid in a study [18] which aimed to assess the effect of crude methanolic and hexane extracts from *Chroococcus turgidus*, characterised by 1,2,3-benzenetricarboxylic acid, against the Gram-positive and Gram-negative bacterial strains *Staphylococcus aureus* (MTCC 3615), *Vibrio parahaemolyticus* (ATCC 17802), *Escherichia coli* (ATCC 25922), *Enterococcus faecalis* (ATCC 29212), *Eubacterium lentum*, *Enterobacter aerogenes* (ATCC 13048) and *Staphylococcus epidermidis* (ATCC12228). It was concluded that the metabolites produced by *Chroococcus turgidus* can be a potential source of antimicrobial and antioxidant agents [18]. Antifungal properties were also proposed [158]. Antiviral efficacy was suggested and it was reported that gas chromatography-mass spectroscopy (GC-MS) analysis of *Chroococcus turgidus* indicated the presence of active volatile organic compounds such as 1,4-benzenedicarboxylic acid, bis(2-methylpropyl)ester, 1,2,3-benzenetricarboxylic acid, and 1,2-benzenedicarboxylic metabolites, which are known to have higher antiviral (against rabies virus), antimicrobial and antioxidant potential [8]. Evidence was provided to support the anti-inflammatory and antioxidant efficacy of 1,2,3-benzenetricarboxylic acid [10]. A part of the benzene nucleus between the two carboxylic groups is thought to enter the plasma membrane and act on acyl-chains in phospholipids in the RBC membrane [106]. For dicarboxylic and tricarboxylic acids, limited numbers of hydrocarbons in molecules are speculated to enter the RBC membrane with the hydrophilic carboxylic groups remaining outside, stabilizing the structure of the cell membrane and resulting in an increase in osmotic resistance of rat RBCs [106].

3.11 Propenoic acid

The antioxidant and anti-inflammatory properties of 2-propenoic acids are demonstrated by its identification in extracts from *Bougainvillea x buttiana* (var. Rose) known for its antioxidant and anti-inflammatory properties [61]. Antimicrobial and antioxidant efficacy is concluded GC-MS measuring 2-propenoic acid in an extract of *Cyperus rotundus L.* (family *Cyperaceae*), a highly bactericidal against *Escherichia coli, Staphylococcus aureus* and *Klebsiella pneumoniae* and fungicidal against *Aspergillus niger,*

Aspergillus flavus and *Candida albicans* [3]. Antioxidant activity of a methanol extract of *Cyperus rotundus* rhizomes was compatible to the standard of a well-known antioxidant, L-ascorbic acid [3], confirmed the presence of 2-propenoic acid on an extraction from the bark of *Araucaria columnaris*. *Araucaria* species are used in traditional medicine for antiulcer and antipyretic (*Araucaria bidwillii*), gastro-protective and wound healing action (*Araucaria ara*ucana), antibacterial activity (*Araucaria angustifolia*) and antimicrobial activity (*Araucaria cunninghamii*) [37]. The anti-inflammatory, antibacterial and fungicidal properties of phytochemical compounds characterised by the presence of 2-propenoic acid is well documented [124].

3.12 Phthalic acid

The antimicrobial application of phthalic acid was demonstrated and described the synthesis, characterisation and antimicrobial activity of mixed ligand complexes of Mn(II) and Zn(II) with phthalic acid [6]. This was demonstrated against Gram-positive (*Bacillus cereus* and *Staphylococcus aureus*) and Gram-negative (*Escherichia coli* and *Shigella sonnei*), as well as the fungal species *Candida albicans, Saccharomyces cerevisiae* (human pathogens) and *Aspergillus niger* (plant pathogens)[6]. The antimicrobial efficacy of plant extracts characterised by phthalic acid and other phytochemicals against *Staphylococcus aureus, Bacillus subtilis, Bacillus megaterium, Sarcina lutea, Escherichia coli, Shigella sonnei, Shigella shiga, Shigella dysenteriae, Aspergillus niger, Aspergillus flavus, Aspergillus fumigatus* and *Fusarium* species was summarized [131]. Dengue (DENV2), chikungunya (CHIKV) and human parainfluenza (hPiV3) viruses have profoundly impacted on human morbidity, mortality and the economy worldwide and as a current therapy option to treat infections of these viruses. An isolated novel phthalate acid ester from *Acrostichum aureum* was tested for antiviral activity against DENV2 and hPiV3 in Vero cells, and against CHIKV virus in Rhesus monkey kidney cells (LLC-MK2) cells. Other researchers [162] also reported antiviral activity against RNA(+) and (-) viruses[162]. A phthalic acid was identified from a *Psidium guajava* Linn root bark extract and proposed that the combination of active carboxylic compounds in *Psidium guajava* may present an opportunity for developing new antiviral drugs as diethyl phthalate and phthalic acid displayed antiviral activity against the White Spot Syndrome Virus [172].

3.13 Arabonic acid

The potential antibacterial properties of arabonic acid were demonstrated against *Escherichia coli* and *Staphylococcus aureus* [159]**.** MIC_{50} values from extracts of *Cistus salviifolius* exhibited potent bacteriostatic effects against *Staphylococcus aureus* when compared with other *Cistus* species tested suggesting that these extracts could serve as an alternative source of antimicrobial ingredients in medical devices or cosmetics [159]. To determine which compounds were responsible for the observed antibacterial activity, a preliminary identification and quantitation of the polyphenolic composition of the different extracts was performed, proving that arabonic acid was found in all *Cistus* extracts [159]. Based on the ancient ethnobotanical use of various *Cistus* species these plants are a good remedy for several microbial related disorders and infections [15].

3.14 Benzenetetracarboxylic acid

1,2,4,5-Benzenetetracarboxylic acid was found to be a potent inhibitor of *Protobothrops flavoviridis* venom-induced haemorrhage [9]. This could be related to the strong chelating properties of 1,2,4,5-benzenetetracarboxylic acid [26]. Antioxidant properties for 1,2,4,5- benzenetetracarboxylic acid (H4btec) was demonstrated [185] and it was found that the half maximal inhibitory concentration (IC50) antitumor effect against the cancer MCF- 7 cell lines was dose dependent and that only a very small dose of H4btec was needed to demonstrate antioxidant efficacy.

3.15 6-Hydroxy-2H-pyran-3(6H)-one

The derivatives of 6-Hydroxy-2H-pyran-3(6H)-one showed to be an inhibitor of *Staphylococcus aureus* and *Streptococcus* species [53]. The anticoccidial and *in vitro* antimicrobial properties of 6-Hydroxy-2H-pyran-3(6H)-one were also investigated and reported [86].

3.16 Pyruvic acid

The anti-inflammatory efficacy of pyruvic acid was demonstrated in the treatment of acne vulgaris, a chronic inflammatory disease of the pilosebaceous follicles and one of the most common skin diseases in humans [67]. It was reported that pyruvic acid is effective in the treatment of mild to moderate acne reducing the number of comedones, papules, and the acne severity index (ASI) over the course of an eight-week treatment period, with no side-effects [67]. These anti-inflammatory properties were confirmed by evaluating the efficacy and tolerability of chemical peeling with pyruvic acid and proved the anti-inflammatory and healing qualities of pyruvic acid with no side-effects observed during or after treatment [28]. The anti-inflammatory and healing properties of pyruvic acid were also demonstrated for other dermatological conditions such as superficial scarring, photo damage and pigmentary disorders [16]. The anti-inflammatory efficacy of sodium pyruvic acid was demonstrated for both acute and chronic inflammation models in carrageenan-induced paw oedema [62]. The antibacterial efficacy of pyruvic acid is demonstrated by the fact that it is an extremely important intermediate [196]. It was reported that pyruvic acid connects the Embden–Meyerhof–Parnas pathway (EMP) and the tricarboxylic cycle (TCA) to the hexose monophosphate shunt pathway (HMP) and an accumulation of pyruvic acid inhibits the normal physiological metabolism, especially the TCA pathway of bacteria. The TCA pathway is crucial in catabolism and anabolism, and blockage of this pathway hinders carbohydrate synthesis of bacteria as demonstrated by its efficacy in killing *Escherichia coli* and *Staphylococcus aureus* [196]. This proof that the accumulation of pyruvic acid and the reduction of the ATP change cell membrane permeability, destroy bacterial respiratory metabolism and ultimately lead to pyknosis and death. Pyruvic acid has excellent antioxidant properties as demonstrated by its critical role in energy metabolism and its capability to non-enzymatically reduce H_2O_2 into H_2O [100]. This is of great importance in neural disorder management as elevated production of hydrogen peroxide (H_2O_2) in the central nervous system has been implicated in the pathogenesis of several neurodegenerative diseases such as Parkinson's disease, ischemic reperfusion, stroke and Alzheimer's disease [100]. The importance of pyruvic acid as a free radical scavenger was also demonstrated by other researchers [93] and it was reported that the formation of oxygen free radicals during heart transplantation could be related to alterations occurring during ischemia and reperfusion and may explain the short preservation time of donor hearts. Because of its acidity, pyruvic acid dissociates immediately to pyruvate in the human body to prevent reperfusion injuries in the isolated heart, probably by its antioxidative properties. The application of pyruvate may contribute to the preservation of hearts for organ transplantation [93]. The antioxidant efficacy of pyruvic acid in the human body was also studied [29] and demonstrated, through lucigenin-enhanced chemiluminescence (LcCL) *in vitro* and *in vivo*, that the antioxidant effect of pyruvate during myocardial reperfusion improved myocardial function during reperfusion.

3.17 Itaconic Acid

Itaconic acid plays an important role in inflammation processes and acts as an endogenously produced antimicrobial compound against pathogens by interfering with enzymes of their central metabolism, including the glyoxylate shunt, 2-methylcitrate cycle and the tricarboxylic cycle (TCA) of bacteria [27]. The anti-inflammatory and antimicrobial mode of action for itaconic acid was demonstrated and explained that itaconic acid inhibits isocitrate lyase, which is the key enzyme in the glyoxylate shunt and a pathway essential for bacterial growth and for bacteria to survive during infection, such as *Pseudomonas indigofera* [101]. This proposed mode of action was confirmed and it was reported that the immune responsive gene 1 (IRG1), which is highly expressed in mammalian macrophages during

inflammation, is the gene that codes for an enzyme producing itaconic acid through the decarboxylation of cis-aconitate, a tricarboxylic acid cycle intermediate [103]. Using a gain-and-loss-of-function approach in both mouse and human immune cells, it was found IRG1 expression levels correlate with the amounts of itaconic acid and purified IRG1 [103]. It showed that itaconic acid inhibits the growth of bacteria expressing isocitrate lyase such as *Salmonella enterica* and *Mycobacterium tuberculosis*. Furthermore, IRG1 gene silencing in macrophages resulted in significantly decreased intracellular itaconic acid levels as well as significantly reduced antimicrobial activity during bacterial infections, suggesting that IRG1 links cellular metabolism with immune defences by catalysing itaconic acid production, and thus confirming that itaconic acid has effective bactericidal properties. The fungicidal efficacy of itaconic acid was demonstrated and found to be an excellent fungicidal for *Candida albicans* [134]. The antiviral properties of itaconic acid were reviewed and investigated the role of mimi-viruses as potential human pathogens [35]. A novel antiviral immunomodulatory pathway was proposed, that is controlled by interferon-β (IFN-β) and mediated by IRG1 and itaconic acid. The discovery was made that the virus can grow on IFN-α2, but not on IFN-β-treated cells [35]. It was proposed that IFN-β preferentially upregulates IRG1 in human macrophages, which in turn produces itaconic acid to link itaconic acid metabolism to antiviral activity as a novel immunomodulatory pathway [35].

3.18 Glutaric Acid

Glutaric acid has strong microbicidal efficacy and is highly effective against representatives of Gram-positive, Gram-negative bacteria and yeasts [121]. Glutaric acid also possesses antiviral activity in addition to the aciduant effect against rhinoviruses [85]. The antiviral activities of glutaric acid, glutaric acid analogs and other mono- and dicarboxylic acids were tested and the antiviral activity for most strains tested, appeared to be due to a combination of low pH and another mechanism of action presumably unrelated to pH [85]. Hand lotion containing glutaric acid was significantly more effective than placebo in inactivating rhinovirus serotype 2 on the fingers of human volunteers [65]. It may be possible to develop safe, cosmetically acceptable hand lotions which have durable antiviral activity and can interrupt the hand-to-hand transmission of the rhinovirus [65].

3.19 D-Glucuronic acid

Many drugs have been conjugated to D-glucuronic acid to obtain the required tools for improving insights on their absorption, metabolism and bioavailability [175]. This is confirmed by the fact that heparin, used clinically as an anticoagulant and antithrombotic agent for over 60 years, consist of repeating disaccharide units containing D-glucuronic acid [177]. The anti-inflammatory properties of D-glucuronic acid was reported and it was discovered that K5NOSepiLMW, an O-sulphated heparin-like semi-synthetic polymer of the D-glucuronic acid, has marked anti-inflammatory effects, as proven by the carrageenan-induced pleurisy rat model commonly used to test NSAID efficacy [23]. The lability of glucuronic acid concentration in the blood of patients with rheumatoid arthritis is interesting as it is known that glucuronic acid compounds such as hyaluronic acid and chondroitin sulfate are important constituents of cartilages and the joint capsules [45]. The question was posed [45], whether arthritis may be accompanied by a disorder of glucuronic acid metabolism. The role in the prevention of inflammatory disorders by D-glucuronic acid was described [137] and reported on the testing of the scavenging effect of ROS. It was found that the synovial fluid from rheumatoid arthritis patients, with added hyaluronic acid, and D-glucuronic acid markedly decreased the O_2-, H_2O_2, OH and chemiluminescence measured in both systems [137]. Hyaluronic acid in combination with its two subcomponents, D-glucuronic acid and N-acetyl-D-glucosamine, and synovial fluid, which are known to be susceptible to degradation by excessive ROS in rheumatoid arthritis patients, also seem to play an active role in protecting articular tissues from oxidative damage. D-glucuronic acid plays a role in xenobiotic liver detoxification, as it can combine with toxin molecules to facilitate their elimination from the organism, which makes it very important as an auxiliary for liver functions [87]. The work, which underscored the antioxidant properties for D-glucuronic acid was supported by other researchers [173], who stated that toxification, is one of the most

extensively debated health issues of the modern age as there are many types of toxins influencing human health such as increased amounts of xenobiotics, toxins of infectious micro-organisms and reactive metabolites. Insufficient detoxification causes "metabolic poisoning", an accumulation of toxic metabolites that are not processed by the liver and thus accumulate within cells, tissues and organs, causing kidney failure and inefficient liver functions. This leads to the accumulation of toxins in blood, impairment of the functions of brain cells and progression of non-communicable diseases, such as cardiovascular, neurodegenerative, chronic respiratory and kidney diseases, diabetes mellitus type 2 and cancer [173]. When D-glucuronic acid, well known as detoxicant will detoxify the human body, eliminate xenobiotics and support fat-soluble endobiotics to facilitate normal metabolism and the prevention of non-communicable diseases [173]. D-glucuronic acid also increases the bioavailability of polyphenols needed to enhance antioxidant activity [173]. Antibacterial activity and antifungal efficacy were also investigated [38,47]. Antiviral efficacy was demonstrated by investigating the *in vitro* anti-HIV-1 activity of natural chondroitin sulphate (CS) copolymers, mainly composed of D-glucuronic acid [72]. The results were compared to the results obtained with compounds of known *in vitro* antiretroviral activity, namely, zidovudine. Chondroitin polysulphate was the most effective polyanionic compound studied in contrast with zidovudine, and highly specific for inhibition of HIV-1 reverse transcriptase. D-glucuronic acid antiviral efficacy was also reported [160] for a D-glucuronic acid solution which potently inhibited dengue virus type 2 (DEN2) replication and significantly inhibited the proliferation of HTLV-1-infected T-cells. Antiviral efficacy for D-glucuronic acid against the HIV, hepatitis C virus and dengue virus, was also supported by other investigations [4,178].

3.20 Gluconic acid

The antibacterial spectra of gluconic acid against *Gaeumannomyces graminis* and the protozoan *Colpoda steinii, Vahlkampfia sp*. and *Neobodo designis* [114], *Gaeumannomyces diazotrophicus* [74] and *Salmonella infantis, Staphylococcus aureus, Clostridium perfringens* and *Clostridium aquaticum* [169] have been defined. The antimicrobial action of honey has been well known for millennia. Gluconic acid was identified as the predominant organic acid in all samples, suggesting that it forms a major part of the chemical structure of honey [154]. These organic acids in the tested honey samples may contribute to the antioxidant capacity of honey, either by their own antioxidant properties or by enhancing the effect of other antioxidant compounds. The effect of gluconic acid on *Paenibacillus* larvae, a pathogen responsible for American foulbrood disease in honeybees was studied and a bactericidal effect was reported. It confirmed [133] the efficacy of gluconic acid against *Paenibacillus larvae* by concluding that dark honey seem to have a higher gluconic acid content and a higher antimicrobial activity suggesting that honey characterisation, including colour and physico-chemical characteristics (e.g. gluconic acid concentration, total phenolic and total flavonoid contents, glucose oxidase activity), could be crucial for the assessment of its use against *Paenibacillus larvae* [133]. The antioxidant properties of gluconic acid are demonstrated by the antioxidant efficacy of honey as gluconic acid is the predominant organic acid in honey [149]. The antioxidant capacity of honey appears to be the result of the combined activity of a wide range of compounds including phenolics, amino acids and the organic acids gluconic, citric and malic acids [99].

3.21 4-Hydroxybenzoic acid or salicylic acid

The anti-inflammatory properties of 4-hydroxybenzoic acid isolated from the ethanolic extract of *Vitex glabrata* (*Verbenaceae*), commonly known as smooth chaste tree was reported [93] and explored in microglial cells stimulated with lipopolysaccharide but it did not show any effect [179]. However, research [76] did show analgesic and anti-inflammatory effects and suggested that repeated daily low oral doses of 4-hydroxybenzoic acid may demonstrate a potent stress response desensitisation almost as significant as aspirin. The study [189] on Dendrobii Herba, a traditional Korean medicine characterised by its 4-hydroxybenzoic acid content, demonstrated that 4-hydroxybenzoic acid had a significant inhibitory effect on TNF-α production and the levels of IL-6 were significantly reduced. 4-hydroxybenzoic acid showed comprehensive antimicrobial activity against Gram-positive and Gram-

negative bacteria and fungi as demonstrated [96] for microbicidal efficacy against *Escherichia coli*, *Bacillus aureus*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Candida albicans Lactobacilli (Lactobacillus paraplantarum*, *Lactobacillus plantarum*, *Lactobacillus fermentum*, *Lactobacillus fermentum*, *Lactobacillusbrevis* and *Lactobacillus cornyformis)*, *Listeria monocytogenes*, *Fusarium culmorum* and *Saccharomyces cervisae.* It was reported [176], that 4-hydroxybenzoic acid and its derivative 3,4,5-trihydroxybenzoic acid also inhibited the growth of two strains of the algae, *Microcystis aeruginosa*. An interesting observation was made [176] when it was found that the sequence of 50 % growth inhibition concentration of 6 compounds for two strains of *Microcystis aeruginosa* followed the same order: gallic acid > 3,5-dihydroxybenzoic acid > 4-hydroxybenzoic acid > salicylic acid > 3-hydroxybenzoic acid > benzoic acid, suggesting that the position and the numbers of hydroxy groups between each hydroxy and carboxyl group influenced the anti-algal effects of the phenolic acids. It was also reported [96] that almost three quarters of contagious diseases in the world are caused by viruses, highlighting the use of ester derivatives of hydroxybenzoic acid for treating infections caused by the hepatitis B virus, human papilloma, herpes simplex virus, condylomata acuminatum, cervicitis and cervical erosions in human and animals. The antioxidant properties of 4-hydroxybenzoic acid was demonstrated [171] and tested the antioxidant properties of 14 different benzoic acid derivatives against the superoxide radical and found that monohydroxybenzoic acid derivatives showed the best antioxidant properties. The neuroprotective capacity of 4-hydroxybenzoic acid was assessed in primary cultures of cerebellar granule neurons and found that 4-hydroxybenzoic acid can mitigate oxidative stress induced by hydrogen peroxide which is thought to be contributing to neuronal cell death in neurodegeneration [179].

3.22 α-Ketoglutaric acid

It was reported [60] that α-Ketoglutaric acid administered in the form of a dietary supplement contributes to inhibition of osteoporosis in women and that it promotes the growth of muscle mass and accelerates wound healing. α-Ketoglutaric acid has a significant impact on the morphology of the gastrointestinal tract in healthy animals and animals with damaged gastrointestinal tract mucosa as well as being a promising substance for the treatment of patients with short bowel syndrome by stimulating beneficial changes in intestinal morphology [60]. α-Ketoglutaric acid also possesses neuroprotective effects [60]. The anti-inflammatory properties of α-Ketoglutaric acid are related to its ability to suppresse the nuclear factor kappa B (NF-κB) mediated inflammatory pathway and enhances the pregnane X receptor (PXR)-regulated detoxification pathway [66] The interaction between the NF-κB mediated inflammatory pathway and PXR-regulated detoxification pathway is described [66] as a check-and-balance mechanism for keeping the homeostatic state of the intestine by preventing the onset of intestinal inflammation which may lead to cancer. α-Ketoglutaric acid -treated intestinal tissues demonstrated a strong inhibitory effect on the NF-κB-mediated inflammatory pathway, by inhibition of LPS-induced NF-κB phosphorylation and supressing TNF-α [66]. It was concluded that α-Ketoglutaric acid improves the intestinal immune system by modulating the interaction between PXR and NF-κB and thus have important implications for the prevention and treatment of intestinal inflammatory diseases in neonates. It was suggested that individuals with high protein intake, bacterial infections, or gastrointestinal dysbiosis may benefit from supplemental α-Ketoglutaric acid to improve metabolic health [107]. The antibacterial properties of α-ketoglutaric acid against *Staphylococcus aureus* and *Pseudomonas aeruginosa* was reported [107]. An elevated concentration of free radicals and resultant oxidative damage such as lipid peroxidation and protein carbonylation have been repeatedly demonstrated in neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, and ischemic stroke [34]. This suggests that α-Ketoglutaric acid, being a natural metabolic intermediate and energy substrate, does exert antioxidant effects in the brain and other tissues. Their study was designed to identify which Krebs cycle intermediates are effective neuroprotective compounds against oxidative stress in neuronal cells. Krebs cycle intermediates activate specific signalling transduction pathways and exert various biological actions such as neuroprotection, anti-inflammation, osteogenesis and anti-aging [34].

3.23 2,3-Dihydroxysuccinic acid or tartaric acid

Tartaric acid is often used in cosmetic formulations and to treat inflammatory skin conditions such as acne [150]. Investigating the anti-inflammatory and antinociceptive effects of total alkaloids extracted from the leaves of *Tamarindus indica*, known for containing several phenolic compounds and other compounds like tartaric acid, mucilage, pectin, uronic acid and triterpenes, it was found that crude and alkaloidal extracts of the plant exhibited significant anti-inflammatory and antinociceptive activities, thus, supporting its folkloric use for the treatment of these conditions [68]. The antibacterial efficacy of 2,3-dihydroxysuccinic acid was demonstrated against *Escherichia coli* [78]. Other studies reported the *in vitro* growth inhibition of some important pathogenic fungi such as *Candida albicans, Aspergillus fumigatus* and *Malassezia furfur* and reported that the combination of citric acid and tartaric acid had fungistatic and fungicidal activities [145].

3.24 Acetyl salicylic acid / aspirin

Aspirin is a unique nonsteroidal anti-inflammatory drug, known for its inhibition of cyclooxygenase and pro-inflammatory signaling pathways including NF-κB [109]. A study [7] demonstrated that aspirin possesses potent antibacterial activity against *Pseudomonas aeruginosa* and *Escherichia coli* as well as *in vitro* fungicidal anti-biofilm activity and could be useful in combined therapy with conventional antifungal agents in the management of some biofilm-associated *Candida* infections. Pathogenic fungi in the genus *Candida* can cause both superficial and serious systemic diseases and are widely recognised as important agents of hospital-acquired infection [7]. Biofilms of *Candida albicans* normally consist of matrix-enclosed micro-colonies of yeasts and hyphae and are resistant to a range of antifungal agent currently in clinical use, including amphotericin B and fluconazole. Aspirin demonstrated broad-spectrum antimicrobial activity for both planktonic and biofilm cultures as it was successful in eradicating *Pseudomonas aeruginosa, Escherichia coli* and *Candida albicans* biofilms established on abiotic surfaces [7]. Aspirin derivatives have also shown antibacterial activities against *Bacillus subtilis* and *Staphylococcus aureus* as well as antitumor and anticancer properties [24,155].

3.25 4-*Hydroxy-1,2-benzenedicarboxylic acid*

The antimicrobial properties of 4-hydroxy-1,2-benzenedicarboxylic acid is demonstrated by isolating 4-hydroxybenzoic acid from rice hulls and showed a 50 % growth inhibition (IC50) against various micro-organisms [26]. Most of the Gram-positive and some Gram-negative bacteria were sensitive to 4-hydroxybenzoic acid confirming its microbicidal efficacy [26]. Novel hydroxybenzoic acid compounds including 4-hydroxybenzoic acid ester, 2,4-dihydroxybenzoic acid ester, 3,4- dihydroxybenzoic acid ester, 2,3,4- trihydroxybenzoic acid ester, 3,4,5-trihydroxybenzoic acid ester and 3,4,6- trihydroxybenzoic acid ester have been synthesised to study the *in vitro* antiviral properties of hydroxybenzoic acid esters and analogues as well as the antiviral properties of hydroxybenzoic acid and analogues. The data indicated that the antiviral effects for hydroxybenzoic acid esters are more effective as demonstrated by the antiviral activity of a hydroxybenzoic acid ester (propyl gallate) being higher than its corresponding acid (gallic acid) [163]. Antioxidant properties of 4-hydroxy-1,2-benzenedicarboxylic acid was demonstrated by investigating the chemical composition and biological activity of the volatile oils from the flowers of three buckwheat species, *Fagopyrum esculentum*, *Fagopyrum tataricum* and *Fagopyrum cymosum* [192]. GC-MS was used to identify 4-hydroxy-1,2-benzenedicarboxylic acid as well as nonanoic acid (7.58 %), (E)-3-hexen-1-ol (6.52 %) and benzothiazole (5.08 %) among the 28 components, suggesting that the volatile oils of buckwheat flowers could be a potential source of natural antimicrobial and antioxidant agents [192]. The antioxidant efficacy of benzoic acid derivatives against the superoxide radical was also reported and tested the antioxidant properties of 14 different benzoic acid derivatives against the superoxide radical [171]. It was stated that the main source of adenosine triphosphate (ATP) in the mammalian cell is the mitochondrial electron transport chain. Although the electron transport chain is essential for life, during energy transduction a small number of electrons leak to oxygen prematurely forming the oxygen free

radical, superoxide, which has been implicated in the pathophysiology of various diseases. Phenolic compounds well known as antioxidants against the superoxide radical and some of their derivatives such as 4-hydroxy-1,2-benzenedicarboxylic acid are very efficient in preventing auto-oxidation [171]. It was proved that the position of the –OH group relative to the carboxylate groups determined the antioxidant efficiency for 4-hydroxy-1,2-benzenedicarboxylic acid [171]. The compounds with the hydroxyl group in the ortho and para position to the carboxylate group showed the best antioxidant properties and derivatives with blocked –OH groups showed very low antioxidant properties in comparison with derivatives with blocked –COOH groups, confirming that the structure and position of a hydroxyl group is very important for antioxidant efficacy.

3.26 Butanoic acid / butyric acid

The antimicrobial activities of butyric acid and its derivates against *Salmonella typhimurium* and *Clostridium perfringens* were studied [112]. The results from the work done [112] showed that butyric acid and derivatives could be used to control *Salmonella thyphimurium* or *Clostridium perfringens* in poultry species. Butyric acid exerts a variety of effects on the intestinal function; it is the preferred source of energy for colonocytes [164]. Butyric acid affects cellular proliferation, differentiation and apoptosis. The anti-inflammatory effects have been well documented. The inhibition of histone deacetylase activity, resulting in hyperacetylation of histones and as a consequence suppression of NF-κB activation, is a likely explanation. It has been proposed that butyric acid reinforces the colonic defence barrier by increasing production of mucins and antimicrobial peptides. It has been shown that butyric acid decreases intestinal epithelial permeability by increasing the expression of tight junction proteins. Anti-inflammatory activities, combined with a strengthening of the mucosal barrier integrity, are ideal properties for therapeutic compounds against inflammatory bowel disease-like syndromes [164]. It was also reported [191] that butyric acid is an important regulator of colonocyte proliferation and apoptosis, gastrointestinal tract motility and bacterial microflora composition in addition to an involvement in many other processes such as immunoregulation and anti-inflammatory activity. It was indicated that butyric acid, acetic acid and propionic acid accounts for approximately 83 % of the short-chain fatty acids in the human colon and reported that the presence of butyric acid suppresses the growth of *Escherichia coli*, *Campylobacter spp*., *Salmonella spp*. and *Shigella spp*. Butyric acid may also play an important role in the treatment of gastrointestinal infections [191]. It was referred to the supplementation of butyric, acetic and propionic acids that reduced congestion, infiltration of inflammatory cells and necrotising features in the mucosa [191]. Butyric acid has also been shown to exert potent anti-inflammatory effects both *in vitro* and *in vivo* [191]. Its immunoregulatory and anti-inflammatory activities are presumably based on the topical inhibition of inflammatory mediators in the epithelium. Butyric acid can decrease concentrations of pro-inflammatory cytokines such as interleukin 8 (IL-8) and tumour necrosis factor-α (TNF-α) [191]. Observations were made on the anti-inflammatory effects of butyric acid in the treatment of ulcerative colitis and radiation proctitis. Reports [174] that the short-chain fatty acids, acetic, propionic and butyric acids play an important role as fuel for intestinal epithelial cells and modulator of different processes in the gastrointestinal tract such as electrolyte and water absorption. These short-chain fatty acids act on leukocytes and endothelial cells through at least two mechanisms: activation of G protein coupled receptors, the inhibition of histone deacetylase and the regulation of several leukocyte functions, including production of cytokines [174]. The short-chain fatty acids, acetic, propionic and butyric acids also influence the ability of leukocytes to migrate to the foci of inflammation and destroy microbial pathogens.

4. Discussion

CHD-FA has anti-inflammatory, antibacterial, antifungal, antiviral and antioxidant properties. The present study provided evidence, based on a comprehensive literature review of the pharmaceutical properties identified for the backbone structures embedded in the supramolecular structure of CHD-FA as reported in relevant scientific publications on the organic acid structures. This research provides

evidence for understanding pharmacological properties related to the complex nature of CHD-FA. The pharmacological activity of the individual components and the synergistic effects of the molecular mechanisms involved in determining the efficacy of these components identified in scientific publications are summarised in Table 5-2.

Table 5-2. Summary of the biological properties of the CHD-FA backbone structures.

Backbone structures of CHD-FA	Pharmacological properties
2-Propenoic acid or Acrylic acid	Anti-inflammatory; antibacterial; antifungal; antiviral; antioxidant
Pyruvic acid	Anti-inflammatory; antibacterial; antioxidant
6-Hydroxy-2*H*-pyran-3(6*H*)-one	Antibacterial; antifungal;
Maleic Acid	Anti-inflammatory; antibacterial; antifungal; antiviral; antioxidant
Levulinic acid	Anti-inflammatory; antibacterial; antifungal; antiviral; antioxidant
Succinic acid	Anti-inflammatory; antibacterial; antifungal; antiviral; antioxidant
Itaconic Acid	Anti-inflammatory; antibacterial; antifungal; antiviral;
Glutaric Acid	Antibacterial; antifungal; antiviral;
Malic Acid	Anti-inflammatory; antibacterial; antifungal; antiviral; antioxidant
Butanoic acid or butyric acid	Anti-inflammatory; antibacterial;
4-Hydroxybenzoic acid or Salicyclic acid	Anti-inflammatory; antibacterial; antifungal; antiviral; antioxidant
α-Ketoglutaric acid	Anti-inflammatory; antibacterial; antioxidant
2-Hydroxyglutaric acid	Antibacterial; antifungal; antiviral;
2,3-Dihydroxysuccinic acid or Tartaric acid	Anti-inflammatory; antibacterial;
Phthalic acid	Antibacterial; antifungal;
2,3,4,5-Tetrahydroxypentanoic acid	Antibacterial; antifungal;
2-Acetoxybenzenecarboxylic acid or Acetylsalicylic acid / Aspirin	Anti-inflammatory; antibacterial; antifungal;
4-Hydroxy-1,2-benzenedicarboxylic acid	Antibacterial; antiviral; antioxidant
d-Glucuronic acid	Anti-inflammatory; antibacterial; antifungal; antiviral; antioxidant
d-Gluconic acid	Antibacterial; antifungal; antioxidant
Benzenetricarboxylic acid	Anti-inflammatory; antibacterial; antifungal; antiviral; antioxidant
Benzenetetracarboxylic acid	Anti-inflammatory; antibacterial; antifungal; antiviral; antioxidant
Bis(dihydroxymethyl) terephthalic acid	Anti-inflammatory; antibacterial; antifungal; antiviral; antioxidant

5. Conclusion

The anti-inflammatory, antibacterial, antifungal, antiviral and antioxidant activities of the backbone structures embedded in CHD-FA reported in relevant scientific publications has provided evidence for the effective and safe use of CHD-FA in pharmaceutical chemistry. The synergistic effect of the components in CHD-FA, characteristic of the unified mechanism of a supramolecular structure, provided evidence to confirm the anti-inflammatory, antibacterial, antifungal, antiviral and antioxidant properties of CHD-FA. Although this study has only provided a literature review on the main peaks identified as backbone structures, all the components are associated with one or more biological properties.

This information provides a basic understanding of the pharmacological properties embedded in CHD-FA and forms a platform for further investigations into the pharmacological properties of CHD-FA.

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CHAPTER 6: CONCLUSIONS AND RECOMMENDATIONS

Chapter 6 is a summary of the research results and the key findings presented in this study, and will be briefly discussed. This study has advanced the current knowledge on fulvic acid and provides suggestions and/or recommendations for future research.

6.1 INTRODUCTION

Natural medicines are biochemically diverse with unique and variable drug-like properties offering incomparable advantages over synthetic medicine for new developments in physicochemical, biochemical, pharmacokinetic research.

A new invention, CHD-FA, is synthetically produced from a carbohydrate source, sucrose. It is a major breakthrough in the production of a sustainable heavy metal free fulvic with batch-to-batch consistency.

The present study has investigated the transformation process of sucrose into fulvic acid during a controlled non-catalytic wet oxidation process and developed a theoretical model for the mechanism by which CHD-FA is formed from sucrose. The characteristic properties of CHD-FA were compared to that of environmental fulvic acids and through various spectroscopic techniques, this study has successfully identified the backbone structures within the complex supramolecular structure of CHD-FA. The clinical properties of these backbone structures embedded in CHD-FA were reviewed to provide insight into the anti-inflammatory, antibacterial, antifungal, antiviral and antioxidant properties of CHD-FA.

6.2 SUMMARY OF RESULTS

Evidence is presented for:

- A detailed description of the theoretical pathway for the synthesis of fulvic acid, based on literature, from a carbohydrate source. The detailed step-by-step analysis of the degradation process of sucrose in the presence of oxygen during Phase 1 of the controlled exothermic reaction at high temperatures, high pressure and controlled sucrose flow rates has confirmed the degradation of sucrose into fructose and glucose with a corresponding reduction in pH. This correlated with a change in the chemical composition of the sucrose molecule changing from carbohydrate to carboxylic acids.
- Colour changes in the reactor from a light yellow to a dark brown colour demonstrated the chemical transformation of sucrose in the reactor to a mixture of compounds.

- NMR and LC-MSMS confirmed the chemical transformation of sucrose in the reactor to CHD-FA.
- No convincing NMR data is presented to confirm the presence of penicillin-derivated fulvic acid or molecular fulvic acid. The NMR data presented are rather vague and general and could be representative of numerous organic compounds.
- GC-MS confirmed the presence of CHD-FA and the existence of an ordered structure within the supramolecular structure of CHD-FA.
- The LC-MSMS spectrum revealed that the most prominent component of CHD-FA is a molecular species with a molecular weight of 290.227 g/mol. This species was identified as 7,8-dihydroxy-3-methyl-10-oxo-1*H*,10*H*-pyrano[4,3-b]chromene-9-carboxylic acid (anhydrofulvic acid) using the National Institute of standards and Technology spectral search program and was predicted with a match probability of 92.5 %. This compound is the dehydrated analogue of penicillin-derivated fulvic acid ($C_{14}H_{12}O_8$) and possibly derived from penicillin-derivated fulvic acid during sample preparation or analysis. The 24 main backbone structures embedded in CHD-FA was further identified with LC-MSMS.
- The major constituent in CHD-FA has a natural abundance of 20.8% and was identified with MALDI-TOF MS. It had a molecular weight-exact mass of 308 g/mol. This confirmed that penicillin-derivated fulvic acid is the most prominent component in the CHD-FA structure ($C_{14}H_{12}O_8$).
- The penicillin-derivated fulvic acid was used as reference standard with an empirical formula of $C_{14}H_{12}O_8$ and a molecular weight of 308.240 g/mol to confirm that fulvic acid with similar structure as penicillin-derived fulvic acid is present in CHD-FA.
- CHD-FA is a supramolecular structure consisting of relatively small heterogeneous molecules (masses ~500 Da). The molecules are held together through hydrogen bonding and other weaker intermolecular forces such as Van der Waals forces.
- Twenty-four principal backbone structures which include malic acid, maleic acid, levulinic acid, succinic acid, propenoic acid, phthalic acid, arabonic acid, itaconic acid, glucuronic acid, glutaric acid, benzene tri- and tetracarboxylic acids were identified in CHD-FA.
- Batch-to-batch manufacturing consistency was demonstrated chromatographic and spectroscopic data of CHD-FA for a number of years.

6.3 NOVEL FINDINGS AND CONCLUSIONS

CHD-FA derived synthetically from sucrose through a non-catalytic wet oxidation process is a pure form of fulvic acid. It has a supramolecular nature. CHD-FA is characterised by a main constituent/parent fulvic acid structure with a molecular mass of 308 g/mol, several carboxylic acids and a number of unidentified structures.

CHD-FA have twenty-four principal backbone structures embedded in it's supramolecular structure, including malic acid, maleic acid, levulinic acid, succinic acid, propenoic acid, phthalic acid, arabonic acid, itaconic acid, glucuronic acid, glutaric acid, benzene tri- and tetracarboxylic acids, each structure known to have one or more biological properties. The composition of the backbone structures is responsible for the anti-inflammatory, antimicrobial and antioxidant properties of CHD-FA.

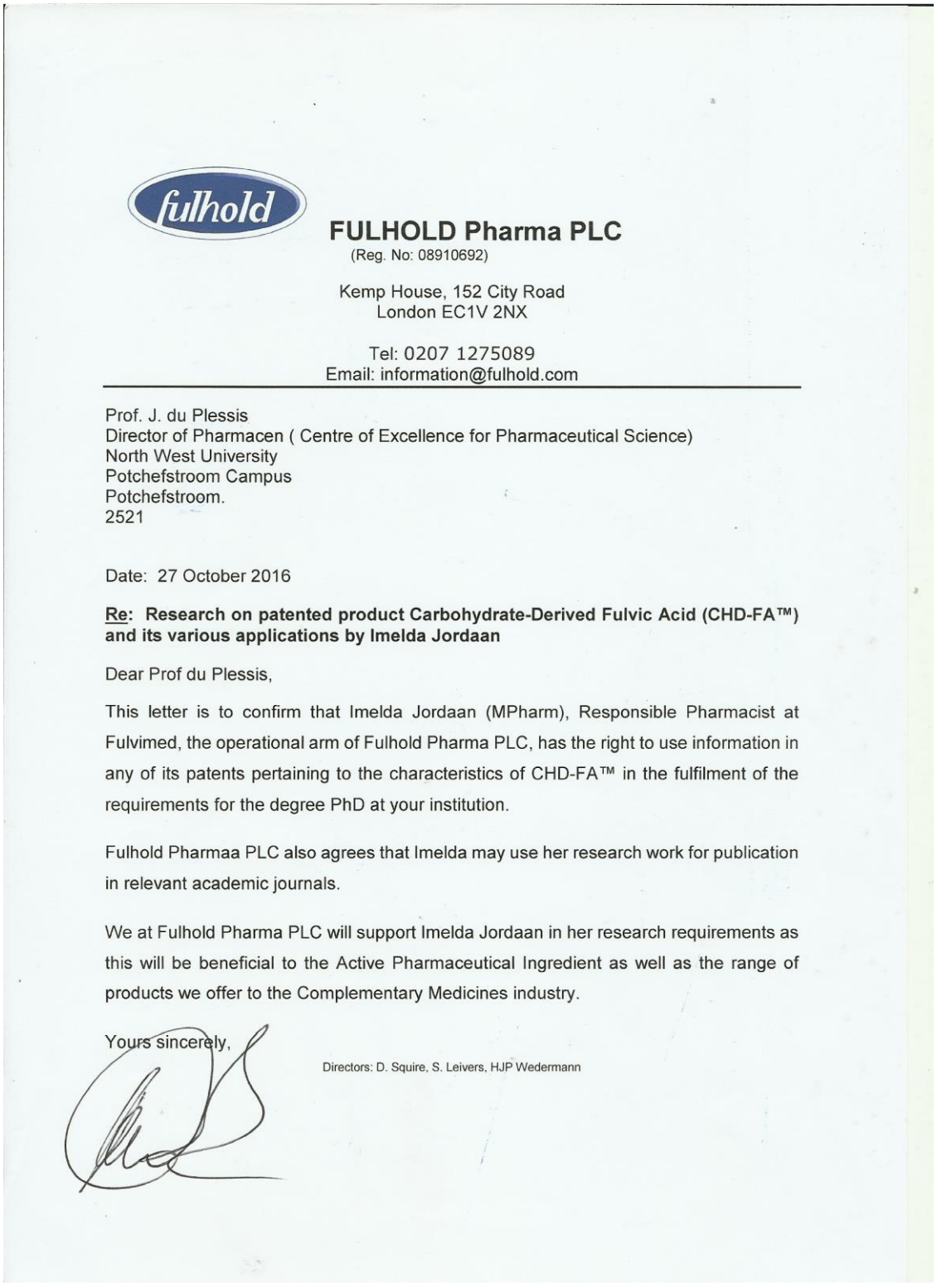
The batch-to-batch consistency for the manufacturing of CHD-FA demonstrated by this study, provide sufficient data for the exploration of the potential use of CHD-FA as a natural pharmaceutical compound in the development of natural medicines.

6.4 RECOMMENDATIONS FOR FUTURE RESEARCH

Comprehensive clinical research trials are needed to assess the efficacy of natural medicines formulated with CHD-FA as the active pharmaceutical ingredient. These studies should aim to utilize the biological properties of CHD-FA to assess its nutritional and health benefits for application in human and veterinary medicines.

ANNEXURE A

Approval received from Fulhold Pharma to perform research on patented product CHD-FA™ and its various applications.



FULHOLD Pharma PLC
(Reg. No: 08910692)

Kemp House, 152 City Road
London EC1V 2NX

Tel: 0207 1275089
Email: information@fulhold.com

Prof. J. du Plessis
Director of Pharmacen (Centre of Excellence for Pharmaceutical Science)
North West University
Potchefstroom Campus
Potchefstroom.
2521

Date: 27 October 2016

Re: Research on patented product Carbohydrate-Derived Fulvic Acid (CHD-FA™) and its various applications by Imelda Jordaan

Dear Prof du Plessis,

This letter is to confirm that Imelda Jordaan (MPharm), Responsible Pharmacist at Fulvimed, the operational arm of Fulhold Pharma PLC, has the right to use information in any of its patents pertaining to the characteristics of CHD-FA™ in the fulfilment of the requirements for the degree PhD at your institution.

Fulhold Pharmaa PLC also agrees that Imelda may use her research work for publication in relevant academic journals.

We at Fulhold Pharma PLC will support Imelda Jordaan in her research requirements as this will be beneficial to the Active Pharmaceutical Ingredient as well as the range of products we offer to the Complementary Medicines industry.

Yours sincerely,

Directors: D. Squire, S. Leivers, HJP Wedermann

ANNEXURE B

Approval received from Prof David S. Perlin, the author for correspondence author to artcle as reference in chapter 2: Zhao, Y., Paderu, P., Delmas, G., Dolgiv, E., Lee, M.H., Senter, M., Park, S., Leivers, S. & Perlin, D.S. 2015. Carbohydate-derived fulvic acid is a highly promising topical agent to enhance healing of wounds infected with drug-resistant pathogens. *Journal Trauma Acute Care Surgery* 79 (4):S121 – S129

Imelda Jordaan

From: David Perlin <perlinds@njms.rutgers.edu>
Sent: 07 November 2018 06:08 PM
To: Imelda Jordaan
Cc: Stefan
Subject: RE: Article published: CHD-FA as promising topical agent

Imelda,

You have my permission. Please feel free to use any and all figures.

David

David S. Perlin, Ph.D.
Professor
Public Health Research Institute
New Jersey Medical School-Rutgers
225 Warren Street, Newark, NJ 07103 USA
973 854-3200 (voice); 973 854-3101 (fax); Email: perlinds@njms.rutgers.edu;
www.phri.org

From: Imelda Jordaan [mailto:imelda@fulvimed.co.za]
Sent: Wednesday, November 07, 2018 5:42 AM
To: David Perlin <perlinds@njms.rutgers.edu>
Cc: Stefan <stefan@fulvimed.co.za>
Subject: Article published: CHD-FA as promising topical agent

Dear David,

Trust that you are doing very well.
I am currently busy with my PHD at the University of Northwest (South-Africa) on the molecular structure of CHD-FA and the literature review section I would like to use your pictures as published in the attached article, however I will need to written approval to do so.

Therefore I would like to request if I may use the pictures with full referencing to your and your team's work done. If you do approve me using it, please can you give me the approval in writing as I need to attached it to my thesis.

Looking forward to hear from you soonest, much appreciated.

Kindest regards

Imelda Jordaan
Responsible Pharmacist (B. Pharm NWU; M.Pharm UP)
Fulvimed SA (PTY) Ltd
Stellenbosch
Tel: 021-881-3600
Cel:0826587222

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