KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY

COLLEGE OF HEALTH SCIENCES

FACULTY OF PHARMACY AND PHARMACEUTICAL SCIENCES

DEPARTMENT OF PHARMACOGNOSY

ANTI-INFLAMMATORY AND ANTIOXIDANT ACTIVITIES OF THE FRUITS OF VITEX DONIANA (HORT. BRIT.) AND THE QUANTIFICATION OF ONE OF ITS BIOACTIVE CONSTITUENTS OLEANOLIC ACID

BY

ADJEI, SILAS

APRIL, 2019

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MASTER OF PHILOSOPHY IN PHARMACOGNOSY

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DECLARATION

I hereby declare that this thesis is my own work towards the MPhil. Pharmacognosy degree and has not been presented for any degree in any university and that, to the best of my knowledge, I have wholly undertaken this study reported therein except where due acknowledgement has been made in the thesis.

…….. ……..

Silas Adjei Date
(PG 9590317)

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…….. ……..

Dr. Isaac Kingsley Amponsah Date
(Supervisor)

…….. ……..

Professor Abraham Yeboah Mensah Date
(Head of Department)

DEDICATION

This work is dedicated to the Almighty God and to my entire family for their prayers, support and encouragement throughout the period of the study.

ACKNOWLEDGEMENT

I would like to express my profound gratitude to the Almighty God for His grace and favour throughout my study.

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ABSTRACT

Inflammation is associated with virtually all diseases with oxidative stress being implicated in its complications. Nature has offered a number of clinically useful antiinflammatory agents and also affords a plethora of antioxidants. *Vitex doniana* fruit, a delicacy of some tribal groups in Northern Ghana, has not been investigated for its beneficial effect although it is used ethno medically in the treatment of various inflammatory conditions. Therefore the present studies aimed at evaluating the fruits for its antioxidant and anti-inflammatory activities and constituents as well as its toxicity. The dried pulverised fruit was extracted with methanol and subsequently partitioned to obtain pet-ether, ethyl acetate and methanol soluble fractions. The methanol fruit extract and subsequently its petroleum ether, ethyl acetate and remaining methanol soluble fractions were evaluated for anti-inflammatory and antioxidant activities using the chick carrageenan anti-inflammatory assay and three antioxidant assays; DPPH radical scavenging, ferric reducing power and total antioxidant capacity. In the antiinflammatory assay using 7-day old chicks, the extracts and fractions were dosed at 30, 100 and 300 mg/kg body with dexamethasone (0.1, 0.3, 1.0 mg/kg) and diclofenac (10, 30, 100 mg/kg) used as reference drugs. The foot volumes were measured using a digital caliper for 5 hours. The acute and subacute toxicity studies of the crude extract dosed at (100, 300 and 3000 mg/kg) and (100, 300 and 1000 mg/kg) respectively were also evaluated. All extracts demonstrated dose-dependent anti-inflammatory activity with the methanol soluble fraction exhibiting the highest activity (ED_{50} = 16.52 ± 4.65 mg/kg) followed by ethyl acetate (ED_{50} = 19.17 ± 2.89mg/kg) and pet ether respectively. The ED_{50} values for dexamethasone and diclofenac were 7.19 ± 4.91 and 7.55 ± 1.09 mg/kg respectively. The ethyl acetate fraction showed the highest antioxidant activity with IC_{50} value of 99.35 ±

0.77 µg/mL for the DPPH radical scavenging assay and a gallic acid equivalence (GAE) of 58.22 ± 3.66 mg/g in the total antioxidant capacity assay. LD_{50} by oral route was estimated to be beyond 3000 mg/kg. No signs of autonomic and CNS stimulation/depression were recorded. Subacute studies revealed an increase in haematological parameters (RBCs, HB PCV) as well as lymphocyte counts. Liver enzymes (ALT, AST, ALP and GGT), serum proteins and bilirubin levels were not increased. Chromatographic investigation of the active ethyl acetate fraction yielded compounds VDF-1, 2 and 3 of which the structure of the former was elucidated as 3βhydroxyolean-12-en-28-oic acid (oleanolic acid). Oleanolic acid and the other compounds showed significant anti-inflammatory and antioxidant activities and contributed to the respective activities displayed by the crude extract. The content of oleanolic acid in the crude extract was determined to be 84.15 mg/g using a validated HPLC method. The results obtained indicate that the methanol fruit extract of *V. doniana* possesses considerable anti-inflammatory, antioxidant and possible immunomodulatory activities and that high performance liquid chromatography (HPLC) remains an essential tool in the quality control of natural products.

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ABBREVIATIONS

5-HPETE	5-hydroperoxyeicosatetraenoic acid
ANOVA	Analysis of variance
APC	Antigen presenting cell
CCL_4	Carbon tetrachloride
CD	Cluster of differentiation
$CHCl_3$	Chloroform
CNS	Central nervous system
COSY	Correlational spectrosscopy
COX	Cyclooxygenase
DEPT	Distortionless enhancement by polarisation transfer
DPPH	2, 2-diphenyl-1-picryhydrazyl
EtoAc	Ethylacetate
FT-IR	Fourier transformed infra- red
GAE	Gallic acid equivalence
GC-MS	Gas chromatography mass spectrometry
GM-CSF	Granulocyte macrophage colony-stem factor
HIV	Human-immunodeficiency virus
HMBC	Heteronuclear multiple bond correlation

HPLC	High performance liquid chromatography
HSQC	Heteronuclear single quantum correlation
ICH	International Conference on Harmonisation
IFN	Interferon
IL	Interleukin
LD	Lethal dose
LOD	Limit of detection
LOQ	Limit of quantification
LT	Leukotriene
MeOH	Methanol
MHC	Major histocompatibility complex
NMR	Nuclear magnetic resonance
NO	Nitric oxide
NSAIDS	Non-steroidal anti-inflammatory drugs
PAF	Platelet activating factor
PDA	Photodiode array
PFC	Plaque forming colony
PG	Prostaglandin
RSD	Relative standard deviation

SAID	Steroidal anti-inflammatory drugs
TNF	Tumour necrosis factor
TPA	12-o-tetracanoil phorbol-13-acetate
UV	Ultra violet
WHO	World Health Organisation

CHAPTER ONE

INTRODUCTION

1.1 GENERAL INTRODUCTION

Throughout the ages, individuals have relied upon nature for their basic necessities such as food, medicine, shelter, clothing and fragrances. The awareness of the significant contributions that natural products from plants, minerals and animals make to the diet and health of mankind cannot be over emphasized. For the majority of the global population, plant-based products continue to play critical roles in the healthcare system and this is very true in developing countries where they have been used in providing primary healthcare (Dar *et al*., 2017; Yuan *et al*., 2016). As indicated by the World Health Organization (WHO), 80% of individuals still depend on plant-based conventional medicines for essential medicinal services (WHO, 2004).

Over the years, medicinal plants have constituted a formidable part of the development and discovery of therapeutic agents. They contain a large repository of molecules which have been used in the management and treatment of diseases. About 80% of 122 plantderived drugs were identified with their unique ethnopharmacological activities including anti-inflammatory, antimicrobial, antioxidant, anticancer, antidiabetic and antihypertensive and with as many as 60% of anti-tumour drugs in use, still obtained from natural sources (Dias *et al*., 2012; Palhares *et al*., 2015). For example, the blood pressure lowering agent, reserpine from *Rauwolfia sp*, the anticancer agents, vinblastine and vincristine from *Catharanthus roseus*, paclitacel from *Taxus sp*, the antimalarial agent, artemisinin from *Artemisia annua* and salicin, an anti-inflammatory agent from

Salix alba are few clinically useful drugs derived from natural products (Dar *et* al., 2017; Yuan *et* al., 2016). This was made possible through drug synthesis. The inception of drug synthesis resulted in the development of modern therapeutic agents which have curbed pathological conditions which plagued humanity for centuries with the consequent reduction in the overdependence of natural products (Yuan *et al*., 2016). That notwithstanding, these modern therapeutic agents present with numerous unwanted side effects. The pharmaceutical industry now face the challenge of producing new efficacious agents with minimal side effects and particular focus is now shifted to natural products including medicinal plants for novel therapeutic leads (Yuan *et al*., 2016; Valavanidis *et al*., 2014; Trosset and Carbonell, 2015). It is estimated that about one-tenth of the global plant population has been studied for their medicinal properties.

Hence, majority of them remain to be screened for novel therapeutic leads (Dias *et al*., 2012).

1.2 JUSTIFICATION OF RESEARCH

The body's defense system, the immune system, is made up of numerous reliant cell types that on the whole shields it from viral, fungal, parasitic, bacterial infections and from the development of tumour cells (Nagarathna *et al*., 2013; Singh and Yadav, 2014).

Modulation (stimulation or suppression) of the body's defense system to relieve it from pathological conditions has been of interest for some time now. Medicinal plants are endowed with diverse groups of secondary metabolites which non-specifically stimulate the function and efficiency of granulocytes, macrophages, lymphocytes and the complement system (para-immunity) as well as the production of effector molecules by activated cells (Sharififar *et al*., 2009; Nagarathna *et al*., 2013). Medicinal plants with established immunomodulatory activity include *Panax ginseng*, *Azadirachta indica* and *Tinospora cordifolia*. (Sharififar *et al*., 2009). Plant-derived natural products such as

steroids, flavonoids, terpenoids and glycosides have attracted extensive interest in recent years due to their diverse pharmacological activities including immunomodulation, antiinflammatory, antimicrobial, anti-cancer and antioxidant (Patil *et al*., 2010, Jose *et al*., 2014). Conventional immunomodulators such as methotrexate, cyclosporine, cyclophosphamide and tacrolimus have been used successfully in immune mediated conditions such as rheumatoid arthritis, idiopathic thrombocytopenic purpura and transplant rejection. These therapies, apart from the fact that are expensive, also presents with some undesirable effects including myelosuppression, systemic immunosuppression and nephrotoxicity (Dhama *et al*., 2015; Patil *et al*., 2012). In this regard, the search for new immunomodulators from medicinal plants can thus constitute a safer inexpensive option and also serve as alternative or adjunct to conventional therapy. Inflammatory mediators including prostaglandins, leukotrienes and cytokines released during inflammation play a critical role as part of the body's combative (immune) mechanism against noxious stimuli. However, if the release and actions of these mediators goes unabated, the result is chronic inflammation.

Inflammation is associated with virtually all kinds of diseases and its mitigation with medicinal plant extracts can be traced throughout ancient times (Dinnarello, 2010). The leading cause of various diseases, debilitations and mortality are inflammatory disorders including rheumatoid arthritis, glomerulonephritis, hepatitis and vasculitis. If, for any particular reason, acute inflammation fails to heal, chronic inflammation ensues. For some time now, chronic inflammation has been a major concern due to the burden of pathological conditions and co-morbidities associated with it (Ahmed, 2011). The mainstay drugs used in managing inflammatory disorders include steroidal

antiinflammatory drugs, the opiates and non-steroidal anti-inflammatory drugs. However, prolong use of these drugs come with a plethora of unwanted side effects such as immunosuppression, Cushing syndrome and hyperlipidaemia which are associated with the steroidal agents; gastric ulcerations, bleeding, renal dysfunction associated with the NSAIDS; and dependence, tolerance and withdrawal effects associated with the opiates.

The challenge therefore, is to search for and develop more effective and less toxic agents in the management of various inflammatory disorders. In this regard, attention is focused on medicinal plants as a source of new drugs owing to the fact that they boast of a large repository of chemically diverse molecules which can lead to the discovery and development of efficacious therapeutic agents to be used in clinical settings. Natural products with anti-inflammatory activities include salicin from *Salix alba*, gingerol from *Zingiber officinale*, colchicine from *Colchicum autumnale*, curcumin from *Curcuma longa*, capsaicin from *Capsicum annum* and allicin from *Allium sp*. (Azab *et al*., 2016; Nworu and Akah, 2015; Murugesan and Deviponnuswamy, 2014). These agents have been used alone or in combination as adjuncts with other medications in the treatment of inflammatory conditions. For this reason, medicinal plants can serve as practical option to regular orthodox medications in the management of inflammatory conditions. The production of free radicals, in excess, as part of the inflammatory cascade contributes to oxidative stress. Oxidative stress is implicated in the complications of inflammatory conditions.

Free radicals are chemical entities which are produced as a result of normal essential cellular metabolism. The production of these highly unstable and reactive chemicals if not controlled in the cell, reacts with membrane lipids, proteins, nucleic acids and enzymes resulting in cellular

damage (Langseth, 1995). The production and release of excess toxic oxygen radicals such as peroxide and superoxide ions by polymophonuclear leukocytes in oxidative burst during inflammation as part of phagocytosis helps in healing of tissue injury. These reactive oxygen species, if, they are not deactivated or controlled, may pile up and overwhelm the body's defense mechanism, aggravate the inflammatory process leading to chronic inflammation (Kumar, 2014, Vergnolle, 2003). Antioxidants, some of which occur in nature, are chemicals which are able to scavenge these destructive reactive oxygen species. The anti-inflammatory activities of some triterpenoids such as oleanolic acid, ursolic acid, betulinic acid and flavonoids such as apigenin, oroxylin A, quercetin, myricetin as a well as the sesquiterpene lactone, cumanin are known to be due to their antioxidant activities (Nworu and Akah, 2015; Azab *et* al., 2016). Hence the search for more natural antioxidants will go a long way in complementing the inflammatory process as well as modulating the body's immune system.

Vitex doniana is a plant commonly used in traditional settings for the treatment of a number of diseases. A decoction of the woody parts of the plant is used in the treatment of inflammatory disorders, stomach pains, diarrhoea, rheumatic pains and dysentery (Dauda *et* al., 2011; Ezekwesili *et* al., 2012). A decoction of the stem bark is administered in the treatment of gastroenteritis (Kilani, 2006). The root bark is administered for the management of backache in women and treatment of gonorrhoea (Orwa *et* al., 2009). The fruit is employed in treating jaundice, leprosy, anaemia, dysentery and fertility related problems (Mohammed *et al.*; 2016, Orwa *et al.*, 2009).

The plant is known for its fruit which serves as a delicacy for majority of people, especially in the Northern and Eastern part of Ghana. The use of the fruit is widespread, however, its biological activities have not been explored. This study therefore aimed at investigating the protective function of the fruit

including its anti-inflammatory and antioxidant activities as well as isolating and quantifying constituent(s) which might be responsible for these activities. Its safety profile will also be ascertained.

1.3 RESEARCH AIM

The aim of the research is to:

Evaluate the anti-inflammatory and antioxidant activities of the fruit extract of *Vitex doniana*, assess its toxicity in animal models and quantify the amount of isolate in the crude extract.

1.4 RESEARCH OBJECTIVES

The research objectives are to:

- investigate the anti-inflammatory activity of the fruit of *Vitex doniana* using the carrageenan induced paw oedema model in chicks.
- assess the total antioxidant activity of the fruit extract using total antioxidant capacity, ferric reducing power and DPPH radical scavenging assays . ➢ isolate bioactive constituents using various chromatographic techniques ➢ characterize isolated constituents using spectroscopic techniques.
- quantify the amount of isolated constituent in the crude extract using HPLC ➢ evaluate bioactive constituents for anti-inflammatory and antioxidant activities and
- evaluate the acute and subacute toxicity of the fruit extract .

CHAPTER TWO

LITERATURE REVIEW

2.1 THE FAMILY LAMIACEAE

Lamiaceae is an important plant family comprising of about 236 genera and in excess of 6000 species (Pio-Leon *et al*., 2014). The largest genera are Scutellaria, Stachys, Salvia Plecthranthus, Hypetis, Vitex, Teucreum, Thymes and Nepeta. Lamiaceae is comprised of nine subfamilies namely Symphorematoideae, Viticoideae, Ajugoideae, Prostantheroideae, Nepetoideae, Scutellarioideae, Callicarpoideae and Tectonoideae (Carovic-Stanko *et al*., 2016; Li and Olmstead, 2017). It is a family of different variety with a wide geographical distribution. Species in this family are easily identified by their square stems and oppositely arranged leaves. Flowers are zygomorphic with five (5) united petals and five (5) joined sepals, typically bisexual and verticillaster (CarovicStanko *et al*., 2016). The majority of the species are sweet-smelling. Plants belonging to this family have extensive variety of phytoconstituents with potent antimicrobial, antioxidant, anti-inflammatory and antiviral properties (Carovic-Stanko *et al*., 2016; PioLeon *et al*., 2014).

2.2 THE GENUS VITEX

The genus Vitex was described by Linnaeus in 1753 and is distinguished within subfamily Viticoideae by its palmate leaves, terminal or axillary inflorescence and 1- 4 seeded fruit (Callmander *et al*., 2014). The genus has between 250-300 species of trees and bushes, mainly distributed in tropical and sub-tropical areas of the world with a few occurring in the temperate regions. *Vitex agnus-castus*, *Vitex negundo, Vitex doniana* and *Vitex cannabifolia* are few species in this genus (Emmanuel *et al*., 2015). The generic name "Vitex" is derived from the latin word 'vieo', meaning to weave, bind or to tie up, a

reference to the use of *Vitex agnus-castus* in basketry (Umberto, 2000). Historically, Vitex *spp* have been employed in a large number of ethnomedical application such as treatment of microbial infections, premenstrual and gynaecological conditions, gastrointestinal problems, inflammatory conditions as well as insect repellent and against stings of venomous animals (Carovic-Stanko *et al*., 2016).

2.3 VITEX DONIANA

Vitex doniana, populary known as African black plum is locally known in Ghana as 'Afetewa' or 'Abiswa' (Twi), 'Afua' (Fante) and 'Toyiti' (Ewe) (Mshana *et* al., 2000). It is also known among the Igbos and Yoruba (Nigeria) as 'ucha koro' and 'oori-nla' respectively, 'dinya' (Hausa), 'mfundu/mfuru' (Swahili), 'plem' (Ethiopia-Amharic), 'mufutu' (Zambia-Bemba), 'galbihi' (Benue-Congo-Fula) and 'munyamazi' (Uganda) (Orwa *et al*., 2009). Synonyms of the plant are; *Vitex umbrosa* G. Don ex Sabine, *Vitex cuneata* Thonn and Schumach, *Vitex cienkowskii* Kotschy and Peyr, *Vitex paludosa* Vatke, *Vitex dewevrei* De Wild & T. Durand, *Vitex pachyphylla* Baker in D. Oliver, *Vitex puberula* Baker in D. Oliver, *Vitex chariensis* A. Chev., *Vitex hombei* De wild and *Vitex hornei* Hemsl (World Checklist of Selected Plant Families).

2.3.1 Morphological description

Vitex doniana is a medium-sized deciduous tree, 8-18 m high, with a heavy rounded crown and a bole clear of branches up to 5m. The pale brown bark is rough with thin vertical fissures. The glabrous compound leaves are oppositely arranged, with 5 leaflets on stalk. The leaflets are ovate, obovate-elliptical with the margins being entire. The apex of the leaves is emarginate or rounded with a cuneate base. The upper surface of the leaves is dark green, leathery with stellate hairs and pale green below. The flower petals are white

with the exception of the largest lobe, which is purple, with dense opposite and axillary cyme. The greenish immature drupe-like fruit is oblong which turns purple or purplish-black on ripening with a starchy black pulp. Each fruit contains 1 hard, conical seed (Orwa *et al.*, 2009).

Figure 2.1*Vitex doniana* plant parts.
A: Whole plant, B: leaves, C: flowers, D. Unripe fruit and E: Ripe fruits

2.3.2 Geographical and ecological distribution

The plant occurs mostly in savannah regions, including Ghana. It is deciduous forest tree of coastal woodland, riverine and swampy forests, reaching as high as upland grassland and requires high water table (Orwa *et al*., 2009).

2.3.3 Ethnomedicinal uses

Various parts of *Vitex doniana*, including the leaves, stems, roots and fruits are used in traditional medicine for the treatment of diseases (Dauda *et* al., 2011). The fruit is employed in treating jaundice, leprosy, anaemia, dysentery and fertility related problems (Mohammed *et al*., 2016, Orwa *et al*., 2009). Again, the fruit is commonly eaten by the people of Northern Ghana and it's believed to stimulate immune function. An infusion of the leaves is used in the treatment of stomach and rheumatic pains, inflammatory disorders, diarrhoea and dysentery (Dauda *et al*., 2011; Ezekwesili *et al*., 2012). The expressed juice from the young immature leaves is used in ocular disorders (Orwa *et al*., 2009). A decoction of the dried leaves and stem bark are used in the management of dizziness (Pio-Leon *et al*., 2014). A decoction of the stem bark is administered in the treatment of gastroenteritis and to improve fertility (James *et al*., 2014; Kilani, 2006). The stem bark is also employed in the management of epilepsy and leprosy (Pio-Leon *et al*., 2014). The root bark is administered for the management of backache in women and treatment of gonorrhoea (Orwa *et al*., 2009). The roots and leaves are used for nausea, colic and epilepsy. The plant is used among the Kanuri's (Nigeria, Niger and Cameroun) in the management of mental illness (Sanni *et al*., 2005).

2.3.4 Biological and pharmacological activities

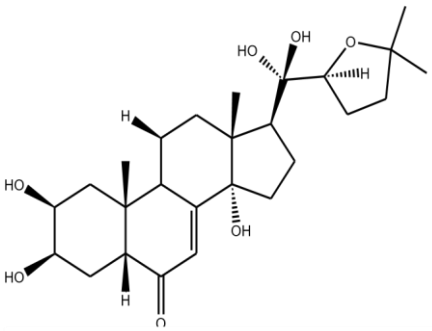
The ethanolic fruit extract demonstrated antidiarrheal action by a concentrationdependent inhibition of both histamine and acetylcholine-induced contractions. The extract, in the same study, repressed gastric peristalsis in mice fed charcoal meal and protected them against castor oil-induced diarrhoea to a large extent (Suleiman and Yusuf, 2008). The root extract exhibited antiviral activity with a concentration of 25µg/mL inhibiting the replication of HIV-1 *in vitro*. The extract also did not show any interference in activity with the standard antiretroviral drug when administered concomitantly (Tietjen *et* al., 2016). The fractions and isolates of a stem bark extract exhibited a dose-dependent anti-plasmodial activity against *Plasmodium falciparum* (Mudi, 2011). The antioxidative potential of the ethanolic extracts of the leaf and root have been reported (James *et al*., 2014). Similarly, the hepatoprotective effect of the aqueous root bark, leaves and stem bark extracts has been reported. All extracts significantly ($P < 0.05$) lowered liver enzyme biomarkers in rats which were initially increased by the administration of CCL_4 (James *et al*. 2014). Phytoecdysteroids isolated from the methanol stem bark (dosed at 100 mg/kg) significantly ($P \leq 0.05$) demonstrated anti-inflammatory activity in carrageenan-induced oedema in Sprague Dawley rats (Ochieng *et al*., 2013). The antidiabetic effect of the aqueous leaves extract in Wistar albino rats has been reported. The extract (50 mg/kg) exhibited significant ($P< 0.001$) decrease in blood sugar level from 492.8 to 84.5 mg/dL (Ezekwesili *et al*., 2012). Kilani (2006) reported the antibacterial activity of the methanol stem bark extract. The extract inhibited clinical strains of *Salmonella typhi*, *Shigella dysentariae* and *Escherichia coli.* Extracts of the fruit have shown reduction in reproductive functioning in female olive baboons (Papio hamadryas Anubis) with

progestogen-like compounds in the fruit been suggested as the notable cause of fertility reduction (Higham *et* al., 2007). Though the fruits are largely consumed locally, its biological activity is not widespread.

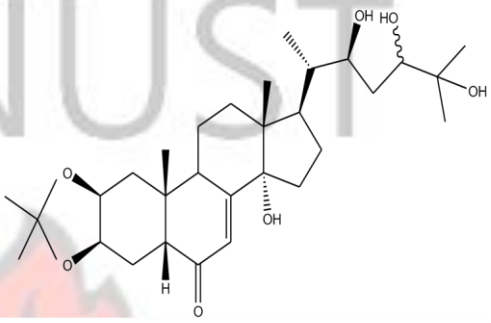
2.3.5 Phytochemistry of *Vitex doniana*

Several phytochemical assessments of *Vitex doniana* have resulted in the isolation of various constituents. From the leaves, three triterpenoids; 1α, 3β-dihydroxybauer-7-en28 oic acid [**1]**, 2β, 3β, 19α, 24-tetrahydroxy-23- norus- 12-en- 28-oic acid **[2]** and (3β, 5a, 7β)-3,7-dihydroxy-4, 4, 14-trimethyl-11, 15-dioxochol-8- en-24-oic acid [**3]** were isolated (Mohammed *et* al., 2016). The GC-MS analysis of the methanol leaf extract led to the characterization of 1-chloro-3-methyl-butane **[4]**, 1-tridecyne **[5]**, 14methylpentadecanoic acid **[6]**, hexadecanoic acid **[7],** 9,12-octadecadienoic acid **[8]**, 6octadecenoic acid **[9]**, octadecanoic acid **[10],** oleic acid **[11],** octylether [**12**] and 2,2,4trimethylpentylvinyl ether [**13**] (Nweke *et* al. , 2015). From the methanol stem bark extract, 20-hydroxyecdysone [**14**] was isolated (Tijjani *et* al., 2017). The methanol soluble fraction of an ethanol stem bark extract resulted in the isolation of a naphthoquinolinone derivative; 3- ethyl-3,4, 4a,5,6,6a,10a,11,12,12a-decahydro-1Hnaphto[2,3,-g]quinolin-2-one [**15]** (Mudi, 2011). Several phytoecdysteroids including, 11β-hydroxy-20-deoxyshidasterone [**16**], 21-hydroxyshidasterone [**17**], 2, 3-acetonide24-hydroxyecdysone [**18**], Ajugasterone [**19**], Shidasterone [**20**], 24-hydroxyecdysone [**21**] and 11β, 24-dihydroxyecdysone [**22**] have been isolated from the methanol stem bark extract of the plant (Ochieng *et* al. , 2013). The isolation of two flavonoid glycosides; 3-O-[α-L-rhamnopyranosyl-(1→6)–β-D-glucopyranosyl]-7-O-(β-Dglucopyranosyl) (Quercetin) [**23**] and 3-O- β-glucosyl- (1→2)-

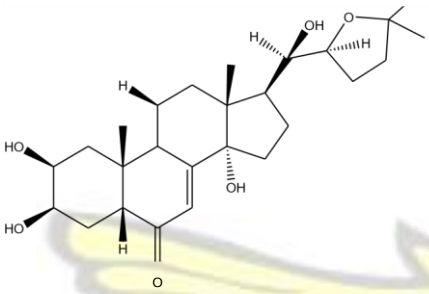
β-glucoside-7-0-βglucosyl- (1→4)-α- rhamnoside (Myricetin) [**24**] from the n-butanol fraction of n-hexane extract of the flowers has been reported by Mohammed *et al*. (2017).



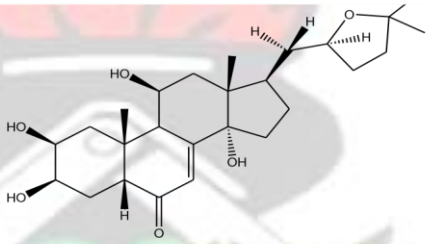
21-hydroxyshidastrone (17)



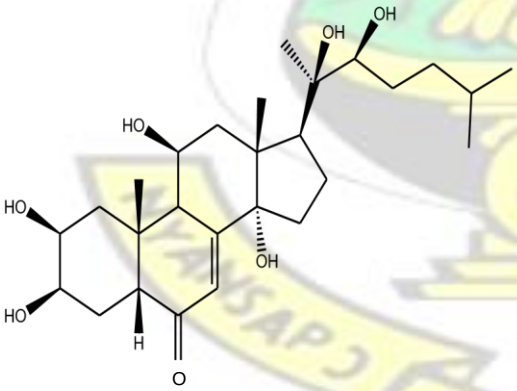
2, 3-acetonide-24-hydroxyecdysone (18)



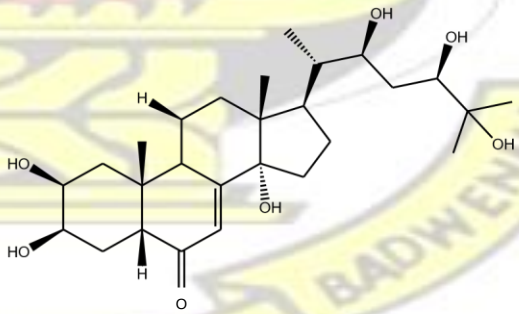
Shidasterone (20)



11-β-hydroxy-20-deoxyshidasterone (16)

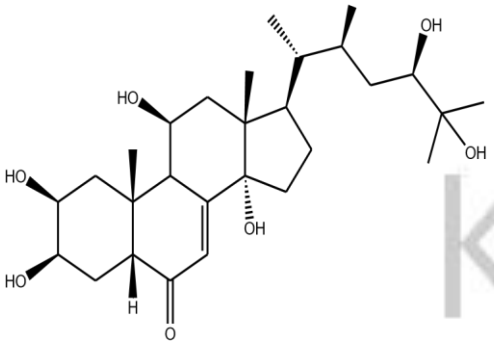


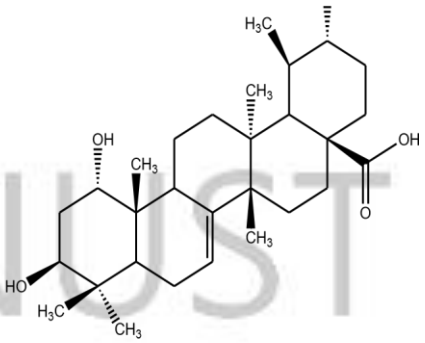
Ajugasterone (19)



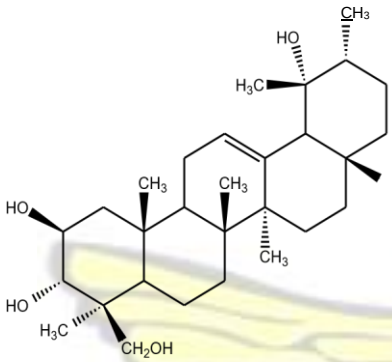
24-hydroxyecdysone (21)

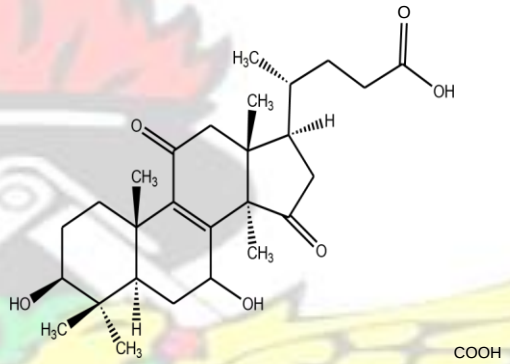
OH CH₃





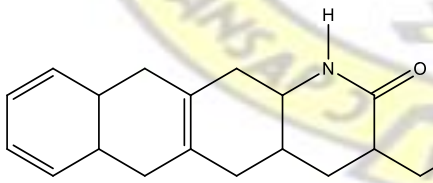
11β, 24-dihydroxyecdysone (22) **1α, 3β-dihydroxybauer-7-en-28-oic acid (1)**

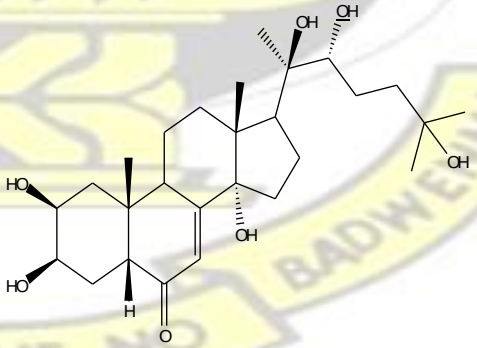




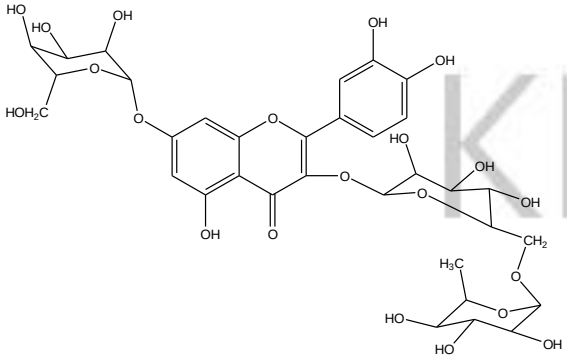
2β, 3β, 19α, 12-en-2824-oic acid-tetrahydroxy (2) **- 11(3β, 5α, , 15-dioxochol7β)-3, 7--dihydroxy8-en-24-oic acid-4, 4, 14 (3)-trimethyl -**

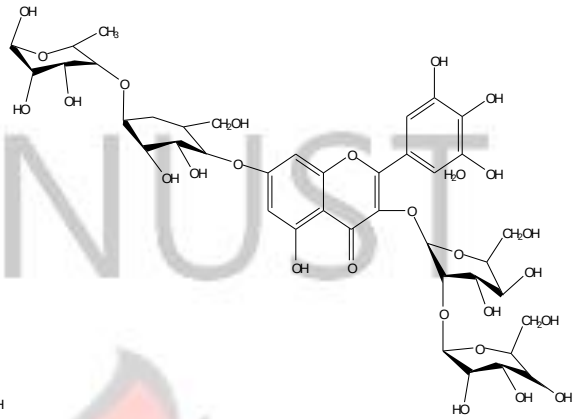
norus-





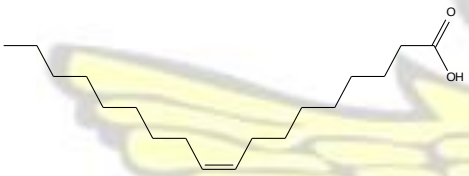
3-ethyl -3, 4, 4a, 5, 6, 6a, 10a, 11, 12, 12a- 2β, 3β, 14α, 20R, 22R, 25decahydro -1H-naphtho [2, 3-g] quinolin- hexahydroxy-cholest-7-ene-6-one (20 2-one (15) hydroxyecdystone) (14)





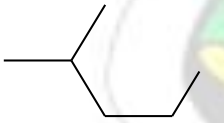
3-O-[α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranosyl]-7-glucopyranosyl)-Myricetin(24)

3-O-β-D-glucosyl(1→2)-β-glucoside-7-O-β-Dglucosyl-(1→4)-*α*-rhamnoside O-(β-D quercetin (23).



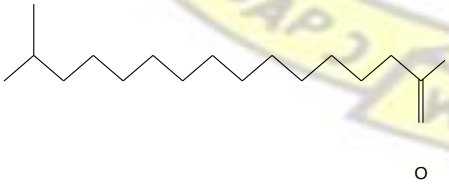
Oleic acid (11)

Octyl ether (12)

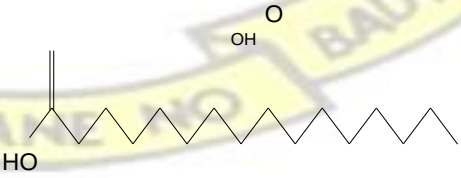


1- chloro-3-methyl-butane (4)

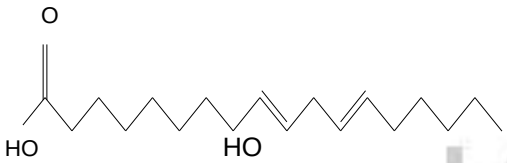
1-tridecyne (5)



14-methylpentadecanoic acid (6)



Hexadecanoic acid (7)

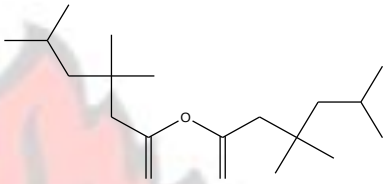


9, 12 octadecadienoic acid (8)

6-octadecenoic acid (9)

HO

Octadecanoic acid (10)



2, 2, 4-trimethylpentylvinyl ether (13)

Figure 2.2 Previously isolated chemical constituents from *Vitex doniana*

2.3.6 Non-medicinal Uses

A teak-like termite resistant timber produced by the tree is essential in making furniture, boats, carvings and light building materials. A dye obtained from the bark is utilised in the clothing industry. Wine, jams and marmalades are manufactured from the fruit. The wood also serves as charcoal and firewood (Orwa *et al*., 2009).

2.4 INFLAMMATION

The response of living tissue to cellular injury caused by various noxious stimuli including microbial infections (fungi and bacteria), viruses, parasites, trauma, sunburns and hypersensitive reactions constitute inflammation. The relevance of inflammation is to essentially reinstate normal tissue homeostasis (Ahmed, 2011; Vane and Botting, 2014). Progressive tissue damage and degradation with subsequent cell death occurs in the

absence of inflammation. On the other hand, artherosclerosis, rheumatoid arthritis, vasculitis and other conditions are the resultant/cumulative effect of an unregulated or unmonitored inflammatory process (Kumar *et al*., 2004). The early and immediate response to tissue injury by noxious agents (microbiological, chemical, physical, etc.) constitutes acute inflammation. This comprises of three (3) different phases namely; vasodilation, vascular leakage and oedema where an increase in vascular permeability causes protein-rich filtrate (exudate) from plasma to replace transudate in the interstitial space resulting in increased osmotic pressure and oedema and the last phase constitutes the emigration of leukocytes predominantly, polymophonuclear leukocytes to site of inflammation where various biochemical reactions take place with the cumulative effect being resolution of tissue injury. The classical signs are redness, swelling, loss of function and pain (Anilkumar, 2010; Ahmed, 2011). If for any particular reason, acute inflammation fails to heal, chronic inflammation ensues. Chronic inflammation is the major determinant of various pathological conditions such as asthma, glomerulonephritis, rheumatoid arthritis, neurodegenerative diseases, atherosclerosis and cancers. It is characterized by granulation of tissues (fibrosis), proliferation and neovascularization where new blood vessels and collagen formation occurs. Cardinal signs of acute inflammation such as heat and redness rarely occur in chronic inflammation (Ahmed, 2011).

2.4.1 Pathophysiology of Inflammation

Numerous chemical mediators/regulators of plasma and cellular origin are involved in the pathophysiology of inflammation. They are produced when tissue damage by noxious

stimuli occurs. They include histamine, bradykinin, serotonin, oxidising agents such as platelet activating factor (PAF), nitric oxide, cytokines (tumour necrosis factor (TNF), interleukins), prostaglandins and its derivatives, leukotrienes, complement system (C5a/3a), proteases (Hageman factor), and growth factors (somatomedins) (Vane and Botting, 2014; Anilkumar, 2010).

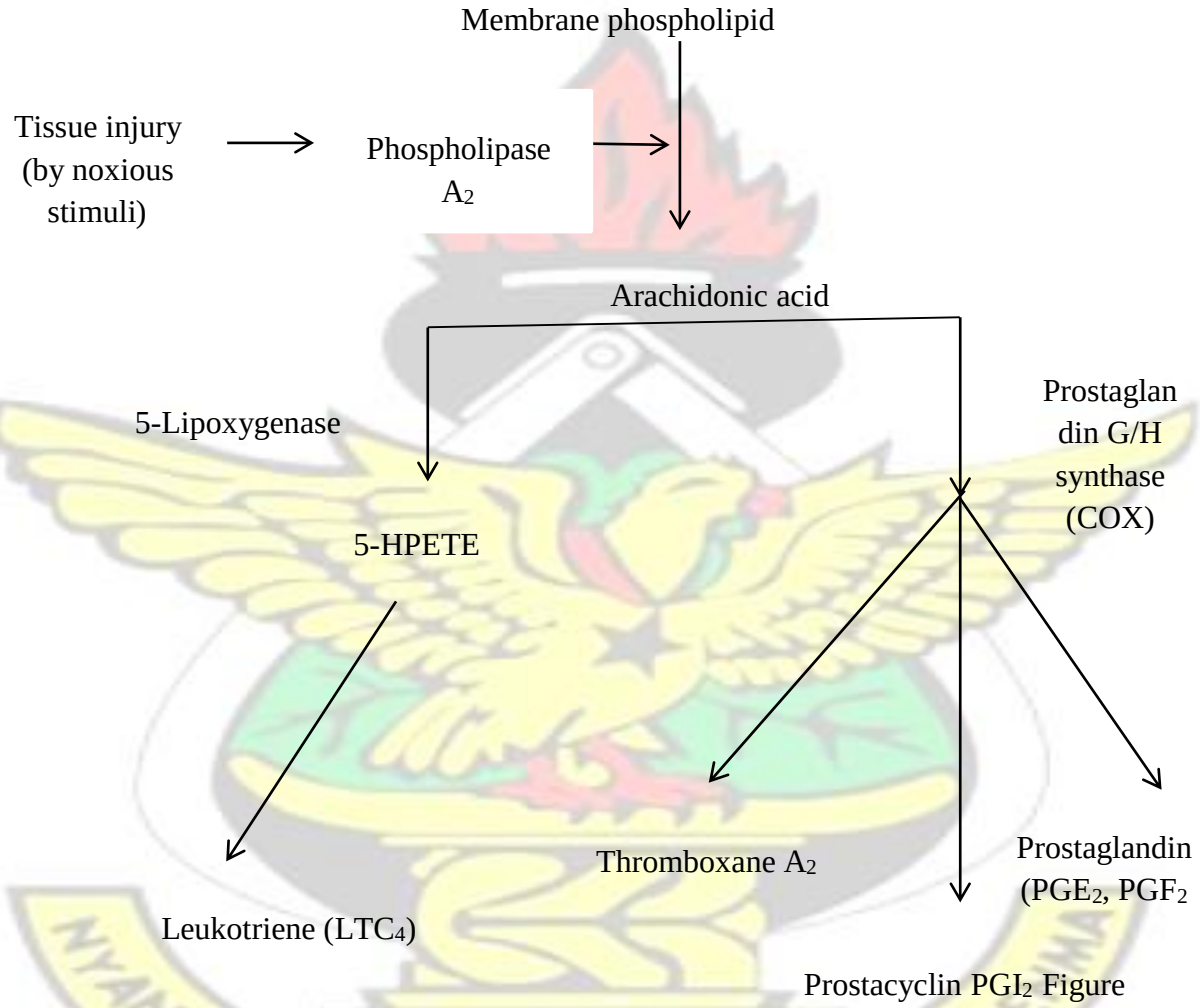
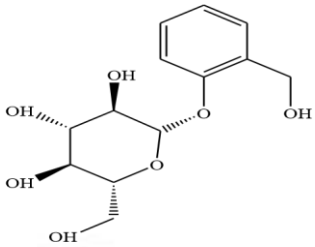


Figure 2.3 Pathway for the generation of various mediators/regulators

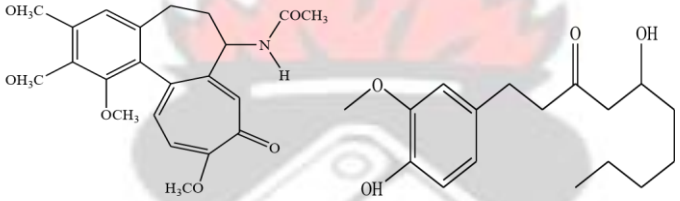
2.4.2 Anti-inflammatory agents from medicinal plants

The general usage of medicinal plants in the treatment of various inflammatory conditions by man is well known. Various researchers have published articles reporting the use of medicinal plants as anti-inflammatory agents.

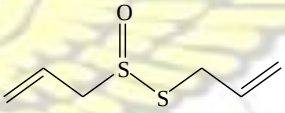
Some of these agents include salicin **(25)**, gingerol **(26)**, colchicine **(27)**, curcumin (**29**), capsaicin (**28**) and allicin **(30)** (Azab *et al*., 2016; Nworu and Akah, 2015; Murugesan and Deviponnuswamy, 2014). Anilkumar (2010) also reported the anti-inflammatory activities of β-boswellic **(32)** acid from *Boswellia serrata*, sylimarin **(31)** from *Silybum marianum*, iridoid glycosides **(34** and **35)** from *Harpagophytum procumbens* and bromelain **(33)** from *Ananas comosus*. These agents have been used alone or in combination as adjuncts with other medications in the treatment of various inflammatory conditions (Anilkumar, 2010).



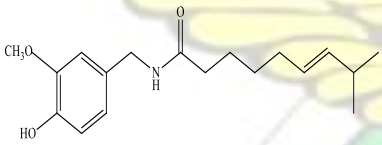
Salicin (25)



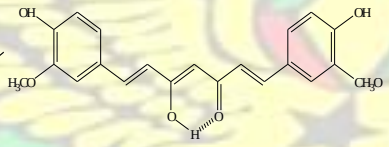
Gingerol (26)



Colchicine (27)

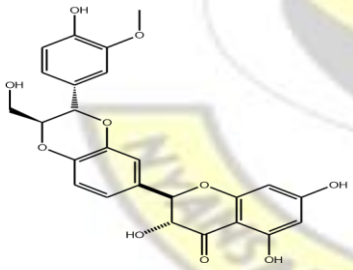


Capsaicin (28)

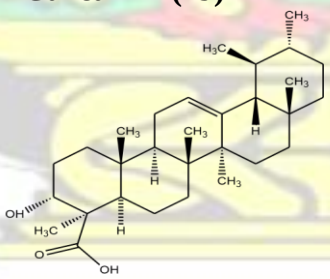


Curcumin (29)

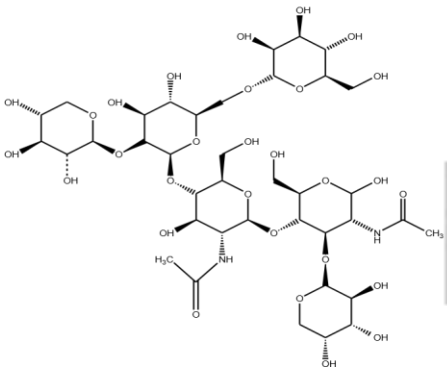
Allicin (30)



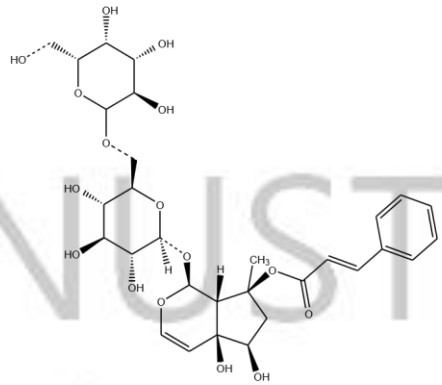
Silymarin (31)



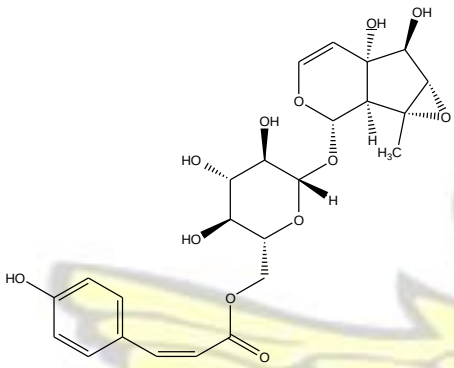
β-boswellic acid (32)



Bromelain (33)



Harprocumbide A (34)



Harprocumbide B (35)

Figure 2.4 Anti-inflammatory agents from plant sources

2.4.3 Experimental Models of Inflammation

Experimental models employed in the evaluation of anti-inflammatory activity include ear and paw oedema, UV-erythema, vascular permeability, pleurisy and granuloma pouch techniques (Patel *et al*., 2012; Umar *et al*., 2010). In the various models, injection of phlogistic agents including egg albumin, oxazolone, croton oil, 12-o-tetracanoil phorbol-13-acetate (TPA), dextran, formaldehyde, brewer's yeast, aerosol, kaolin and sulphated polysaccaharides such as carrageenan or naphthoylheparamine induce acute inflammatory responses. Vasoactive amines such as prostaglandins, bradykinin, histamine and mast cell

degranulators are also employed in the pleurisy model of inflammation (Patel *et al*., 2012; Umar *et al*., 2010).

2.4.4 Models of Acute Inflammation

Acute inflammatory response is evaluated using various techniques such as measurement of chemical inflammatory regulators/mediators in exudative fluids present in plasma, increased permeability of capillaries (hyperemia), leukocyte and phagocytic infiltration, increase in ear and foot volume as a result of oedema formation, accumulation of lymphocytes and evaluation of some plasma markers (Patel *et al*., 2012). The commonest method employed among the various assays is the carrageenaninduced footpad oedema in animal models (Patel *et al*., 2012) which has also been used for the current study.

2.4.5 Carrageenan-induced paw oedema

Carrageenan causes the formation of oedema by releasing chemical regulators/mediators following its injection into the cell and this underlies the basic principle behind this model. The formation of oedema by carrageenan administration into the paw of mice occurs in two phases (biphasic). The first phase is constituted by the release of histamine and serotonin. The second phase is caused by the release of bradykinin, prostaglandins, proteases and lysosome (Hafeez *et al*., 2013). Administration of carrageenan into the rat paw produces inflammation as a result of protein and fluid extravasation and leukocytes accumulation at sites of inflammation (Posadas *et al*., 2004). Following carrageenan injection, the first phase ensues. The second phase begins immediately, following the decline of the first phase.

Study animals (chicks/rats) are separated into groups of six each prior to the day of the work. The control group orally receives the vehicle and test groups are administered with

test and standard drugs. The paw (left) at the level of the lateral malleolus is marked with an ink; using the plethysmometer in the volume displacement method, the basal (initial) paw volume is measured by immersing the paw till the level of the lateral malleolus. Drugs are then administered to the animals. Test animals are subcutaneously injected with 0.1 mL of 1% solution of carrageenan into the left hind paw at the subplantar side after one hour of dosing. After 1, 2, 3, 4, and 5 hours of the challenge, the paw volume at the different time interval is determined again. The increase in paw volume is calculated by subtracting the basal paw volume from the volumes measured at each time interval. The percentage inhibition is then calculated (Winter *et al*., 1962; Amponsah *et al*., 2013).

2.5 OXIDATIVE STRESS

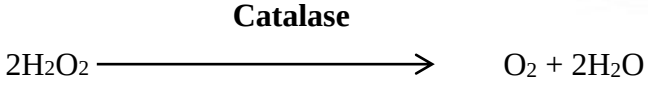
Free radicals are chemical entities which are perpetually produced as a result of normal cellular metabolism. Within an atomic orbital, molecules containing unpaired electrons and capable of existing independently constitute free radicals. These radicals are weakly attracted to the magnetic field and they either give off electrons to or attract electrons from other molecules in order to neutralize themselves, exhibiting particular characteristics of oxidants and reductants (Young and Woodside, 2001). These radicals are highly reactive and mostly within biological systems, have short half-life, although, some survive much longer. The commonest of these molecules include various reactive oxygen and nitrogen derivatives such as superoxide radical, singlet oxygen, hydroxyl radical, hypochlorite radical, hydrogen peroxide, hydroperoxyl radical, nitric oxide, peroxynitrite, s-nitrosothiols and other lipid peroxides (Percival, 1996). These radicals are produced in various pathways including normal aerobic metabolism, oxidative burst from phagocytes as part of mechanism by which microorganisms and antigens are denatured, xenobiotic

metabolism, enzyme reactions, autooxidation reactions and environmental sources (cigarette smoke, ultraviolet light and ionizing radiation) (Yadav *et al*., 2016; Young and Woodside, 2001). The production of these highly unstable and reactive chemicals if not controlled in the cell, reacts with membrane lipids, proteins, nucleic acids and enzymes resulting in cellular damage (Langseth, 1995). To curb the deleterious effects produced by these radicals, biological systems have developed antioxidant systems made up of enzymes and non-enzymatic systems. This system deactivates or stabilizes these free radicals. The enzymatic system includes catalase, superoxide dismutase and glutathione peroxidase and reductase. The non-enzymatic system is usually of exogenous nature and derived from natural sources (ascorbic acid, α-tocopherol, carotenoids, flavonoids, etc.) or synthetic origin (butylhydroxyanisole, butylhydroxytoluene, etc.). When the capacity of this antioxidant system is overwhelmed by free radical production, oxidative stress ensues which is responsible for the etiology of various disease conditions such as atherosclerosis, diabetes, pulmonary dysfunction, rheumatism, cancers, Alzheimer's disease, Parkinson's disease, multiple sclerosis, renal and liver diseases (Kumar, 2014; Breitenbach and Eckl, 2015).

2.5.1 Antioxidants

Several defense mechanisms exist in the human body for protection against the deleterious repercussions of free radicals and its derivatives. These defense systems complement each other because they act on different cellular compartments or oxidants (Langseth, 1995).

One class of antioxidants is the endogenous enzyme system made in the body. They include catalase, glutathione peroxidase and superoxide dismutase which reduce the concentration of these harmful free radicals. Superoxide dismutases catalyse the breakdown of superoxide radical to hydrogen peroxide and oxygen using metal ion cofactors such as zinc, copper and manganese (Yadav *et al.*, 2016; Langseth, 1995). Water and oxygen are the catalytic by-products of the breakdown of hydrogen peroxide in a two stage process by catalase. Glutathione peroxidases contain selenium and are involved in the reduction of by-products of lipid oxidation such as hydroperoxides (Shebis *et al.*, 2013; Langseth, 1995; Young and Woodside, 2001). Compounds with low-molecular weights such as uric acid, ubiquinone and glutathione produced as part of cellular metabolism also act as antioxidants. Proteins with metal binding affinity such as ferritin, lactoferrin, caeruloplasmin and albumin are also involved in various catalytic antioxidant reactions (Kumar, 2014; Langseth, 1995; Young and Woodside, 2001).



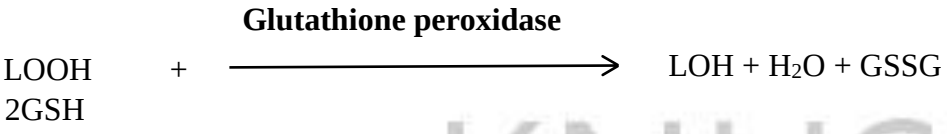


Figure 2.5 Enzymatic antioxidant defense systems

Other antioxidant systems referred to as exogenous antioxidants systems, usually of dietary source include vitamins C, E and carotenoids. Foods serving as antioxidants are mostly made up of phenolic or polyphenolic compounds. Although, these phenolics have no nutritive value, they are essential to human health due to their antioxidant activity (Kumar, 2014; Langseth, 1995; Young and Woodside, 2001).

2.5.2 Antioxidant evaluation methods

Several *in vitro* analytical assays have been employed in evaluating the antioxidant potential of natural products. They include ferric reducing power, DPPH radical scavenging, lipid peroxidation, NO^- scavenging, total antioxidant capacity and total phenol assays. These assays differ in the mechanism of radical species and/or target molecules generation as well as the way end-products are quantified (Pisoschi and Negulescu, 2011). It is essential to employ more than one assay in evaluating the radical scavenging potential of phytoconstituents due to their sophisticated nature as well as the varied mechanisms by which free radicals are generated. Hence, in this study, the phosphomolybdenum total antioxidant capacity assay, ferric reducing power and DPPH radical scavenging assays were employed.

2.5.2.1 Total antioxidant capacity

This assay quantifies the overall antioxidant activity of antioxidants in a biological system and not just for an individual compound. The reduction of phosphate Mo^{6+} to phosphate Mo^{5+} by an extract and the subsequent formation of a green phosphate Mo^{5+} complex at an acidic pH (Prieto *et al.*, 1999) is the underlying principle of this assay. A standard curve is generated using gallic acid as the standard and the total antioxidant values indicated as milligram per gram (mg/g) of gallic acid equivalents (GAE).

2.5.2.2 Ferric reducing power

Antioxidants in test samples form coloured complex with potassium ferricyanide, ferric chloride and trichloroacetic acid and the absorbance measured at 700 nm. The ferricyanide complex (Fe^{3+}) is reduced by antioxidants in test drugs to its ferrous (Fe^{2+}) form and this forms the basis of the assay. Electron transfer reactions are involved in this assay (Oyaizu, 1986; Pisoschi and Negulescu, 2011). A higher absorbance of a test drug indicates a higher reducing power which is indicative of its antioxidant potential. This assay is simple and inexpensive. It however cannot detect molecular entities that work by radical quenching such as proteins and glutathione (Benzie and Strain, 1999).

2.5.2.3 DPPH radical scavenging assay

The most commonly and widely reported method employed in evaluating the antioxidant potential of test samples is the DPPH radical scavenging assay. The delocalisation of the spare electron on the DPPH (2, 2-diphenyl-1-picryhydrazyl) molecule makes it a stable free radical, hence, does not dimerise contrary to most free radicals. When this (DPPH) stable free radical with a characteristic violet colour reacts with hydrogen donating specie,

it is converted to the reduced non-radical form, 1, 1-diphenyl-2-picrylhydrazine (yellow colour) and the residual DPPH which shows maximum absorption at 517 nm is measured (Pisoschi and Negulescu, 2011). An increased free radical scavenging activity of a sample is depicted by a decreased absorption. The result is expressed as the concentration at which 50% of the free radical (DPPH) is scavenged by the test drug (IC_{50}).

2.6 IMMUNE SYSTEM

Within vertebrates exists a remarkably complex defence machinery called the immune system which acts against deleterious stimuli. Its basic role is to differentiate between the host cell and foreign entities (McBride *et al*., 1996). The immune system recognises and eliminates countless number of invading agents by its interdependent and complementary cell types and immunoglobulins. These groups of cells perform specialised functions including killing of tumour cells and parasites, engulfing bacteria as well as eliminating viral infected cells (Nagarathna *et al*., 2013). These cells are formed throughout the body and reside in specialised organs such as thymus and bone marrow (primary immunoorgans) together with the spleen, tonsils, Peyer's patches, lymph nodes and lymphatic vessels (secondary immunoorgans). The immune system is made up of two (2) arms; innate and adaptive (Anarthe *et al*., 2014; McComb *et al*., 2013).

2.6.1 Innate immune system

The innate immune system is composed of several host defences such as barriers (skin), complement system and specialised cells that function interactively to offer an immediate and quick response to invading pathogens. The response is non-specific and does not improve upon repeated exposure (Patil *et al*., 2012). An unbreached skin remains the first line of defense barrier against attacking pathogens. When it is breached, bacteria

lysozymes break the cell wall causing the activation of the complement system. Complement proteins (C1-C9) bind to pathogens and by proteolytic cleavage, are activated. Multimeric complexes are formed disrupting cell membranes of bacteria and directly killing invading pathogens. Complement protein 3b enhances phagocytosis by acting as opsonin (McComb *et al*., 2013). The cell types include macrophages, mast cells, neutrophils, eosinophils and basophils (polymophonuclear cells), natural killer cells and dendritic cells. In response to infections and invading antigens, mast cells quickly release histamine and heparin. This response is crucial and critical in inflammation and wound healing as well as allergic responses (Katzung, 2009). During inflammatory responses, polymophonuclear cells arrive at sites of inflammation from peripheral circulation and initiate an acute wound inflammation. Neighbouring blood vessels are dilated causing an influx of immune cells mediated by chemokines. Polymophonuclear cells are involved in phagocytosis as well as secreting inflammatory mediators such as cytokines and other soluble peptides that help degrade and eliminate offending pathogens (McComb *et al*., 2013; Katzung, 2009). Dendritic cells are also capable of phagocytosis but together with macrophages act as antigen presenting cells (APC). They activate the adaptive arm of the immune system by processing and presenting peptides of pathogens on the major histocompatibility complex II receptor (MHC-II). Natural killer cells are also involved in tissue monitoring for tumour and viral invasion as well as suppression of autoimmune responses that are cell mediated (McComb *et al*., 2013).

2.6.2 Adaptive immune system

When the innate immune system fails in dealing with any noxious stimuli, the adaptive arm of immunity is mobilised to eliminate them. The adaptive immune system is antigen

specific, distinguishes between host and foreign antigens and also improves upon previous exposure by developing memory cells. The adaptive arm is mediated by cellular factors specifically T lymphocytes as well as humoral factors (antibodies) (Katzung, 2009).

The processing and presentation of antigens to major histocompatibility complex (MHC) molecules is enhanced by antigen presenting cells (dendritic cells, B cells and macrophages). The bound antigen to MHC complex encounters T cell receptors in the cell surface. Proteins obtained from intracellular pathogens (bacteria and viruses) bind to MHC-I and those from extracellular pathogens (parasites and bacteria) bind to MHC-II (McComb *et al*., 2013). Dendritic cells due to their stimulatory role and ability to efficiently move through lymphoid tissues to meet T cells, activates and initiates the generation of naive T and B cells. T cells with specific T cell receptors for a particular pathogen peptide/antigen as well as cytokine signals from inflammation become activated. Once they are activated through various biochemical signalling complexes, they are differentiated to cells that exert cytolytic killing or cytokine production (McComb *et al*., 2013; Nagarathna *et al*., 2013). Different cellular functions are performed based on the subtype of a T cell. The CD8 T cells bind to MHC-I receptor and when they are activated, release cytolytic granules causing death of their target cell.

CD4 T cells when activated by interleukin-12 (IL-12) and interferon γ (IF γ), aids in pathogen elimination via CD8 T cells activation (McComb *et al*., 2013; Patel *et al*., 2012). This is referred to as type-1 T-helper (Th1) response usually caused by intracellular bacteria, virus and protozoan. In the presence of interleukin-4 (IL-4), CD4 T cells activate B cell mediated antibody response. This is known as type-II T helper (Th 2) response (McComb *et al*., 2013; Katzung, 2009). When B cells are activated, their receptors are

released as an antibody. The B cell is now known as plasma cell and produces enough antibodies which specifically bind to and inactivate pathogens when released into the blood.When lymphocytes are activated and invading pathogens are eliminated, most of them die while the few surviving cells serve as memory cells which react rapidly to similar pathogens in the case of reinfection (McComb *et al*., 2013).

2.6.3 Immunomodulators

Immune system modulating agents cause an alteration in various phases of immune response by way of expressing and inducing certain genes and receptors, amplification of some signalling biochemical molecules and inactivating or inhibiting the expression of cytokines and other molecules that mediate various aspects of the immune process (Abood, 2017; Nagarathna *et al*., 2013). Modulation of the immune system provides it the required arsenal to fend off any threat that beckons it and to also moderate hyperactive immune responses. Many natural compounds, proteins and amino acids have demonstrated marked ability to influence host immunity. They include interleukins and interferons (IFN-γ), steroids and sterolins. These agents modulate both or either arms of the immune system. Immunomodulators generally stimulate or suppress any of the stages of the immune response (Patel *et al*., 2012; Nagarathna *et al*., 2013).

2.6.3.1 Immunostimulators

Immunostimulators enhance host defense against invading pathogens by stimulating both the innate and adaptive forms of immunity. They serve as promoter agents and prophylactics in healthy individuals as well as potentiating immune system of individuals whose systems are breached (Nagarathna *et al*., 2013).

2.6.3.2 Immunosuppresants

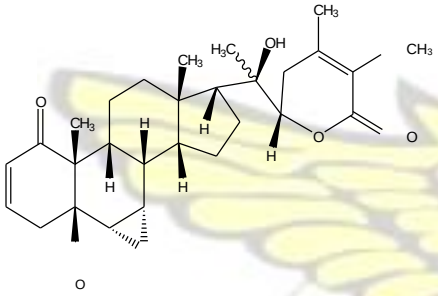
Immunosuppresive agents block unnecessary and unhealthy responses/events in host defense systems as well as moderating overactive responses. These agents are usually administered in combination therapies to treat various disorders including autoimmune disorders and organ transplant rejection (Nagarathna *et al*., 2013).

2.6.3 Immunomodulatory leads from medicinal plants

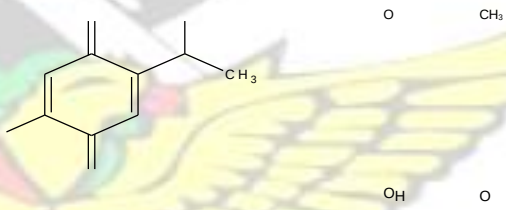
Medicinal plants have been used in various cultures to manage and treat various pathological conditions. These medicinally important plants have demonstrated immunomodulatory potentials by altering both innate and adaptive immune responses.

Withanolide **(36)**, glycowithanolide and sitoindosides **(37)** isolated from the Indian ginseng, *Withania somnifera* have demonstrated increased levels of IL-2 and IFN-γ and granulocyte macrophage colony-stem factor (GM-CSF). Quercetin-3-O-rutinoside, kaemferol 3-O- rutinoside and isorhamnetin-3-O-glucoside from *Urtica dioica* demonstrated immunomodulatory activity by intracellular killing and chemotaxis (Mukherjee *et al*., 2014). Thymoquinone **(38)** from *Nigella sativa* showed activity by augmenting natural killer cell (NK-cell) and T cell mediated immune response as well as supressing leukotrienes and prostaglandins. *Trigonella foenum-graecum* has shown increased phagocytic capacity of macrophages. Hyperforin **(39)**, isolated from *Hypericum perforatum*, has been reported to stimulate the expression of IL-8 in human intestinal epithelial cells as well as mRNA. Aucubin, luteolin, baicaleon and ferulic acid **(40)** from *Plantago major* has exhibited immunomodulatory activity by stimulating IFNγ secretion and lymphocyte transformation (Mukherjee *et al*., 2014; Nagarathna *et al*., 2013). Oenothein B, isolated from *Epilobium angustifolium*, has shown to modulate phagocyte

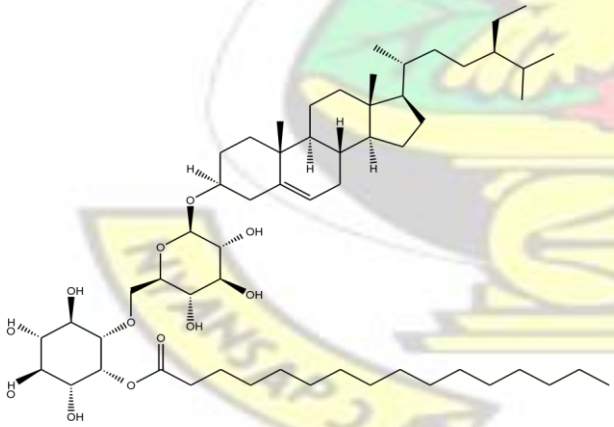
function (Schepetkin *et al*., 2009). Biapigenin, apigenin 7-O-β-Dneohesperidoside, vitexin **(42)** and orientin **(41)** *from Jatropha curcas*, have shown to stimulate both humoral and cell mediated immune responses. Glycyrrhizin and βglycyrrhetic acid from *Glycyrrhiza glabra*, curcumin from *Curcuma longa*, boswellic acid from *Boswellia serrata*, Eupalitin-3-O- β-D galactopyranoside from *Boerhavia diffusa*, dioqenyl saponin from *Paris polyphylla* have all exhibited immunostimulatory effects. Other plants reported to demonstrate significant immunomodulatory activity include *Momordica charantia, Allium sativa, Calendula officinalis*, *Azadirachta indica*, *Aloe vera*, *Bidens pilosa*, *Morinda citrifolia* and *Matricalia chamomile* (Mukherjee *et al*., 2014; Nagarathna *et al*., 2013; Abood, 2017).



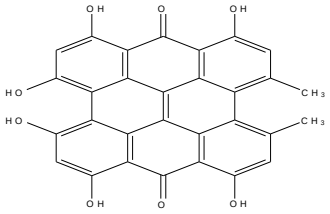
Withanolide (36)



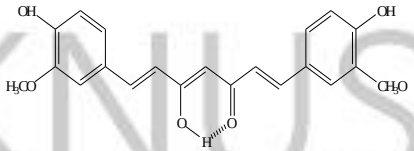
Thymoquinone (38)

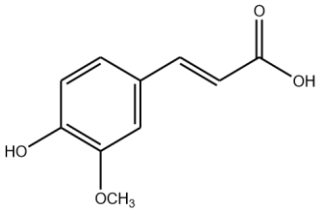


Sitoindoside (37)

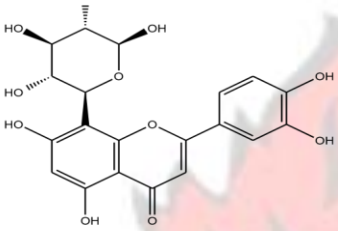


Hyperforin (39)

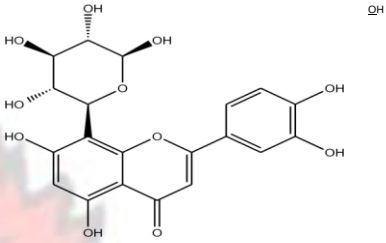




Ferulic acid (40)



Orientin (41)



Curcumin (29)

Vitexin (42)

Figure 2.6 Immunomodulatory agents from medicinal plants

2.6.4 Methods for evaluating immunologic factors

To maintain a disease-free state, immunomodulators non-specifically act on cellular and/or humoral arms of the immune system. To study the immunomodulatory activity of biological samples, several methods both *in vitro* and *in vivo* are employed.

2.6.4.1 In-vitro methods

In vitro methods employed in immunomodulatory studies include mitogen induced lymphocyte proliferation, inhibition of histamine release from mast cells, plaque forming colony (PFC) test, inhibition of T cell proliferation, chemiluminescence in macrophages and inhibition of dihydro-orotate dehydrogenase assays (Nagarathna *et al*., 2013).

2.6.4.2 In vivo methods

Hemagglutination antibody titre assay, acute systemic anaphylaxis in rats, arthus type immediate hypersensitivity, delayed type hypersensitivity, anti-anaphylactic activity

(Schultz-Dale reaction), passive cutaneous anaphylaxis, reverse passive arthus reaction, adjuvant arthritis in rats, collagen type II induced arthritis in rats, experimental autoimmune thyroiditis and proteoglycan-induced progressive polyarthritis in mice are commonly used *in vivo* assays in evaluating immunomodulatory activity of biological samples (Nagarathna *et al*., 2013).

2.7 TOXICOLOGICAL EVALUATION

The concept of toxicology is based on the basic principle that ultimate toxicity is as a result of the molecular interaction of an active agent with a cellular target (DNA, enzyme, membrane phospholipid and structural protein). The extent to which an agent and how much of it causes harm to an organism (man or animals) constitutes its toxicity.

The ultimate toxicity of any agent is death, however, changes in organ/body weights and altered water and food consumption are some mild forms. The toxicological process can be classified under two (2) distinct but related phases; kinetic and dynamic. The kinetic phase encompasses all the processes which determine the relationship between agent exposure and the concentration of the potentially active form at the target site. Factors including lipid solubility, macromolecule binding, metabolism and membrane transport largely affect the kinetic phase. The response of the cellular target to the active agent essentially constitutes the dynamic phase. The dynamic phase is also affected by factors such as physiological state, intracellular metabolism, nature of interaction at the molecular site and detoxification mechanisms. Toxicity study is necessary because informed decisions are taken for acceptance or rejection of a novel agent to be used clinically. Toxicological study is classified as acute, subacute/subchronic and chronic depending on the exposure time of test animals to an agent (Frazier, 1986; Chanda *et al*., 2015).

2.7.1 Acute toxicity

The toxic effects produced by a single exposure of a drug(s) by any route for a short period of time usually, 24 hours constitute its acute toxicity. The principal objective of acute toxicity studies is to establish the single dose causing life threatening toxicity- essentially causing lethality or death. The oral and intraperitoneal routes of administration are the two most commonest routes used in acute toxicity The acute toxicity study is usually carried out using high doses that are sufficient to cause death. It is used to establish the LD_{50} of a drug as well as serving as a guideline in the selection of doses for chronic and subchronic toxicity studies (Chanda *et al*., 2015).

2.7.2 Chronic toxicity

The carcinogenic and mutagenic potentials of therapeutic agents are usually assessed by the chronic toxicity studies where different doses of the agent are administered over a period of 3 months to a year. Hematological, biochemical and histopathological parameters are assessed using chronic toxicity studies. Chronic toxicity studies of an agent provide information such as the specific target organ/s affected as well as possible accumulation at the target site. Periods between acute and chronic toxicity studies are referred to as subchronic/subacute. Subacute and subchronic however, differ in exposure time. The undesirable effects that occur after multiple or continuous exposure between 24 and 28 days is the subacute toxicity. Subchronic toxicity however, is the toxic effects that occur after the repeated or continuous administration of an agent for up to 90 days or not exceeding 10 % of the animal's lifespan (Chanda *et al*., 2015; De Jong *et al*., 2012).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Chemicals and Reagents

All solvents (organic); chloroform ($CHCl_3$), ethyl acetate (EtOAc), petroleum ether (Pet. ether), methanol (MeOH) used for the studiess were of analytical grade and obtained from BDH laboratory Ltd. (UK). Vanillin, anisaldehyde, ammonium molybdate, sulphuric acid, sodium hydroxide, trichloroacetic acid was obtained from BDH laboratory Ltd. (Poole, England). 2, 2-diphenyl-1-picrylhydrayl (DPPH), disodium hydrogen phosphate, gallic acid, ascorbic acid, sodium dihydrogen phosphate. monohydrate, potassium ferricyanide, ferric chloride, polysorbate 80, were obtained from Sigma Aldrich (St. Louis, MO. USA).

Silica gel (70-230 mesh) was bought from Loba Chemie Pvt. Ltd. (Mumbai, India). Sephadex LH-20 beads were obtained from Amersham Biosiences (Buckinghamshire, England). Aluminium precoated silica gel plates GF254 (0.25mm thick) used for analytical TLC was obtained from Alpha laboratories (UK). Non calibrated glass capillary tubes were bought from Heinz Herenz Medizinalbedarf Gmbh (Hamburg, Germany). Diclofenac (IV) and dexamethasone (IV), normal saline were obtained from Juliponia Pharmacy (Kumasi, Ghana).

3.2 Equipments

The absorbance-based assays were measured using the Jenway UV-VIS spectrophotometer (Germany). Heraeus Biofuge Primo Centrifuge, Jos. Hansen and Soehne GmbH (Hamburg, Germany) was employed in assays that required the use of a centrifuge. The infrared (IR) spectra of compounds were determined by the Bruker Fourier transform infrared (FT-IR) spectrometer Hansen and Soehne GmbH (Hamburg,

Germany). S15 Elmasonic sonicator, Jos. Hansen and Soehne GmbH (Hamburg, Germany) was used to ensure complete dissolution of extracts in various solvents. The Stuart SMP10 digital melting point apparatus (Bibby Scientific Ltd. Stone, Staffordshire, STIS 0SA, UK) was used in determining the melting point of compounds. Extracts were concentrated to small volumes using SEO5 rotary evaporator (Australia). Semisolid extract were obtained after drying in Gallenkamp oven. The foot volumes of experimental animals (chicks) were measured using a VINCA DCLA-0605 electronic digital caliper.

3.3 COLLECTION AND AUTHENTICATION OF MATERIAL

The fresh fruits of *Vitex doniana* were harvested from a farmland in Tamale, in the Northern region of Ghana in 2017. The fruits were identified and authenticated by Mr. Clifford Asare of the Department of Herbal Medicine, Faculty of Pharmacy and Pharmaceutical Sciences, KNUST. Herbarium specimen with voucher number KNUST/HM/2017/F001 has been deposited at the herbarium of the Faculty of Pharmacy, KNUST, Kumasi.

3.4 PROCESSING OF MATERIAL

The fresh fruits were washed with water and the seed removed. The fleshy pericarp was then oven-dried at 60 °C for 48 hours. The dried pericarp was then pulverised into coarse powder. The powdered material was then packaged in a brown paper bag until was needed for use.

3.5 EXTRACTION OF PLANT MATERIAL

2.8 kg of the powdered fruit was weighed and soxhlet-extracted using 10 litres of methanol for 72 hours until the material was exhausted. The filtrate thus obtained, was concentrated under vacuum to a small volume using the rotary evaporator at 50˚C. The extract obtained was evaporated to dryness using an oven at a temperature of 50˚C which yielded a semi-solid extract weighing 185 g (yield = 6.61%$^{w}/_{w}$). The extract was then successively fractionated with petroleum ether, ethyl acetate and methanol. The different solvent soluble fractions obtained were concentrated to small volumes and evaporated to dryness to afford petroleum ether (9.61 g), ethyl acetate (38 g) and methanol (86 g) extracts respectively.

3.6 ANTI-INFLAMMATORY ASSAY OF EXRACTS AND ISOLATES

3.6.1 Experimental animals

Cockerels (*Gallus gallus*, from Akate farms, Kumasi) were obtained 1-day after hatching and were kept in stainless steel cages lined with soft wood shavings at a population density of 13 - 15 chicks per cage in standard environmental conditions (temperature 25 ± 2˚C and overhead incandescent illumination was maintained at a 12hour light-dark cycle) within the Pharmacology Department's animal house, Faculty of Pharmacy and Pharmaceutical Sciences. They had access to feed (Chick mash, from Maridav Ghana Ltd., Tema) and water *ad libitum*. The study was conducted on the chicks after 7 days of age. Groups of sample size 5 were used for the experiment.

3.6.2 Carrageenan-induced oedema in chicks

Modification of the carrageenan foot oedema model of inflammation in chicks described by Roach and Sufka (Roach and Sufka, 2003) by Woode *et al.*, (2009), was employed in

evaluating the anti-inflammatory activities of the extracts and compared to diclofenac and dexamethasone as reference drugs. Carrageenan (10 µL of a 2% suspension in normal saline) was injected subplantar into the left footpads of the chicks. The foot volume was measured before injection and for 5 hours at hourly intervals after injection by means of a vernier caliper. The oedema component of inflammation was determined by measuring the difference in foot volume before carrageenan injection and at the various time intervals.

3.6.3 Preliminary bioassay of crude extracts

The study was aimed at evaluating the effects of the extracts and standard drugs (diclofenac and dexamethasone) on oedema 1 hour after carrageenan challenge and continuing up to 5 hours. The extracts were administered by oral route and the drugs through the intraperitoneal (*i.p)* route. The experimental chicks received the extracts dosed at (30, 100 and 300 mg/kg, *p.o.),* dexamethasone (0.1, 1.0 and 3.0 mg/kg, *i.p.),* and diclofenac (10, 30 and 100 mg /kg, *i.p.)* whereas the control animals received only normal saline which served as the vehicle. The doses for the methanol extract and fractions were prepared by dissolving a known weight of the extract in 2% tragacanth mucilage whereas the petroleum ether and ethyl acetate fractions were triturated in 2% polysorbate 80 using 2% tragacanth mucilage as the diluent.

3.6.4 Anti-inflammatory assay of isolates

The isolated compounds were evaluated for anti-inflammatory activities using the method stated in section 3.6.3. The isolates were dosed orally at 10, 30 and 100 mg/kg body weight.

3.7 ANTIOXIDANT ASSAY OF EXTRACTS OF *V. DONIANA*

3.7.1 Total antioxidant capacity assay

Molybdenum reduction, Mo^{+6} to Mo^{+5}, by the extracts and subsequent formation of a green phosphate-molybdate (Mo^{+5}) complex at acidic pH is the underlying principle behind this assay (Prieto *et al.,* 1999). The reagent solution was prepared by adding ammonium molybdate (4 mM), sulphuric acid (0.6 M) and disodium hydrogen phosphate (28 mM). One millilitre (1 mL) each of the different concentrations of the extracts (2000-62.5 µg/mL) and three millilitres (3 mL) of the reagent solution in test tubes were incubated at 95˚C for an hour and thirty minutes. The process was repeated for concentrations of the reference drug, gallic acid (100 -1.56 µg/mL). A blank solution was prepared by adding every other solution excluding the extract and the reference drug. After the mixture had cooled to room temperature, the absorbance was read at 695 nm using the UV-visible spectrophotometer. A calibration curve using the concentrations of gallic acid was drawn. The anti-oxidant capacity was quoted as a milligram (mg) of gallic acid equivalent (GAE) per gram (g) of extract.

3.7.2 Ferric reducing antioxidant power (FRAP)

The method by Oyaizu (1986) was used in investigating the ferric reducing activity of all the extracts. Different concentrations of the extracts (2000 – 15.63 µg/mL) as well as the reference drug, gallic acid (100 – 0.78 µg/mL) were prepared in distilled water and 1 mL each taken into test tubes in triplicates. A volume mixture of 2.5 mL each of 1% potassium ferric cyanide solution and sodium phosphate buffer (pH=7) was added to the test tubes. The mixture was then incubated at 50 °C for 20 minutes. After incubation, the mixture was centrifuged at 3000 revolutions per minute for 10 minutes after 2.5 mL of

10% trichloroacetic acid was added to the mixture. A volume of 2.5 mL distilled water was added to 2.5 mL of the supernatant obtained. The absorbance of the mixture was taken after 1 mL of 0.1% ferric chloride was added at 700 nm. Increased absorbance of an extract is equivalent to higher reducing power.

3.7.3 DPPH radical scavenging assay

The scavenging activity of DPPH by extracts was determined as described by Govindarajan *et al.,* (2003). To 3 mL methanol solution of DPPH solution (20 mg/L) in a test tube, 1 mL each of the extracts (2000 - 62.5 µg/mL in methanol) was added. The absorbance of the reaction mixture was read after incubating in the dark at 25°C for half an hour at 517 nm. One milliliter (1 mL) of methanol (100%) was added to 3 mL DPPH solution, incubated at 25 °C for 30 minutes and used as the blank. Gallic acid (100 – 0.78 µg/mL) was used as the standard drug. The absorbance decreases with increasing free radical scavenging ability. The concentration required to cause a 50% decrease in the absorbance of the initial DPPH concentration was calculated (IC_{50}). Test samples were carried out in triplicates. The percentage (%) DPPH scavenging effect (% of control) of the antioxidant was estimated as follows:

% DPPH scavenging effects = $(Ac - At)/Ac \times 100$

Where Ac = Absorbance of the control, At = Absorbance of the test drugs/ extracts

3.8 ANTIOXIDANT ASSAY OF ISOLATES

3.8.1 Ferric reducing antioxidant power (FRAP)

The isolated compounds and reference drug, gallic acid at concentrations (100 – 0.78 µg/mL) were evaluated for their ferric reducing activity using the method described in section 3.7.2.

3.8.2 DPPH free radical scavenging assay

The isolates and the reference drug, gallic acid at concentrations (100 – 0.78 µg/mL) were evaluated for their DPPH free radical scavenging activity using the method described in section 3.7.3.

3.9 Acute toxicity of crude extract

Swiss albino mice were purchased a week before the experiment for acclimatization from the animal house of the Department of Pharmacology, KNUST. Mice of either sex weighing 20-30 g were housed in groups of four (4) under standard laboratory conditions with standard pellet food and water as required. The overnight fasted experimental animals (water *ad libitum*) were transferred to the laboratory 1 hour before the experiment. They were divided into four groups with each group having 4 animals. The crude extract was dosed at 100, 300 and 3000 mg/kg body weight. Control animals were administered with normal saline. The experimental animals were observed for autonomic, CNS and behavioural effects as well as mortality.

3.10. SUBACUTE TOXICITY STUDIES

Subacute oral toxicity study was carried out according to the Organisation of Eonomic Co-operation and Development (OECD) guidelines (407) for testing of chemicals (OECD, 2008).

Swiss albino mice were purchased a week before the experiment for acclimatization from the animal house of the Department of Pharmacology, KNUST. Mice of either sex weighing 18-25 g were housed in groups of four (4) under standard laboratory conditions with standard pellet food and water *ad libitum*. On the basis of results obtained from the oral acute toxicity study, the crude extract was dosed at 100, 300 and 1000 mg/kg body weight. Groups of four comprising of four (4) animals each were used for the study. Doses were administered every 24 hours for 28 days. Control animals received normal saline. Surviving animals were overnight fasted and anaesthesized after day 28. Heparinized blood samples were collected for haematological studies. Nonheparinized blood from serum was also collected for clinical biochemical studies.

Mortality was also monitored on daily basis (Chanda *et al*., 2015).

3.11 DATA ANALYSES

All results are presented as mean ± SEM. Data analysis was carried out using one-way analysis of variance (ANOVA). Multiple treatment groups comparisons were carried out by the Dunnet's *post hoc* test. $p< 0.05$ was considered statistically significant. All statistical analyses were carried out with GraphPad Prism 5 for windows (GraphPad Prism Software, San Diego, USA).

3.12 CHROMATOGRAPHY

The ethyl acetate fraction of the fruit of *V. doniana* demonstrated the maximum antioxidant effect and considerable anti-inflammatory activity, hence, was chosen for further phytochemical investigation to isolate its constituents.

3.12.1 Chromatographic techniques

Column chromatography (CC) using sephadex LH-20 or silica gel as stationary phase and thin layer chromatography (TLC) are the techniques employed in the isolation of phytoconstituents from the ethyl acetate fraction of *V. doniana*.

3.12.1.1 Column chromatography (CC)

Using silica gel (70-230 mesh ASTM) as the stationary phase, a cleaned dried glass column was packed with an amount of the blank silica gel. The extract (35 g) was reconstituted in methanol and adsorbed onto a minimum amount (10 g) of the blank silica gel. The slurry obtained was dried completely. The dried mixture was then loaded on top of the previously packed content of the glass column. The eluent (gradient mixtures) of methanol, ethyl acetate and petroleum ether in increasing polarity was then placed on top of the column and the extract eluted into different fractions. The fractions were then collected in glass containers, appropriately labelled and bulked according to their TLC chromatogram.

In the elution process using sephadex LH-20 as the stationary phase, the beads of sephadex were soaked overnight in methanol. The solvent was allowed to drain off after the slurry obtained was packed onto a glass column. The extract, solubilised in methanol, was carefully loaded onto the content of the glass column and eluted isocratically with a mixture of chloroform and methanol (1:1).

3.12.1.2 Thin layer chromatography (TLC)

The fractions were analysed in one way ascending technique by TLC. Samples were solubilised in suitable solvents and by means of capillary tubes, were applied as series of spots, 2 cm above the edge and away from the margin of the TLC plates. The plates were developed in an air-tight container (chromatank) containing appropriate solvent systems. By capillary action and in a one way ascending manner, the solvent front ran along the TLC plate with the components of the extracts. When a reasonable solvent distance was reached, the operation was stopped and the chromatogram allowed to dry. The chromatogram was observed under the UV lamp at short wavelength (254 nm) and long wavelength (365 nm) for characteristic fluorescence followed by detection with vanillin or anisaldehyde in conc H_2SO_4 after they were heated.

3.13 ISOLATION OF CONSTITUENTS FROM THE ETHYL ACETATE FRACTION

Thirty five grams (35 g) of the ethyl acetate fraction of the fruit of *V. doniana* was subjected to column chromatography using silica and gradiently eluted with 100% petroleum ether, then, 15%, 25%, 35%, 45%, 60%, 70%, 80% and 100% ethyl acetate, followed by gradient mixtures of ethyl acetate and methanol to obtain sixty (60) fractions (80 mL each). These aliquots were bulked, following TLC analyses, to obtain six (6) fractions (BF1 - BF6).

Fraction three (BF3) (5.0 g) was column chromatographed using silica and gradiently eluted with petroleum ether 90%, then 15%, 20%, 30%, 40%, 60% and 100% ethyl acetate to obtain forty-six (46) fractions (30 mL each). The eluates were monitored with TLC and bulked into four (4) sub-fractions (BF3A – BF3D) based on their TLC profiles. Sub-

fraction three (BF3C) was further column chromatographed on sephadex LH-20 eluting isocratically with $CHCl_3$:MeOH (1:1) to give compound **VDF1** (1000 mg) as a white amorphous powder.

Fraction four (BF4) (18.5 g) was column chromatographed on sephadex LH-20 with $CHCl_3$: MeOH (1:1) as mobile phase to obtain eleven (11) eluates (50 mL each). These were bulked into three (3) sub-fractions ($BF4_A$ –$BF4_C$) based on their TLC analysis. Sub-fraction two ($BF4_B$) (8.0 g) was further subjected to column chromatography using silica and gradiently eluted starting with 80% petroleum ether, followed by 30%, 40%, 50%, 60% and 100% ethyl acetate to obtain 36 eluates (20 mL aliquot). The eluates upon monitoring with TLC were bulked into three (3) sub-fractions ($BF4_{B1}$ - BF_{B3}).

Sub-fraction $BF4_{B2}$ was column chromatographed on sephadex LH-20 with $CHCl_3$: MeOH (1:1) mobile phase to obtain **VDF2** (130 mg), an orange crystalline compound.

Fraction five (BF5) (6.0 g) was column chromatographed on silica and gradiently eluted starting with petroleum ether 80 % , then 10%, 30%, 40%, 50%, 60%, 70% and 100% ethyl acetate, followed by 20%, 40%, 60% and 100% methanol to obtain 42 eluates, 30 mL aliquot each. These were bulked into four (4) sub-fractions ($BF5_A$ – $BF5_D$) following monitoring with TLC.

Sub-fraction three ($BF5_C$) (0.8 g) was column chromatographed on sephadex LH-20 eluting isocratically with $CHCl_3$: MeOH (1 :1) mobile phase to obtain **VDF3** (120 mg), an off - white amorphous powder (Figure 3.1).

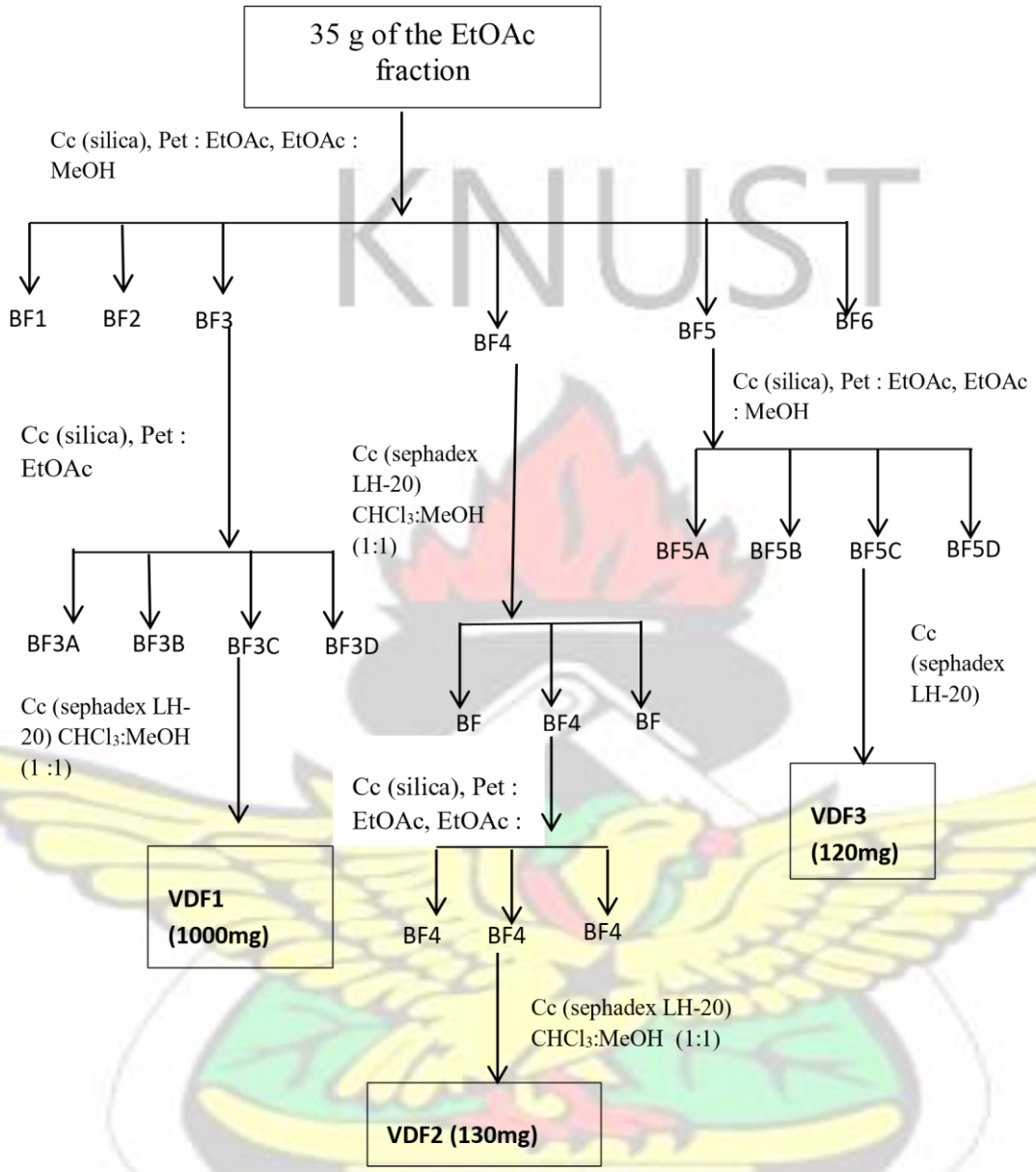


Figure 3.1 Schematic representation of isolation of compounds from the ethyl acetate fraction of *V. doniana* fruit

3.14 IDENTIFICATION OF ISOLATED COMPOUNDS

A combination of different techniques is used in the identification of compounds. They include mass spectrometry, nuclear magnetic resonance, infrared (IR) and ultraviolet (UV) spectroscopy. The identities of compounds isolated in this study were determined by nuclear magnetic resonance and infrared spectroscopy as well as their melting point.

3.14.1 Nuclear magnetic resonance spectroscopy (NMR)

One dimensional (1D) NMR spectroscopic techniques including ^{1}H and Distortionless enhancement by polarisation transfer (DEPT) as well as two dimensional 2D (NMR) techniques such as Hetero-nuclear single quantum correlation (HSQC), Correlational spectroscopy (COSY) and Hetero-nuclear multiple bond correlation (HMBC) were employed in the elucidation of the structure of the isolated compound. These provided comprehensive information in assigning the planar structures of the compounds. Information on the nature and number of protons was deduced from ^{1}H-NMR based on the absorption of the different proton signals and their chemical shifts. DEPT 135 provided information on the number of carbons with methine (CH) and methyl (CH_3) carbons occuring in the positive phase and methylene (CH_2) as well as non- protonated/quaternary (C) carbons in the negative phase. Homonuclear ^{1}H correlations were determined by COSY. One bond ^{1}H– ^{13}C correlation was established by HSQC and that for double and triple bonds ^{1}H – ^{13}C were determined by HMBC. Deuterated chloroform ($CDCl_3$) was the solvent used for the NMR analysis. The pure compounds were dissolved completely in the solvent and carefully transferred into an NMR tube for measurements. The spectra were recorded at 25°C on a NMR spectrophotometer (500 MHz). Chemical shifts (δ) were expressed in parts per million (ppm) using tetramethylsilane as internal standard (Mitchell and Costisella, 2007).

3.14.2 Infrared (IR) spectroscopy

The functional groups present in the isolated compounds were identified using the Bruker Fourier transform infrared (FT-IR) spectrometer scanned between 4000-400 cm^{-1} with a resolving power of 4 cm^{-1} and a cumulative scanning limitation of 24 times.

3.14.3 Melting point

The melting point of the isolated compound was determined using the Stuart SMP10 digital melting point apparatus.

3.15 HPLC QUANTIFICATION OF OLEANOLIC ACID (VDf1) IN *VITEX DONIANA* FRUIT

3.15.1 Chemicals and reagents

Analytical grade reagents and HPLC grade solvents were employed for the preparation of the plant samples and for chromatographic analyses. The chemicals used include methanol (HPLC gradient grade (100%); Sigma Aldrich (St. Louis, MO. USA); SZBE0858V; analytical grade (100%); BDH laboratory Ltd. (UK); L648424), trifluoroacetic acid (HPLC gradient grade; Sigma Aldrich (St. Louis, MO. USA); 1090558), acetonitrile (HPLC gradient grade; Sigma Aldrich (St. Louis, MO. USA); 17B101328), acetic acid (HPLC gradient grade (100%); Sigma Aldrich (St. Louis, MO. USA); 4576990J). Distilled water was freshly produced in-house from a water purification system (Deionised water from Siemens ultra-clear by reversed osmosis was used in the preparation).

3.15.2 Materials

Working standards of isolated oleanolic acid and crude extract from the fruit of *Vitex doniana* with no further purification were used for the experiment.

3.15.3 Instrumentation

Chromatographic measurements were performed on a Perkin Elmar Flexar HPLC system (Jos Hansen & Soehne GmbH, Germany), consisting of a Quaternary Flexar Pump FX-20 (N2910535), Flexar PDA detector (N2950010), UHPLC autosampler (N2930664) and standard column oven (N2601681). The mobile phase was filtered before use. A reversed-phase µBondapak C18 analytical column (300mm x 2.5mm, 5 µm, Waters Corporation, USA) was used for chromatographic separation of the plant isolates

3.15.4 Softwares

Chromatographic analysis and data integration were recorded on a windows computer system using (Chromera Speciation software version 4.1.0.6386., 2006); Jos Hansen & Soehne GmbH, Germany). GraphPad Prism 5 for Windows (version 5.01, 2007; GraphPad Prism Software, San Diego, USA) was used to perform validation relatedcalculations, which included standard deviations, relative standard deviations, standard errors of means, regression analysis, construction of calibration plots for plant isolates, among others

3.15.5 Preparation of standard solution

Stock solution (1000 ppm) of oleanolic acid was prepared by dissolving in 10 mL methanol, an accurately weighed 10 mg of oleanolic acid in a volumetric flask. The prepared stock solution was stored at 48°C and protected from light. Working standard solutions were freshly prepared by serially diluting the stock solution to obtain 800, 400,

200, 100 and 50 ppm for method suitability studies. Solutions were filtered through 0.45 µm membrane filter prior to their injection into the chromatographic system.

3.15.6 Preparation of extract

Stock solution (2000 ppm) of the crude extract was prepared by dissolving 20 mg of the accurately weighed extract in 10 mL of methanol in a volumetric flask. Solution was filtered through 0.45 µm membrane filter prior to its injection into the chromatographic system for analysis.

3.15.7 Chromatographic method development for oleanolic acid estimation

Chromatographic separations adopted a gradient elution approach on a reverse-phase µBondapak C18 analytical column (300mm x 2.5mm, 5 µm, Waters Corporation, USA). A binary mobile phase consisting of a mixture of 0.05% trifluoroacetic acid (A) and 100% methanol (B) was employed for the study. A wavelength of 210 nm was used for detection. At t=0, the mobile phase consisted of 50% A and 50% B and it changed with a linear gradient during 13 min to 100% B. At min 14.1, it returned to the initial conditions (50% A and 50% B) until t = 20 min. The injection volume of the sample was maintained at 20 µL throughout the study. The flow rate was 1 mL/min. The entire chromatographic system was also maintained in an air-conditioned laboratory atmosphere (25°C).

3.15.8 Validation of chromatographic method

The validation was performed for the chromatographic method developed to determine the content of oleanolic acid in the sample. The validation was performed as per International Conference on Harmonisation (ICH) for analytical method validation (Q2 (R1)) (ICH, 2003) by examining the efficiency and/or performance of the analytical

method in respect to the following parameters: precision, linearity, accuracy, robustness, limit of detection and limit of quantification.

3.15.8.1 Precision

Precision may be a measure of degree either of reproducibility or of repeatability of the analytical procedure under normal operating conditions. The interday and intraday precision of the developed method was evaluated by analysing the content of reference oleanolic acid at 200 ppm working concentration level, in six (6) replicate determinations (ICH, 2003).

3.15.8.2 Linearity and Range

Linearity was measured by analysing standard oleanolic acid sample at five (5) concentration levels ranging from 50 – 800 ppm and plotting the peak areas against concentration. The linearity of the plot was expressed as the regression coefficient (R^2). Each of the working concentrations was injected in triplicate (ICH, 2003).

3.15.8.3 Accuracy/recovery

The accuracy study was evaluated at three (3) concentration levels (150, 250 and 350 ppm) by calculating the recovery of oleanolic acid by the method of standard addition. The mean percentage recovery for oleanolic acid obtained from the peak area was then calculated (ICH, 2003).

3.15.8.4 Robustness

It is an indicator of the reliability of a method in normal routine use. The robustness of the developed method was monitored by comparing the relative standard deviation (RSD) of the peak areas of oleanolic acid when the flow rate at (1.0 mL/min) and wavelength (210

nm) were varied from (0.9 to 1.1 mL/min) and (201 and 215 nm) respectively (ICH, 2003).

3.15.8.5 Limit of detection (LOD) and limit of quantification (LOQ)

The limits of detection and quantification of oleanoic acid of the developed chromatographic method were estimated using the linear regression equation of the calibration curve. The limits of detection and quantification were calculated based on the standard deviation (S. D) of the y-intercept and the slope (S) as 3.3 S. D/S and 10 S.D/S respectively (ICH, 2003).

3.15.8.6 Determination of oleanolic acid content in the fruit extract

Sample injection of 200 ppm concentration of crude extract was carried out in the chromatographic system. The injection was made in triplicate. The amount of oleanolic acid in each determination was estimated from the linear equation, $y = 1.12492 \times 10^4 \mathbf{x} - 2.39441 \times 10^5$. The mean and standard deviations of the contents were also determined

CHAPTER FOUR

RESULTS

4.1 ANTI-INFLAMMATORY ACTIVITY OF EXTRACTS

Preliminary anti-inflammatory activity of extracts and fractions of *V. doniana* fruit was assessed using carrageenan induced oedema model in chicks. The extracts were administered to chicks at 30 mg/kg, 100 mg/kg and 300 mg/kg body weight orally after an hour following oedema induction with carrageenan. Dexamethasone (0.1, 1 and 3 mg/kg, i.p) and diclofenac (10, 30, 100 mg/kg, i. p) were used as standard drugs. Acute inflammation induction in control chicks resulted in a bulbous swelling of foot pads which

began an hour following intraplantar carrageenan injection and reached a peak of inflammation after 2 hours and a gradual decline for the next 3 hours.

The extracts; crude extract ($P < 0.0167$), petroleum ether fraction, ethyl acetate fraction and methanol soluble fraction exhibited a significant ($P < 0.0001$) dose dependent reduction of inflammation induced by carrageenan in the chicks with the observed effect occurring 2 hours after carrageenan injection (Figure 4.1- 4.2). Dexamethasone ($P < 0.0003$) and diclofenac ($P < 0.001$) demonstrated significant effects on the time course curve and reduced the total oedema dose dependently (Figure 4.3). Values are indicated as mean + SEM (n = 5) *** $P < 0.001$ ** $P < 0.01$; * $P < 0.05$, ns = not significant compared to vehicle treated group (one way ANOVA) followed by Dunnet's *post hoc* test. The higher the anti-inflammatory activity, the lesser the quantity of drug required to inhibit the oedema by 50%. This is expressed as ED_{50} (mg/kg) values. Dexamethasone demonstrated the greatest activity, followed by diclofenac, methanol soluble fraction, ethyl acetate fraction, petroleum ether fraction and the crude methanol extract respectively (Table 4.1).

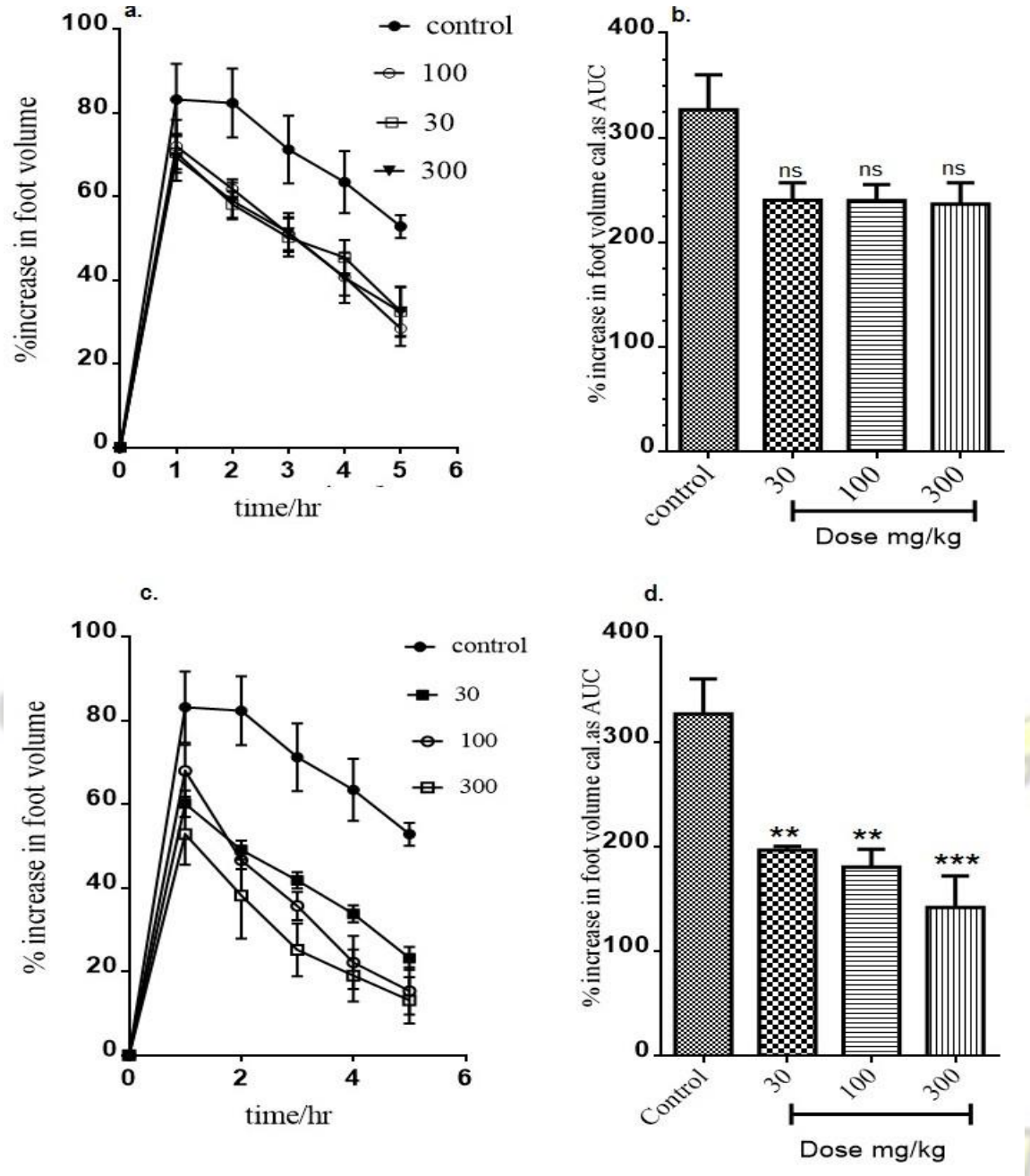


Figure 4.1Effects of the crude extract and petroleum ether fraction (30 – 300 mg/kg, oral) on the time course curve (a and c respectively) and the percentage increase in foot volume (oedema) response expressed as AUC, (b and d respectively) for 5 hours in carrageenan-induced paw oedema in chicks

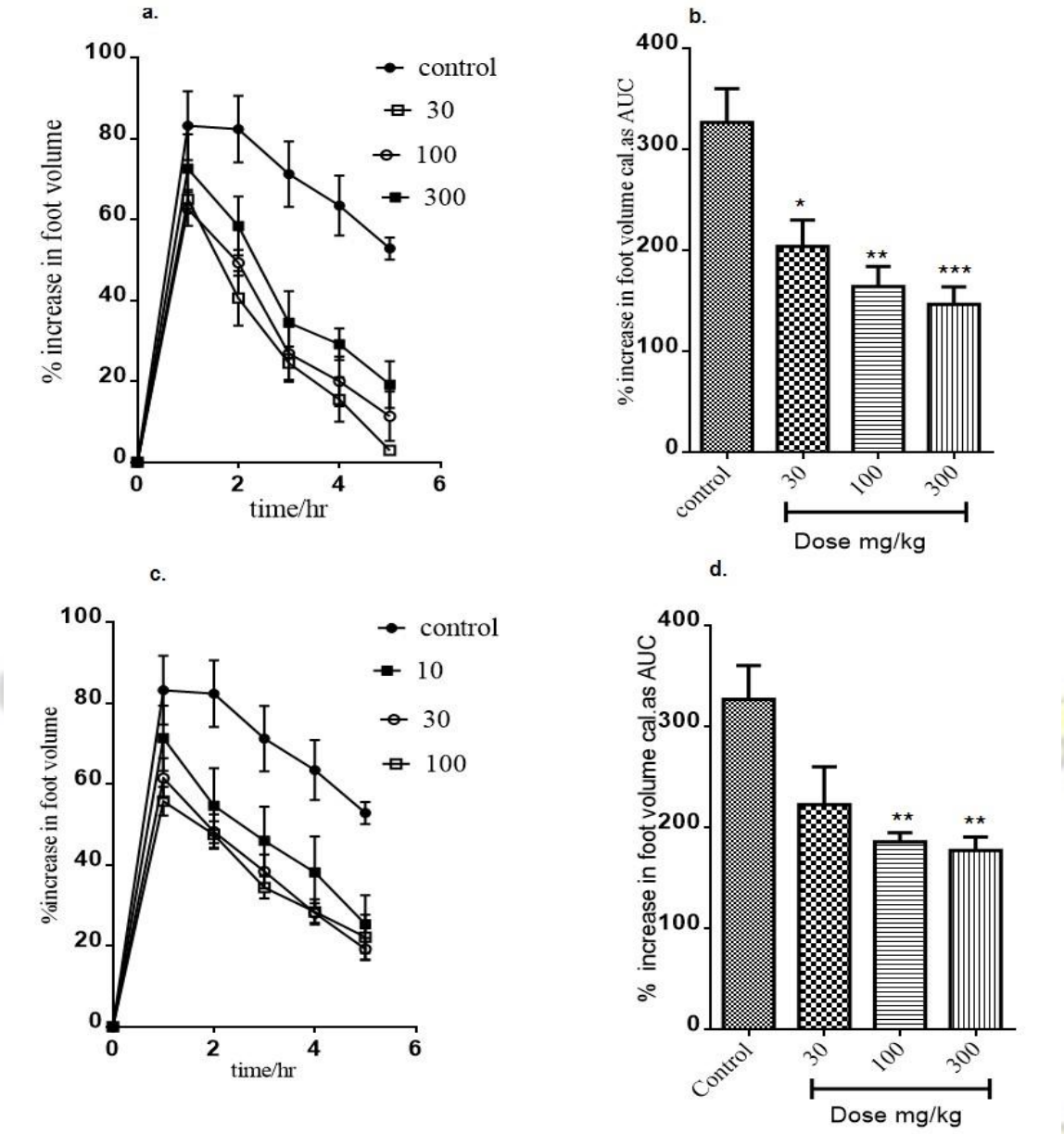


Figure 4.2 Effect of the ethyl acetate extract and methanol fraction (30 – 300 mg/kg, oral) on the time course curve (a and c respectively) and the percentage increase in foot volume (oedema) response expressed as AUC, (b and d respectively) for 5 hours in carrageenan-induced paw oedema in chicks.

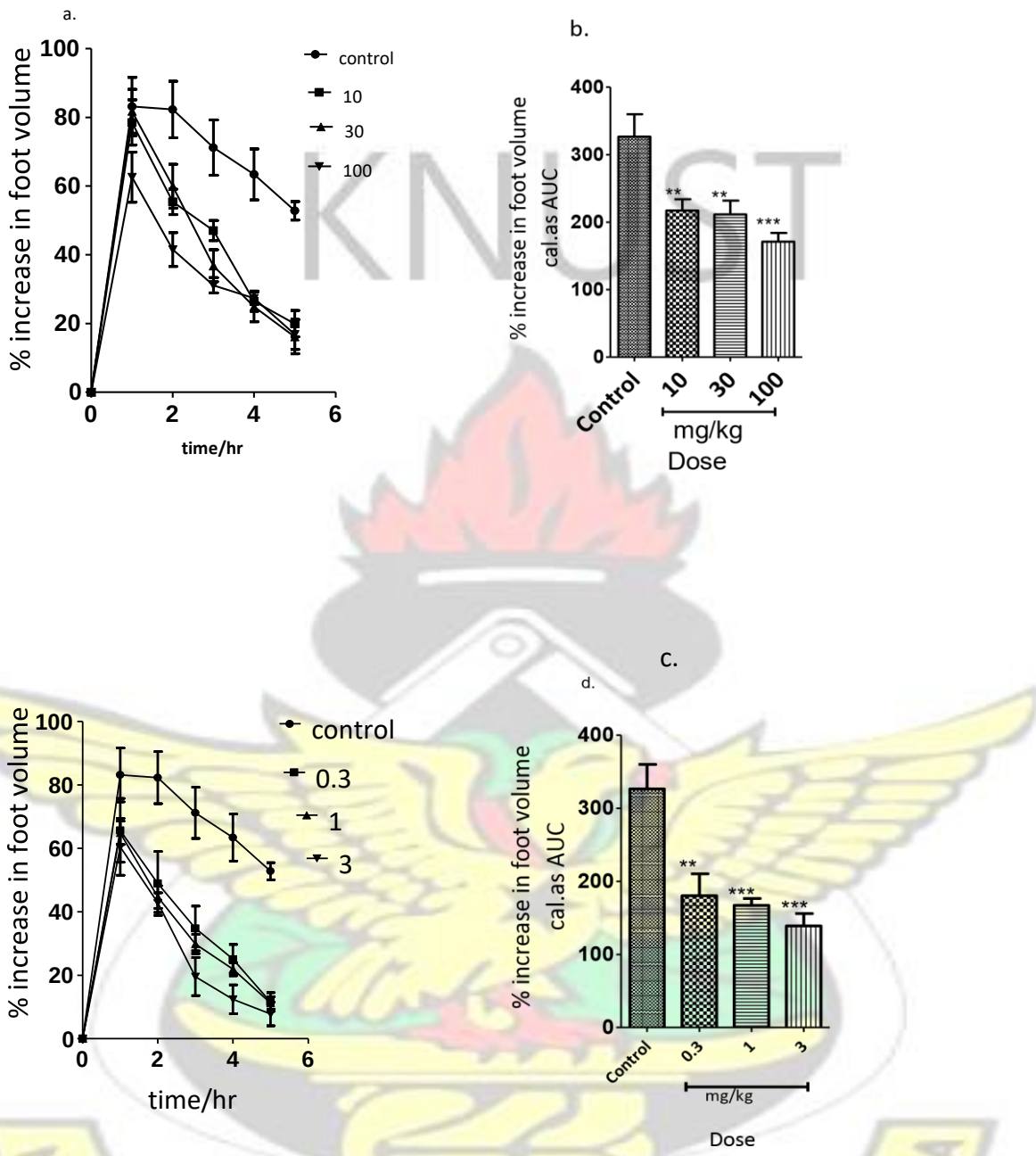


Figure 4.3 Effect of diclofenac (10 -100 mg/kg; *i.p*) and dexamethasone (0.1 – 3 mg/kg; *i.p*) on the time course curve (a and c respectively) and the percentage increase in foot volume (total oedema) response expressed as AUC, (b and d respectively) for 5 hours in carrageenan-induced paw oedema in chicks.

Table 4.1 Effect of fruit extracts and standard drugs on carrageenan-induced oedema

Plant extract/drug	ED_{50} (mg/kg) ± SEM
Crude extract	67 ± 0.65
Petroleum ether	46.21 ± o.67
Ethyl acetate	19.17 ± 2.89
Methanol fraction	16.52 ± 4.65
Dexamethasone	7.19 ± 4.91
Diclofenac	7.55 ± 1.09

4.2 ANTI-INFLAMMATORY ACTIVITY OF ISOLATES

Chromatographic separation of ethyl acetate fraction resulted in the isolation of compounds VDF1, VDF2 and VDF3 which were tested for anti-inflammatory activity

Oral administration of VDF1, VDF2 and VDF3 (10, 30 and 100 mg/kg) exhibited a dose-dependent reduction of oedema in 7-day old chicks. VDF1 showed maximum reduction of 41.01 ± 10.32% at (100 mg/kg) with an ED_{50} of 19.24 ± 0.87. VDF2 exhibited maximum reduction of 45.97 ± 16.19% at (100 mg/kg) with an ED_{50} of 17.47 ± 0.61. Also, VDF3 showed maximum inhibition of 54.22 ± 11.25% at dose (100 mg/kg) with an ED50 value of 30.41± 2.20. Thus, VDF2 exhibited the highest antiinflammatory activity followed by VDF1 and VDF3 respectively (Figure 4.4).

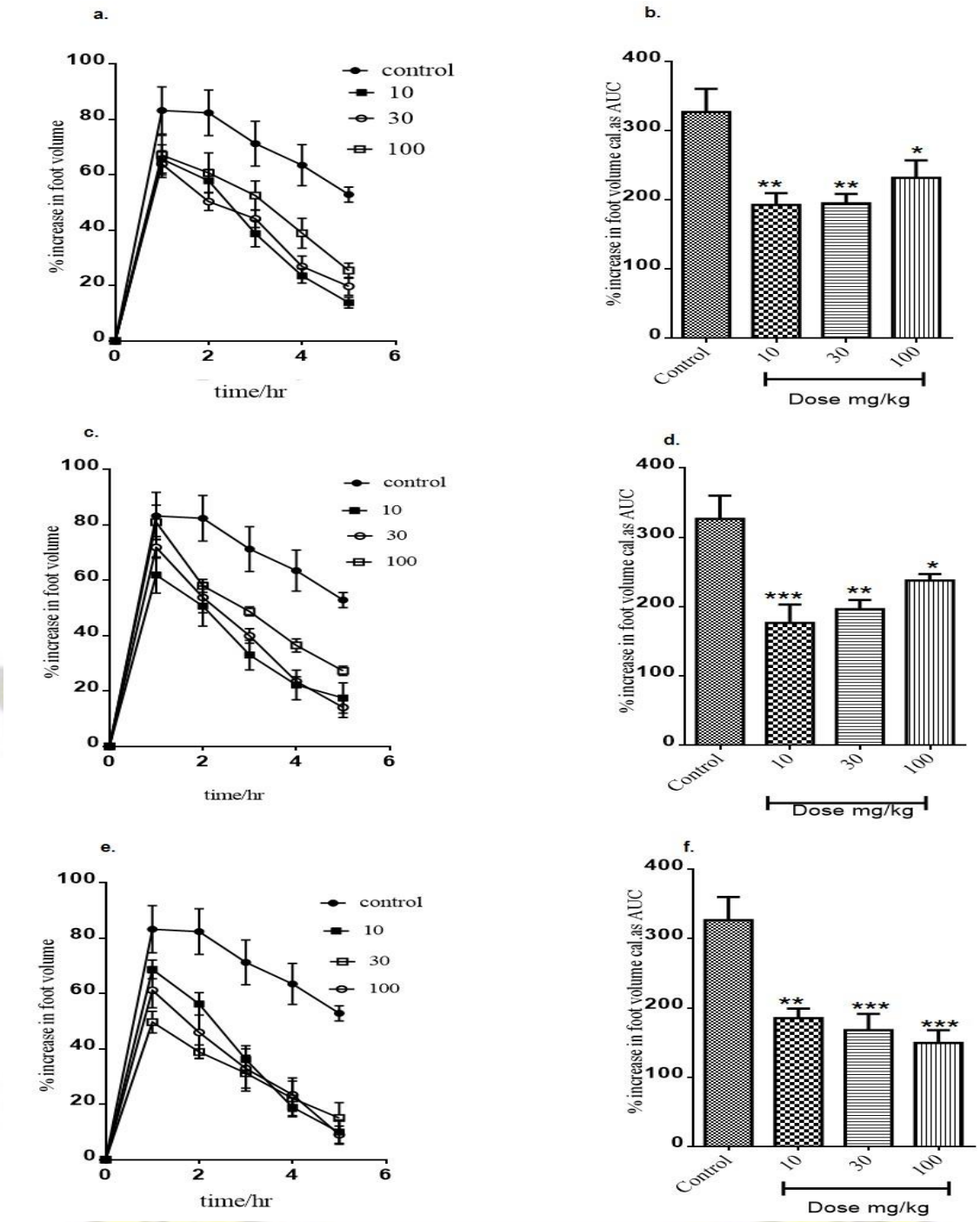


Figure 4.4 Effect of compounds VDF1 VDF2 and VDF3 (10 – 100 mg/kg, oral) on the time course curve (a, c and e respectively) and the percentage increase in foot volume (oedema) response expressed as AUC, (b, d and f respectively) for 5 hours in carrageenan-induced paw oedema in chicks.

4.2.1 Comparable anti-inflammatory activities of isolates, ethyl acetate fraction and crude extract

The isolated compounds showed a comparable dose dependent anti-inflammatory activity to the ethyl acetate fraction from which they were isolated. They however exhibited higher activity than the total crude extract. The order of activity as depicted by the ED_{50} is as follows; VDF2 > ethyl acetate >VDF1 >VDF3 >crude extract. (Table 4.2)

Table 4.2 Comparable anti-inflammatory activities of isolates, ethyl acetate fraction and crude extract

Plant extract/compound	**ED_{50} (mg/kg) ± SEM**
Crude extract	67 ± 0.65
Ethyl acetate	19.17 ± 2.89
VDF1	19.24 ± 0.87
VDF2	17.47 ± 0.61
VDF3	30.41 ± 2.20

4.3 ANTIOXIDANT ACTIVITY OF EXTRACTS

4.3.1 Quantitative antioxidant assay of extracts

Several methods were employed in the evaluation of the antioxidant activity of the extracts. They included DPPH radical scavenging, total antioxidant capacity and ferric reducing power assays.

4.3.1.1 Total antioxidant capacity

Gallic acid was used as the reference drug in the total antioxidant capacity assay. The total antioxidant activity was expressed as milligram (mg) of gallic acid per gram (g) of extract. A calibration curve was drawn for gallic acid (Figure 4.5). Extracts demonstrated increase in antioxidant activity with increase in concentration. The crude methanol extract exhibited the highest antioxidant capacity. The ethyl acetate fraction showed the highest activity followed by the methanol and petroleum ether fractions respectively (Table 4.3)

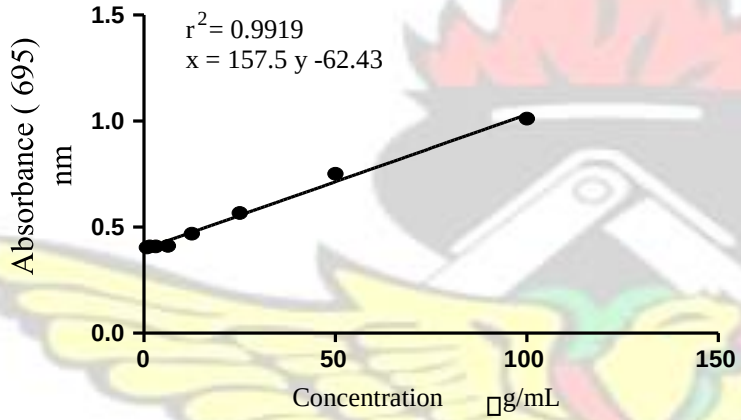


Figure 4.5 Calibration curve of gallic acid

Table

4.3 Total antioxidant capacity of extracts of *V. doniana* fruit

Extract	**Mean Absorbance (mg/GAE/G) ± SD**
Crude extract	64.58 ± 1.12
Ethyl acetate	58.22 ± 3.86
Petroleum ether	31.58 ± 9.72
Methanol fraction	52.53 ± 2.19

4.3.1.2 DPPH radical scavenging assay

The reference drug used in this assay was gallic. The extracts and fractions demonstrated varying degrees of DPPH radical scavenging (Table 4.4) activity, with the ethyl acetate fraction showing the highest activity followed by methanol and petroleum ether fractions. The order of decreasing activity as depicted by IC_{50} is gallic acid > ethyl acetate > methanol fraction > crude extract > petroleum ether.

Table 4.4 DPPH scavenging activity of extracts and reference compound

Extract/compound	**IC_{50} (µg/mL) ± SD**
Crude extract	116 ± 0.57
Petroleum ether	117 ± 0.54
Methanol fraction	104.4 ± 0.65
Ethyl acetate	99.35 ± 0.77
Gallic acid	0.71 ± 0.62

4.3.1.3 Ferric reducing power assay

The extracts and the reference compound, gallic acid exhibited a concentrationdependent reduction of Fe^{3+} to Fe^{2+} (Table 4.5), with the ethyl acetate fraction showing the greatest reducing power followed by the extract, methanol fraction and petroleum ether fractions respectively. The order of decreasing reducing power is gallic acid > ethyl acetate > crude extract > methanol > petroleum ether fraction

Table 4.5 Reducing power of extracts and reference compound

Extract	**Mean absorbance (nm) ± SD**
Crude extract	1.66 ± 0.001
Petroleum ether	0.76 ± 0.002
Ethyl acetate	2.11 ± 0.003
Methanol fraction	1.30 ± 0.02
Gallic acid	1.11 ± 0.002

4.4 ANTIOXIDANT ACTIVTY OF ISOLATES

4.4.1 DPPH radical scavenging assay of isolates

Gallic acid was used as the reference drug in this assay. The compounds; VDF1, VDF2 and VDF3 demonstrated varying DPPH radical scavenging activities (Table 4.6), with VDF2 showing the highest activity followed by VDF3 and VDF1 respectively. The order of activity as depicted by the IC_{50} is gallic acid >VDF2 > VDF3 > VDF1 (Table 4.6).

Table

4.6 DPPH scavenging activity of compounds isolated from *V. doniana*

Compounds	**IC_{50} (µg/mL) ± SD**
VDF1	2.80 ± 0.56
VDF2	1.20 ± 0.94
VDF3	1.83 ± 0.53
Gallic acid	0.71 ± 0.62

4.4.2 Ferric reducing power assay

The isolates (VDF1,VDF2 and VDF3) and the reference compound, gallic acid (at concentrations (0.78 - 100 µg/mL) exhibited a concentration-dependent reduction of Fe^{3+} to Fe^{2+} (Table 4.7) with isolate VDF2 showing the highest reducing power followed by VDF1 and VDF3 respectively. The order of reducing power is VDF2 > gallic acid > VDF1 > VDF3.

Table 4.7 Reducing power of isolates and reference compound

Compound	**Mean absorbance (nm) ± SD**
VDF1	0.26 ± 0.001
VDF2	2.20 ± 0.01
VDF3	0.21 ± 0.002
Gallic acid	1.11 ± 0.002

4.4.3 Comparable antioxidant activities of isolates, ethyl acetate fraction and crude extracts

The isolated compounds showed a higher antioxidant activity than the total crude extract and the ethyl acetate fraction with isolate VDF2 showing the highest activity (Table 4.8)

Table 4.8 Antioxidant activities of Isolates, ethyl acetate fraction and crude extract

Extract/compound	**IC_{50} (µg/mL) ± SD**	**FRAP (Mean abs nm) ± SD**
Crude extract	116 ± 0.57	1.66 ± 0.001
Ethyl acetate	99.35 ± 0.77	2.11 ± 0.003
VDF1	2.80 ± 0.56	0.26 ± 0.001
VDF2	1.20 ± 0.94	2.20 ± 0.01
VDF3	1.83 ± 0.53	0.21 ± 0.002

4.5 ACUTE TOXICITY OF CRUDE EXTRACT

There were no signs of behavioural, autonomic and CNS stimulation/depression in all the treated doses of the extract. LD_{50} by oral route was also estimated to be beyond 3000 mg/kg.

4.6 SUBACUTE TOXICITY OF CRUDE EXTRACT

Subacute studies revealed an increase in haematological parameters (RBCs, HB PCV). as well as lymphocyte counts. It didn't affect MCH, MCHC and MCV. Neutrophils decreased significantly at all doses. Platelet count was however, not affected Liver

Table

enzymes (ALT, AST, ALP and GGT), serum proteins and bilirubin levels were not increased. Renal function was also not significantly affected (Table 4.9-10).

4.9 Effect of crude extract (100,300 and 1000 mg/kg) on haematological parameters

Hematological parameter	**0**	**100**	**300**	**1000**
WBC	**25.40±1.52**	**37.96±5.99**	**26.68±5.03**	**19.52±2.23**
RBC	6.93±0.09	7.90±0.43	7.63±0.65	7.10±0.17
HB	**14.17±0.17**	**17.00±0.79***	**16.32±1.19**	**15.18±0.27**
HCT	**39.88±0.73**	**47.48±1.53***	**44.16±2.92**	**41.62±0.90**
MCV	57.99±0.25	56.80±1.11	58.38±1.28	58.78±1.42
MCH	21.90±0.11	21.50±0.29	21.46±0.37	21.38±0.38
MCHC	37.60±0.20	35.92±2.01	36.88±0.57	36.45±0.23
PLATELET	562.1±21.25	421.8±42.42	584.8±54.82	630.6±68.53
LYMPHOCYTE	**81.84±0.72**	**86.84±1.29***	**86.18±0.83**	**84.52±2.01**
NEUTROPHIL	**8.27±0.54**	**5.02±0.77***	**5.14±0.34***	**5.84±1.12**

Table 4.10 Effect of crude extract (100,300 and 1000 mg/kg) on serum biochemistry parameters

Serum biochemistry	**0**	**100**	**300**	**1000**
ALT	133.8±5.11	116.2±8.53	132.8±31.17	95.58±9.20
AST	249.7±9.00	203.0±13.75	210.4±22.62	265.0±18.76
ALP	212.0±7.11	233.4±23.46	186.30±27.61	206.80±28.07
GGT	5.00±0.38	6.83±0.64	4.32±0.46	3.70±0.55
Total protein	72.95±0.99	69.10±5.73	72.36±4.57	68.73±3.73
Albumin	30.51±0.63	29.20±1.77	35.32±3.90	29.55±2.58
Globulin	42.44±1.09	39.90±4.49	37.04±6.56	39.18±1.63
Bilirubin	6.27±0.19	6.70±0.73	7.40±1.04	5.73±0.47
Direct bilirubin	2.87±0.14	2.86±0.52	3.82±0.29	2.10±0.29
Indirect bilirubin	3.40±0.18	3.84±0.41	3.58±0.92	3.63±0.48
Urea	11.30±0.34	9.56±1.45	10.06±1.51	10.10±1.29
Creatinine	67.17±1.88	79.06±5.07	75.68±5.49	68.00±4.86

Table

4.7 CHARACTERISATION AND IDENTIFICATION OF ISOLATED COMPOUNDS

Chromatographic separation of the crude methanol fruit extract of *V. doniana* over sephadex LH-20 and silica gel led to the isolation of three (3) compounds. The characterisation of the compound was carried out by spectroscopic analysis including NMR and IR and data compared with reported literature.

4.7.1 Characterisation of VDF1 as 3β-hydroxyolean-12-en-28-oic acid

Compound **VDF1** was obtained as a white amorphous powder whose melting point was determined to be 298-300˚C. The IR spectrum (Appendix 1F) revealed characteristic signals for hydroxyl, carbonyl and olefinic groups at *Vmax* of 3457 cm^{-1}, 2924 cm^{-1}, 2852 cm^{-1} and 1687 cm^{-1} . The molecular formular was determined as $C_{30}H_{48}O_3$ suggesting that VDF1 is a triterpenoid.

The ^1H NMR spectrum of VDF1 (500 MHz, $CDCl_3$) (Appendix 1A) indicated the presence of seven aliphatic methyl singlets at $^{\delta}H$ 0.75 (H-26), $^{\delta}H$ 0.77 (H-24), $^{\delta}H$ 0.90 (H-29), $^{\delta}H$ 0.91 (H-25), $^{\delta}H$ 0.92 (H-30), $^{\delta}H$ 0.98 (H-23) and $^{\delta}H$ 1.13 (H-27); and an olefinic proton at $^{\delta}H$ 5.28 (H-12, b, s). The spectrum also revealed signals at $^{\delta}H$ 3.21 (1H, d) and $^{\delta}H$ 3.23 (1H, m) which were assigned to methine protons on oxygenated carbons. The DEPT 135 spectrum (Appendix 1B) revealed thirty (30) carbon signals that included seven (7) methyl carbons $^{\delta}C$ 28.10 (C-23), $^{\delta}C$ 15.55 (C-24), $^{\delta}C$ 15.32 (C-25), $^{\delta}C$ 17.14 (C-26), $^{\delta}C$ 25.96 (C-27), $^{\delta}C$ 33.09 (C-29) and $^{\delta}C$ 23.60 (C-30) as well as an acidic carbon at $^{\delta}C$ 183.47 (C-28). It also showed ten (10) methylene, five (5) methine and seven (7) quaternary carbons. A broad singlet at $^{\delta}H$ 5.28 in the ^1H NMR was corroborated by resonances at $^{\delta}C$ 122.67 and $^{\delta}C$ 143.61 in the DEPT spectrum. These were assigned to the carbon-carbon double olefinic bond at C-12 and C-13 respectively. The spectrum also revealed a signal for one (1) oxymethine carbon at $^{\delta}C$ 79.04 (C-3). The carboxylic acid functionality at C-17 was confirmed based on HMBC correlations between the resonances at $^{\delta}C$ 2.83 (H-18) with $^{\delta}C$ 46.53 (C-17) and the carboxyl carbon signal at $^{\delta}C$ 183.47 (C-28). These data indicated that VDF1 has an olean-12-28-oic acid nucleus with the

hydroxyl group in its structure (Figure 4.10) (Vlahov *et al.,* 2008; Mahato and Kundu, 1994).

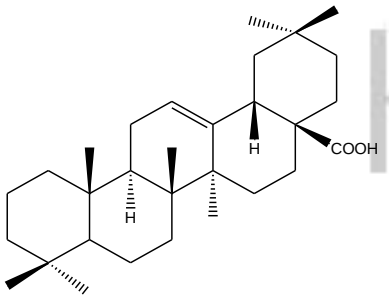


Figure 4.6 An oleanane-type triterpenoid nucleus

The hydroxyl group at C-3 of the oleanane structure was confirmed from the chemical shift and resonance of the proton attributed to H-3 (3.21, d) which showed an HSQC (Appendix 1D) cross peak with the oxymethine carbon at $^{\delta}$C 79.04 (C-3).

The HMBC spectrum (Appendix 1E) showed a correlation between methyl proton peak at $^{\delta}$H 0.77 (H-24) with carbon signals at $^{\delta}$C 79.04 (C-3), $^{\delta}$C 38.75 (C-4), $^{\delta}$C 55.19 (C-5) and $^{\delta}$C 28.10 (C-23). Again, an HMBC correlation existed between the methyl proton peak at $^{\delta}$H 0.98 (H-23) with carbon signals at $^{\delta}$C 79.04 (C-3), $^{\delta}$C 38.75 (C-4), $^{\delta}$C 55.19 (C-5) and $^{\delta}$C 15.32 (C-24). Correlations between methyl proton peaks at $^{\delta}$H 0.75 (H-26) and carbon signals at $^{\delta}$C 47.62 (C-9) and $^{\delta}$C 41.57 (C- 14) as well as $^{\delta}$H 0.92 (H-30) and carbon signals at $^{\delta}$C 45.88 (C-19) and $^{\delta}$C 33.79 (C-21) from the HMBC spectrum was also evident. Correlation between H-18 ($^{\delta}$H 2.83) and $^{\delta}$C 143.61 (C-13) and long correlations from H-18 and H-27 to $^{\delta}$C 122.67 (C-12) together with ^{1}H-^{1}H COSY correlation (Appendix 1C) between H-11 and H-12 ($^{\delta}$C 5.23) confirmed the olefinic group at C-12 and C-13 (Ngoc and My., 2018).

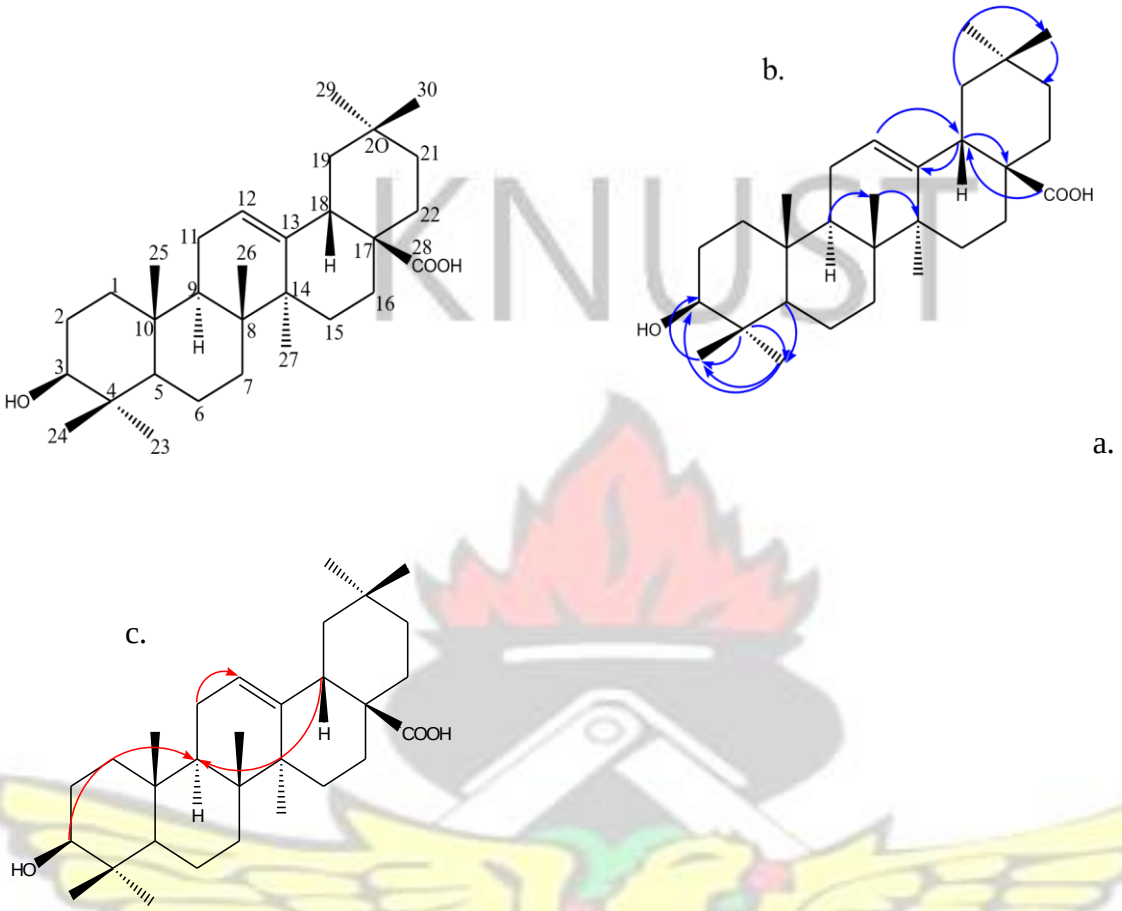


Figure 4.7 a. Oleanolic acid b. HMBC correlation c. COSY correlation

Table 4.11 ^{1}H and ^{13}C NMR data of VDF1 and oleanolic acid in deuterated chloroform ($CDCl_3$)

Position	**Oleanolic acid (**Vlahov *et al.*, 2008**)**		**VDF1**	
	$^{\delta}$ H (mult)	$^{\delta}$ C	$^{\delta}$ H (mult)	$^{\delta}$ C
1		38.40		38.38 (CH_2)
2		27.20		27.17 (CH_2)
3	3.22, d	79.0	3.21, d	79.04 (CH)
4	-	38.70	-	38.75 (C)
5	0.74	55.20	0.73, d	55.19 (CH)
6		18.30		18.29 (CH_2)
7		32.60		32.60 (CH_2)
8	-	39.30	-	39.25 (C)
9	1.54	47.60	1.55	47.62 (CH)
10	-	37.10	-	37.08 (C)
11		22.90		22.91 (CH_2)
12	5.29, br, s	122.60	5.28, br, s	122.67 (CH)
13	-	143.60	-	143.61 (C)
14	-	41.60	-	41.57 (C)
15		27.70		27.67 (CH_2)
16		23.4		23.39 (CH_2)
17	-	46.50	-	46.53 (C)
18	2.83, dd	41.00	2.83, dd	40.96 (CH)
19		45.90		45.88 (CH_2)
20	-	30.60	-	30.69 (C)
21		33.80		33.79 (CH_2)
22		32.40		32.44 (CH_2)
23	0.99,s	28.10	0.98, s	28.10 (CH_3)
24	0.74, s	15.30	0.77, s	15.55 (CH_3)
25	0.91, s	15.50	0.91, s	15.32 (CH_3)
26	0.77, s	17.10	0.75, s	17.14 (CH_3)
27	1.14, s	25.90	1.13, s	25.96 (CH_3)
28	-	182.30	-	183.47 (C)
29	0.91, s	33.00	0.90,s	33.09 (CH_3)
30	0.93, s	23.50	0.92, s	23.60 (CH_3)

The structures of compounds VDF2 and VDF3 have not being elucidated.

4.8 HPLC quantification of oleanolic acid

4.8.1 Validation results

4.8.1.1 Precision-Reapeatability

Investigation considered six replicate injections of 200 ppm concentration of analyte for both intraday and interday precision studies (The RSD < 2%)

Table 4.12 Results obtained from intraday and interday precision studies

Sample	**Intraday precision studies**	**Interday precision studies**
	Oleanolic acid (200 ppm)	**Oleanolic acid (200 ppm)**
	Peak area	**Peak area**
1	1913516.1	1935211.8
2	1910611.3	1943907.1
3	1917198.6	1955401.4
4	1922602.2	1952882.4
5	1919255.3	1951519.9
6	1919054.9	1952209.9
Mean ± SD	1917040 ± 4328.47	1948522 ± 7581.21
RSD	0.23%	0.39%

4.8.1.2 Linearity and range

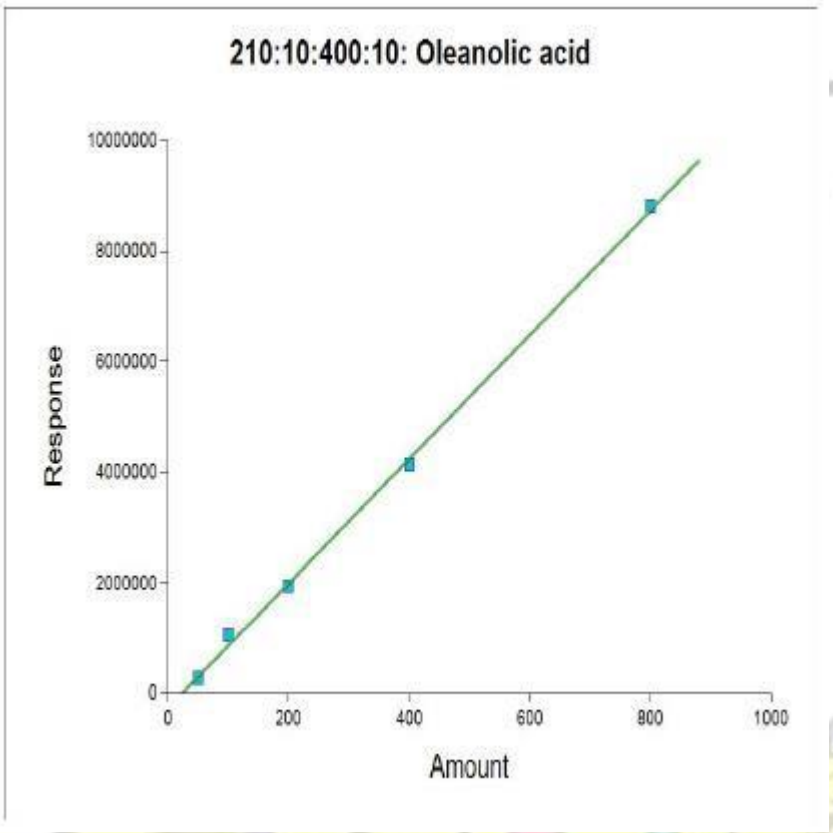


Figure4.8 Linearity curve for oleanolic acid

Table 4.13 Statistical data from linearity studies for oleanolic acid

Parameter	**Assay**
Slope	1.12492×10^{4}
Y-intercept	-2.39441×10^{5}
r^2	0.998861

A linear dynamic range of 50 ppm to 800 ppm was obtained with a correlation coefficient (r^2) of 0.9989.

4.8.1.3 Accuracy

A mean recovery for oleanolic acid was determined to be 80.97%.

4.8.1.4 Robustness

Table 4.14 Results obtained from robustness studies

Sample	**Flow rate**	**Wavelength**
	Peak area	**Peak area**
1	5136803.7	5185096.8
2	5063456.5	5063456.5
3	4949600.8	5091135.7
Mean ± SD	5049954 ± 94329.27	5091136 ± 83630.12
RSD	1.87%	1.64%

4.8.1.5 Limit of detection and limit of quantification

Limits of detection and quantification for oleanolic acid were determined as 26.70 ppm and 80.92 ppm respectively.

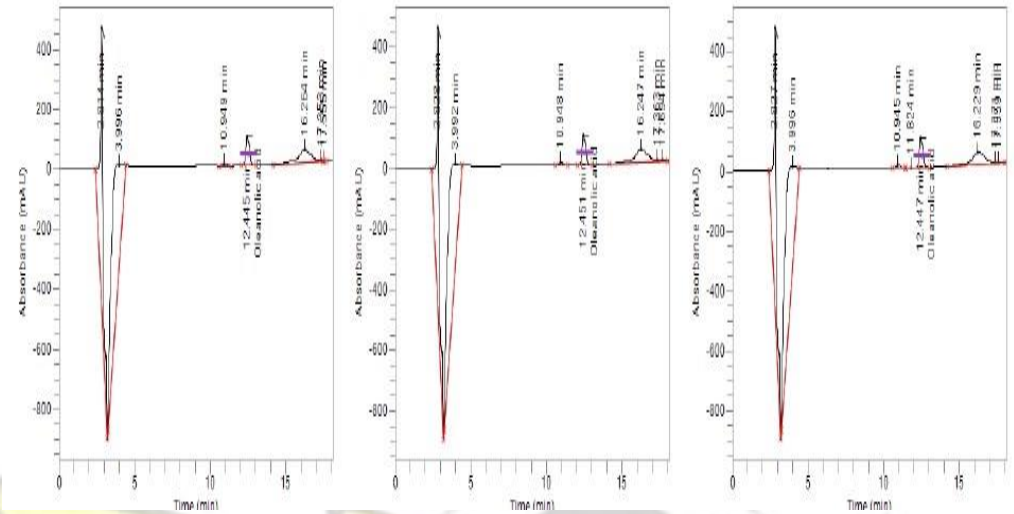
4.8.1.6 Determination of oleanolic acid content of crude extract

Table 4.15 Amount of oleanolic acid in *Vitex doniana* fruit extract

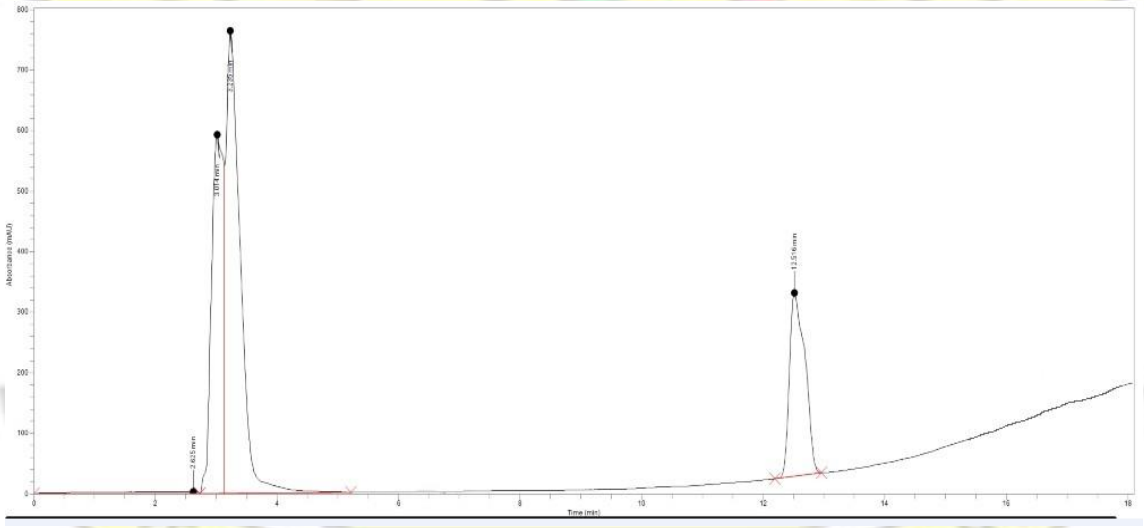
Sample injection	**Amount of oleanolic acid (ppm)**
1	168.047262
2	167.710357
3	169.202875
Mean ± SD	168.3202 ± 0.78

168.3 ppm = 84.15 mg/g, Hence the amount of oleanolic acid in the crude extract was estimated to be 84.15 mg/g.

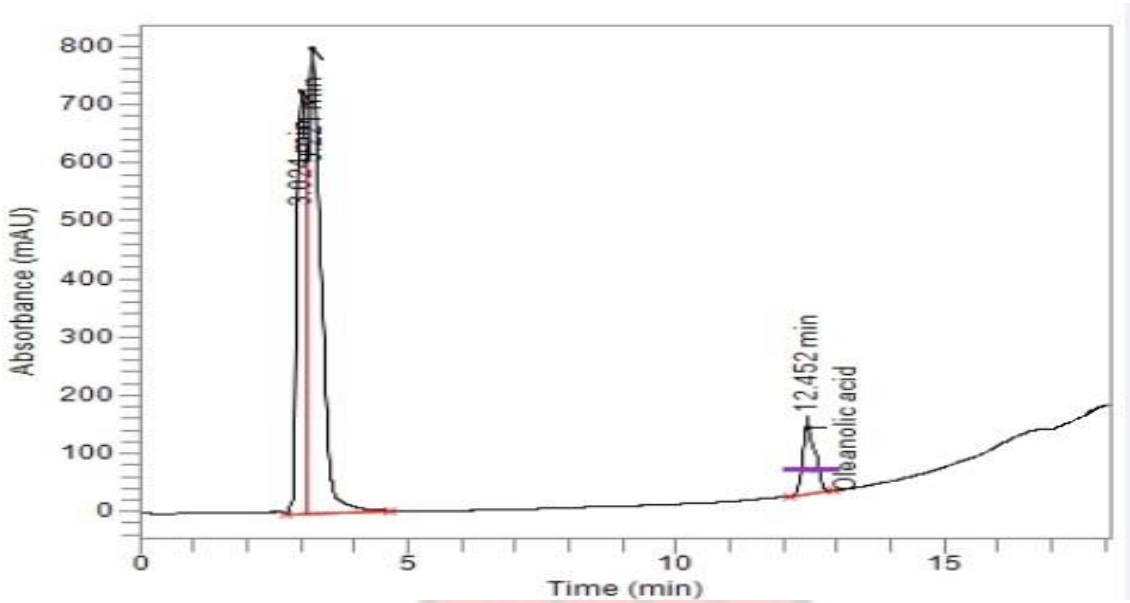
a.



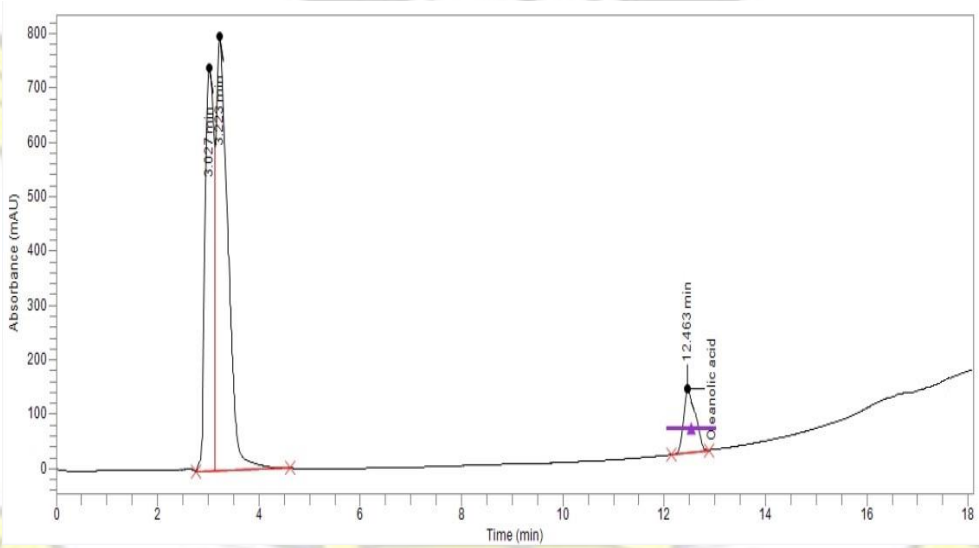
b.



c.



d.



e.

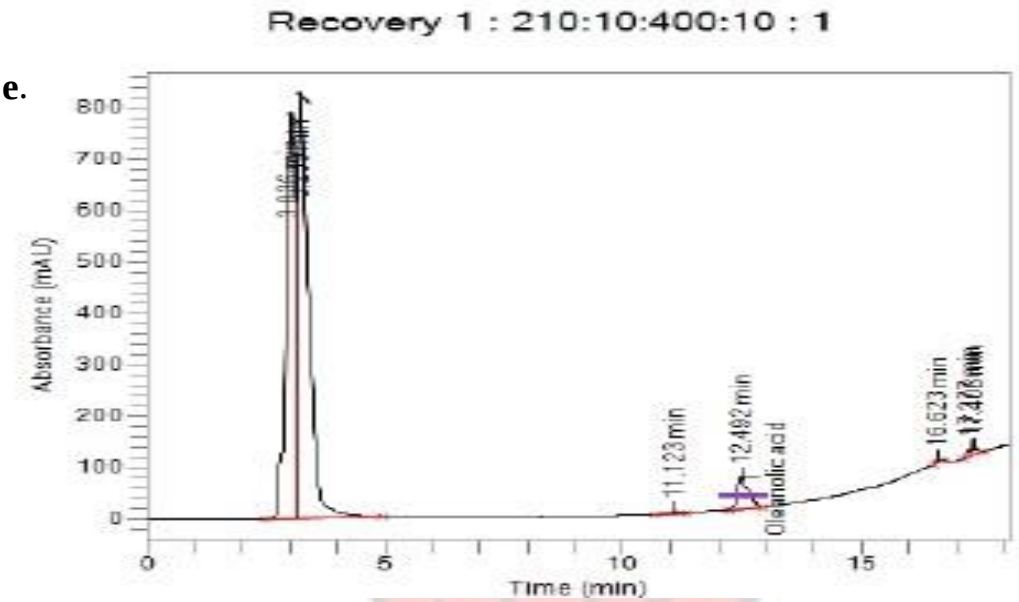


Figure 4.9 Chromtogram for a. crude extract b. oleanolic acid c. intraday precision studies d. interday precision studies e. recovery studies

CHAPTER FIVE

DISCUSSION

5.1 INTRODUCTION

Globally, the significant role herbal medicines play in the delivery of primary health care in developing countries cannot be overemphasize. Providing scientific justification for their use would not only promote/enhance their possible integration into mainstream healthcare systems but also ensure safety of consumers. Moreover, establishing the phytoconstituents responsible for their activity will serve as standardization tool as well as lead compounds in the discovery and development of new therapeutic agents.

5.2 ANTI-INFLAMMATORY ACTIVITY.

This study aimed at evaluating the anti-inflammatory potential of the fruit extracts of *Vitex doniana* using the carrageenan-induced paw oedema model in 7-day old chicks and to isolate phytoconstituents which might be responsible for the observed activity. Carrageenan causes the formation of oedema by releasing chemical regulators/mediators following its injection into the cell. The formation of oedema by carrageenan administration into the paw of mice occurs in two phases (biphasic). The release of histamine and serotonin constitutes the first phase (1 hour). The second phase (over an hour) is caused by the release of bradykinin, prostaglandins, proteases and lysosome (Hafeez *et al*., 2013). All extracts demonstrated a significant ($P< 0.05$) reduction of oedema after the first hour (Figures 4.1-2) and thus, probably, blocked the synthesis and release of prostaglandins, lysozyme and proteases (Hafeez *et al*., 2013). In a similar study reported by Ochieng *et al*, (2013), the methanol crude extract of the stem bark of the same plant showed significant anti-inflammatory activity with the butanol and ethyl acetate fractions showing maximum activity. The observed anti-inflammatory activity of the extracts (Table 4.1) in this study gives credence to the use of the plant in treating inflammatory disorders in traditional medicine. This is the first report of the antiinflammatory activity of the fruits of *V. doniana*. Fractionation of the crude extract afforded a more active methanol (ED_{50}=16.52 ± 4.65 mg/kg) and ethyl acetate fractions with the pet-ether fraction showing the least activity (Table 4.1). This study has demonstrated that the fruit extracts of *Vitex doniana* could be of immense benefit to communities where this plant grows or is used.

5.3 ANTIOXIDANT ACTIVITY

Overproduction of reactive oxygen species lead to oxidative stress which is implicated in the complications of inflammatory conditions. Thus, the antioxidant capacity of plant extracts may play a key role in mitigating the damaging effects of free radicals. This study aimed at evaluating the antioxidant activity of the fruit extract of *Vitex doniana* employing *in-vitro* assays including total antioxidant capacity, DPPH radical scavenging and ferric reducing power. Purification of the total extract afforded a higher DPPH scavenging as well as ferric reducing activity as determined for the ethyl acetate and methanol fractions (Table 4.4-5) respectively. A higher total antioxidant capacity was recorded for the crude methanol extract compared with the fractions (Table 4.3) hence, individual components of the total extract may have worked synergistically to give the combined higher total antioxidant activity. It can then be inferred that the fruit extract of *Vitex doniana* has a considerable antioxidant property and may play an important role in mitigating the damaging effects of free radicals released during the inflammatory cascade.

5.4 TOXICITY STUDIES

The fruit extract of *Vitex doniana* appears to be safe even at 3000 mg/kg body weight and have beneficial effects on haematological and biochemical parameters (Table 4.910). It significantly increased the number of red blood cells (RBC) as wells as their hemoglobin (Hb) content and the pack cell volume (PCV). The increase in lymphocyte count at all doses coupled with the mild increase in white blood cells (WBC) especially in low doses of the extract (Table 4.9-10) is suggestive of a possible immunestimulatory effect. It however did not affect platelet count. Similar findings on haematopoeitic system have been shown by other authors (Abdulrahman *et al.*, 2010). Vitex did not increase liver

function enzymes, serum proteins as wells as bilirubin levels. Renal function was also not affected significantly by the plant extract. Elsewhere, high doses of the aqueous root bark extract was found to cause mild necrosis of the liver and kidney (Abdulrahman *et al*., 2007). The oral and intraperitoneal (*i.p).* LD_{50} values of the methanol stem bark extract in a similar study reported by Ochieng *et al*., (2013) was found to be greater than 5000 mg/kg body weight. Thus, per this study, consumption of the fruits of *Vitex doniana* has no toxic effect.

5.5 BIOACTIVITY OF ISOLATED COMPOUNDS

Three (3) compounds, oleanolic acid (VDF1), VDF2 and VDF3 were isolated from the ethyl acetate extract of the fruit of *Vitex doniana*. VDF2 and VDF3 have not been characterised. These compounds were evaluated for their anti-inflammatory and antioxidant activities. All compounds demonstrated a dose-dependent anti-inflammatory activity (Table 4.2 and Figure 4.4) and a concentration dependent DPPH scavenging and ferric reducing activities (Table 4.6-7). The observed higher activities of the isolated constituents highlight the importance of purification of some medicinal plant extracts to afford higher efficacies.

The triterpenoid, oleanolic acid and its isomers occur abundantly in nature either as aglycones or free acids. Oleanolic acid has been isolated from many plants including *Olea europeae, Syzygium aromaticum, Calendula officinalis, Eucalyptus globulus, Salvia officinalis, Satureja montana, Rosmarinus officinalis* and *Ocimum basilicum* (Jager *et al*., 2009). The compound has been reported to exhibit a dose-dependent inhibition of croton-oil induced ear oedema in mice. The basic carbon nucleus is thought not to have any significant influence on the activity. However, a carboxyl or alcoholic group at carbon-20

and 30 increased the activity. It also selectively inhibited COX-2 catalyzed prostaglandin biosynthesis with an IC_{50} of 295 µM and a selectivity ratio of 0.8. The *meta* position of the carboxyl group is reported to essentially influence the inhibitory activity of the complement system. At IC_{50} of 6.42 µM, oleanolic acid is reported to inhibit elastase, an enzyme which hydrolyses elastin to promote the migration of stimulated pro-inflammatory cells. This activity is reported to be dependent on the presence and orientation of two (2) reactive groups on the molecule. The inhibition of nitric oxide (NO) production by oleanolic acid has also been reported. In an experimentally-induced gastric ulcer model in rats, oleanolic acid exhibited a gastroprotective effect. Oxidation of hydroxyl (OH) group at cabon-3 is thought to decrease the activity. Studies have shown that oleanolic acid possess antitumour activity (IC_{50}= 26.74 µg/mL) by inhibiting the growth of human leukaemia and lymphoma cells specifically, P3HRI. The hepatoprotective, antidiabetic and antihypertensive activities have also been reported (Sun *et al*., 2006). The observed activity in this study and that reported in literature indicate that oleanolic acid and the uncharacterised compounds VDF2 and VDF3 exhibit considerable anti-inflammatory and antioxidant activities and might be responsible for the bioactivity of the fruit of *Vitex doniana*.

5.6 HPLC QUANTIFICATION OF OLEANOLIC ACID

Quality control of herbal products present with challenges due to the myriads of phytochemicals present. The compound approach in the standardisation of herbal or natural products is reportedly one of the best ways of ensuring their quality and purity. This informed the development of a gradient HPLC method for the detection and

quantification of one of the bioactive components to serve as a quality assurance parameter for the fruit of *Vitex doniana.*

A C18 reversed phase stationary phase successfully ensured the efficient separation and resolution of the isolated oleanolic acid. An amount of 84.15 mg/g of oleanolic acid with a retention time of 12.450 ± 0.03 minutes was obtained (Table 4.15). Two (2) other unknown components within the crude extract were also separated and resolved at 10.949 min and 16.264 min respectively. This indicates that the sensitive method developed could be used to identify and quantify oleanolic acid even in the presence of other components. The method was also validated as per ICH guidelines using the isolated oleanolic acid. Validation parameters include; precision (interday and intraday), linearity, recovery, robustness, limit of detection and limit of quantification.

A dynamic linear range of 50 ppm to 800 ppm with a correlation coefficient (r^2) of 0.9989 provides the needed evidence of a good correlation between various concentrations of isolated oleanolic acid with peak areas obtained. The interday and intraday precision obtained (Table 4.12) were all less than 2.0 % as specified per ICH guidelines. This shows the repeatability of the developed HPLC method. Other validation parameters like recovery and robustness provided reliable data that can enhance the qualitative and quantitative determination of pure oleanolic acid or when present in crude extracts. A mean of 80.97% was obtained for oleanolic acid for the recovery determination. By varying the wavelength of maximum absorbance (from 210 nm to 201 and 215 nm), a RSD of 1.64% was obtained and this shows the robustness of the developed method as the RSD < 2.0%. A change in flow rate from 1.0 mL/min to 0.9 and 1.1 mL/min all gave a RSD of < 2.0% (Table 4.14). A sensitive reverse phase method with LOD and LOQ of

26.70 and 80.97 ppm respectively were determined. This provides evidence for the highly sensitive method developed.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 CONCLUSIONS

This study has demonstrated that extracts of fruit of *Vitex doniana* shows considerable anti-inflammatory and antioxidant activities. The bioactivities of the fruit were highest in the ethyl acetate and methanol extracts. The fruit extract may also have immunomodulatory activity. Oleanolic acid, VDF2 and VDF3 (uncharacterized) were isolated from the ethyl acetate extract. The compounds exhibited anti-inflammatory and antioxidant activities and thus contributes immensely to the bioactivity of *Vitex doniana* observed in this study. The amount of oleanolic acid in the crude extract was estimated to be 84.15 mg/g. Again, high performance liquid chromatography (HPLC) remains an essential tool in establishing the assurance of quality and the determination of oleanolic acid in its isolated and crude forms. The present studies have also shown the fruits of *Vitex doniana* to be safe.

6.2 RECOMMENDATIONS

Immunomodulatory studies of the fruit extract should be considered in future work. Future studies should also focus on isolating anti-inflammatory and antioxidant constituents from the methanol fraction of the fruits.

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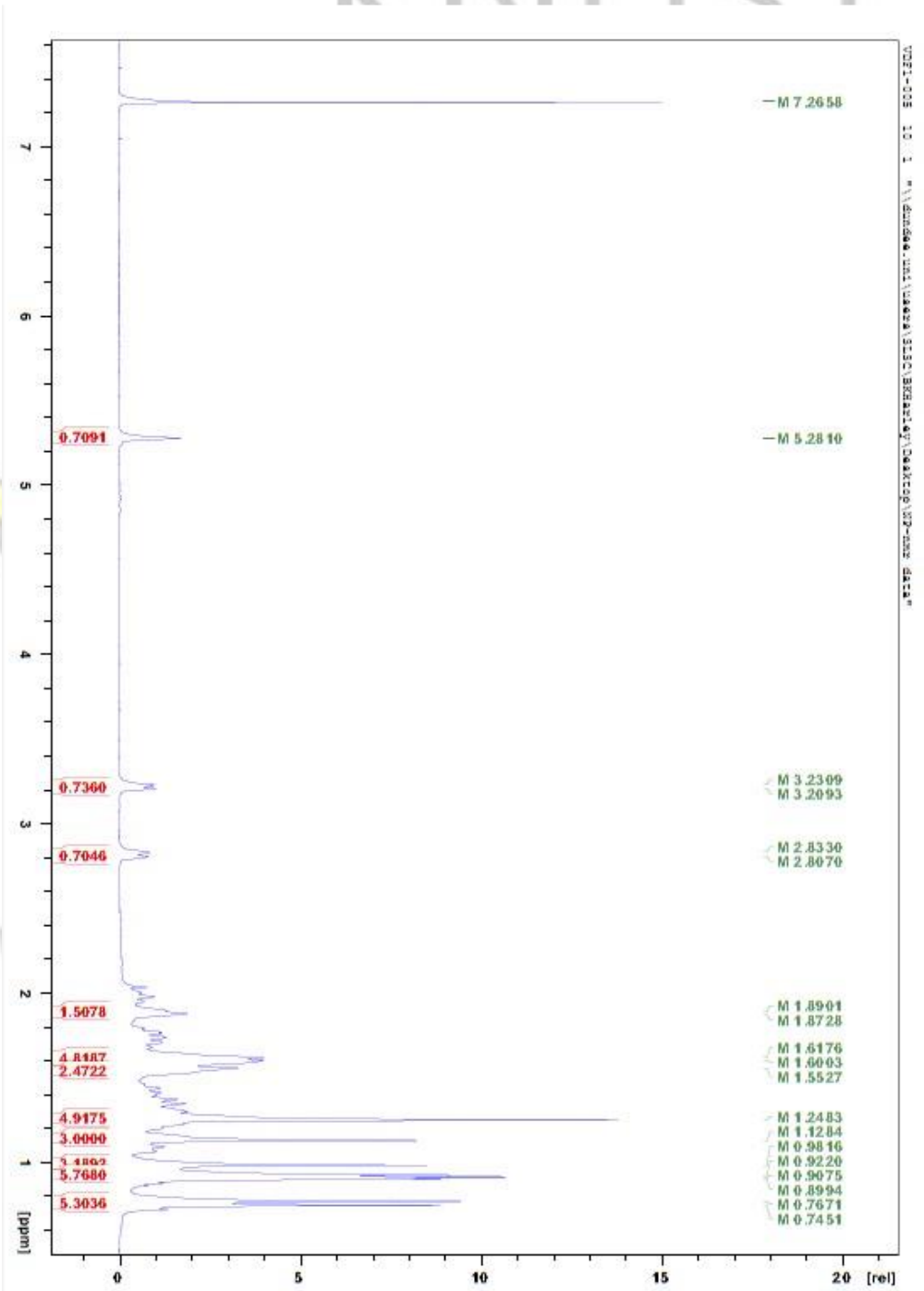
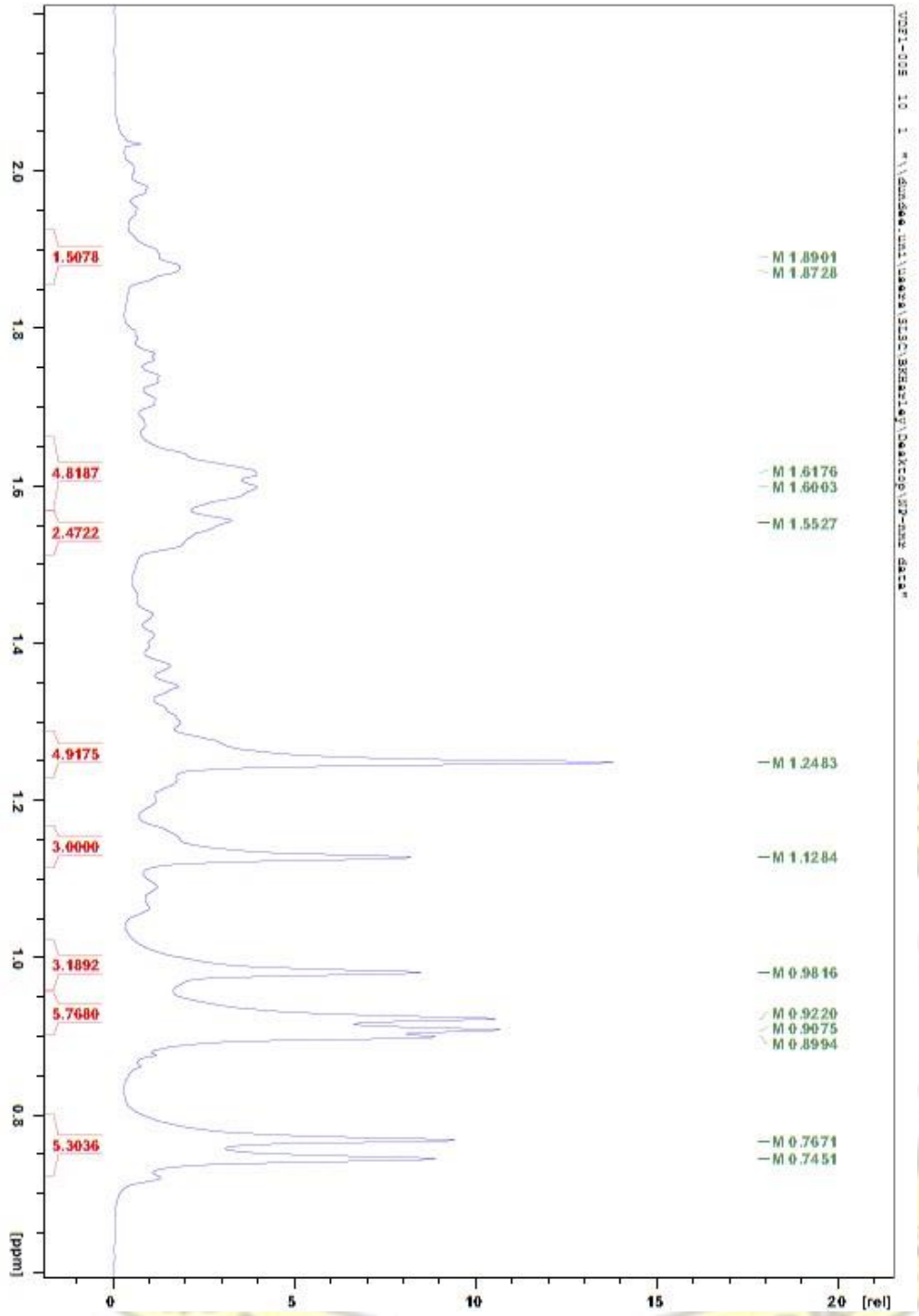
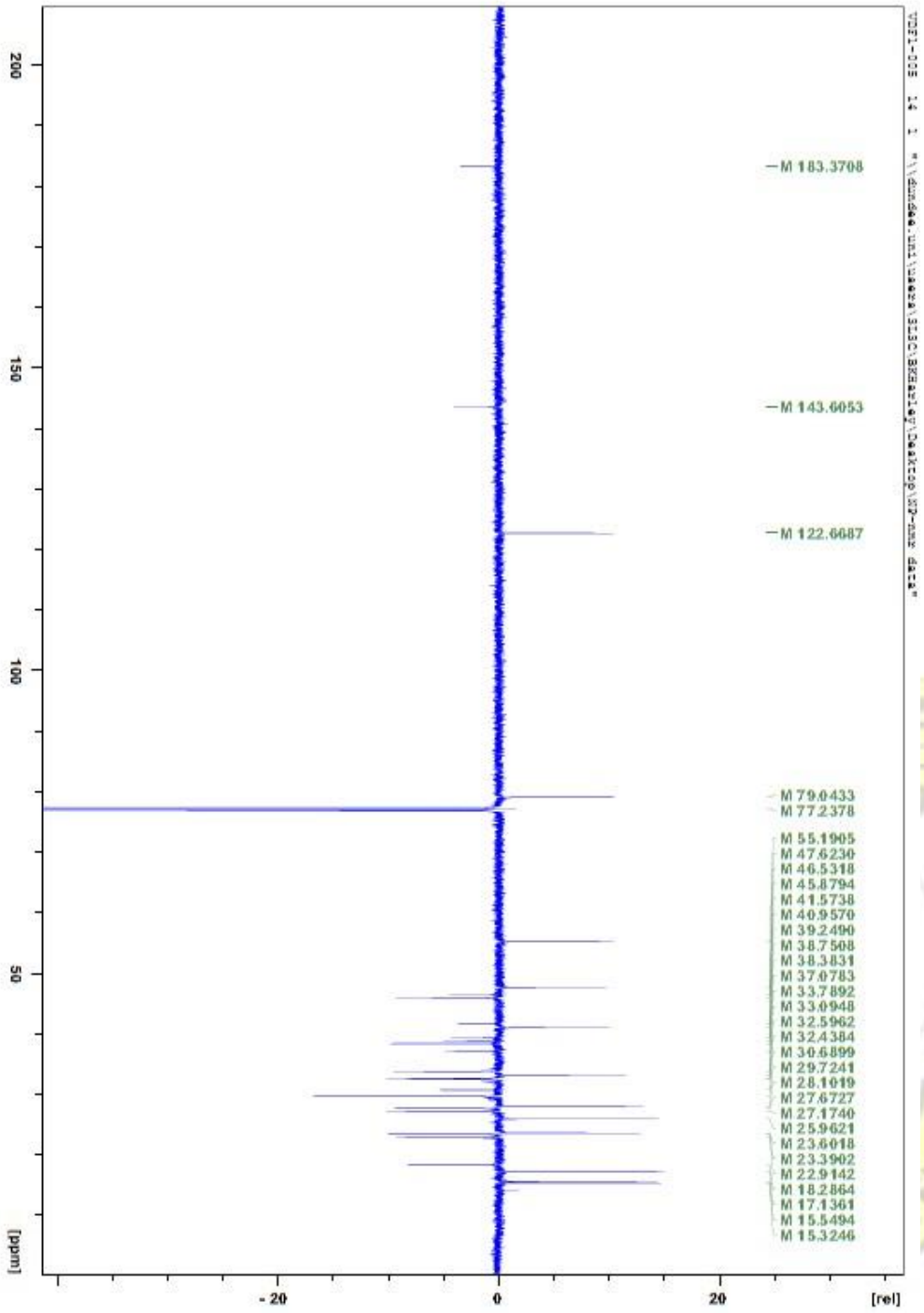
APPENDICES

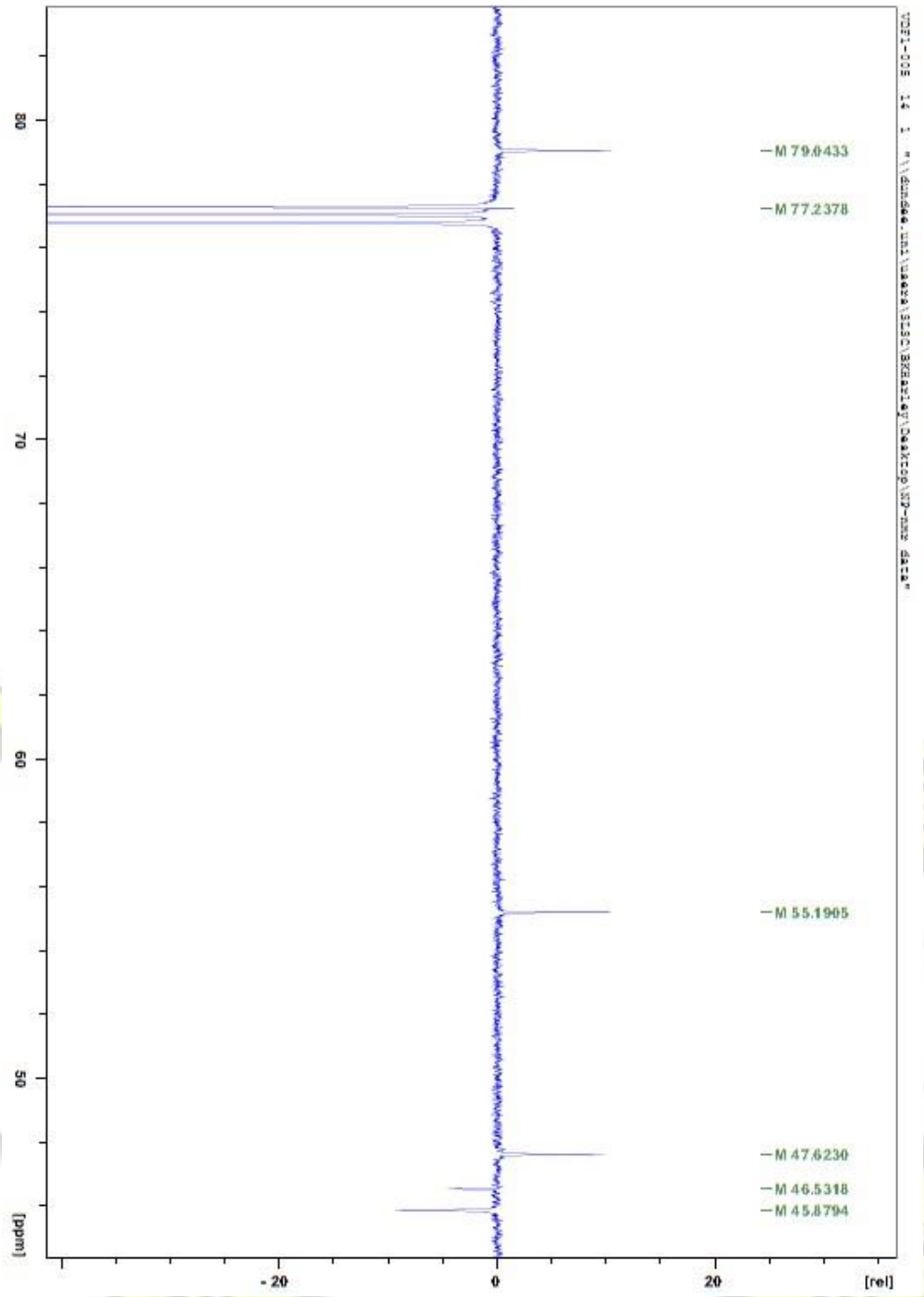
1A. ^{1}H NMR spectra of VDF1 in $CDCl_3$ at 500MHz

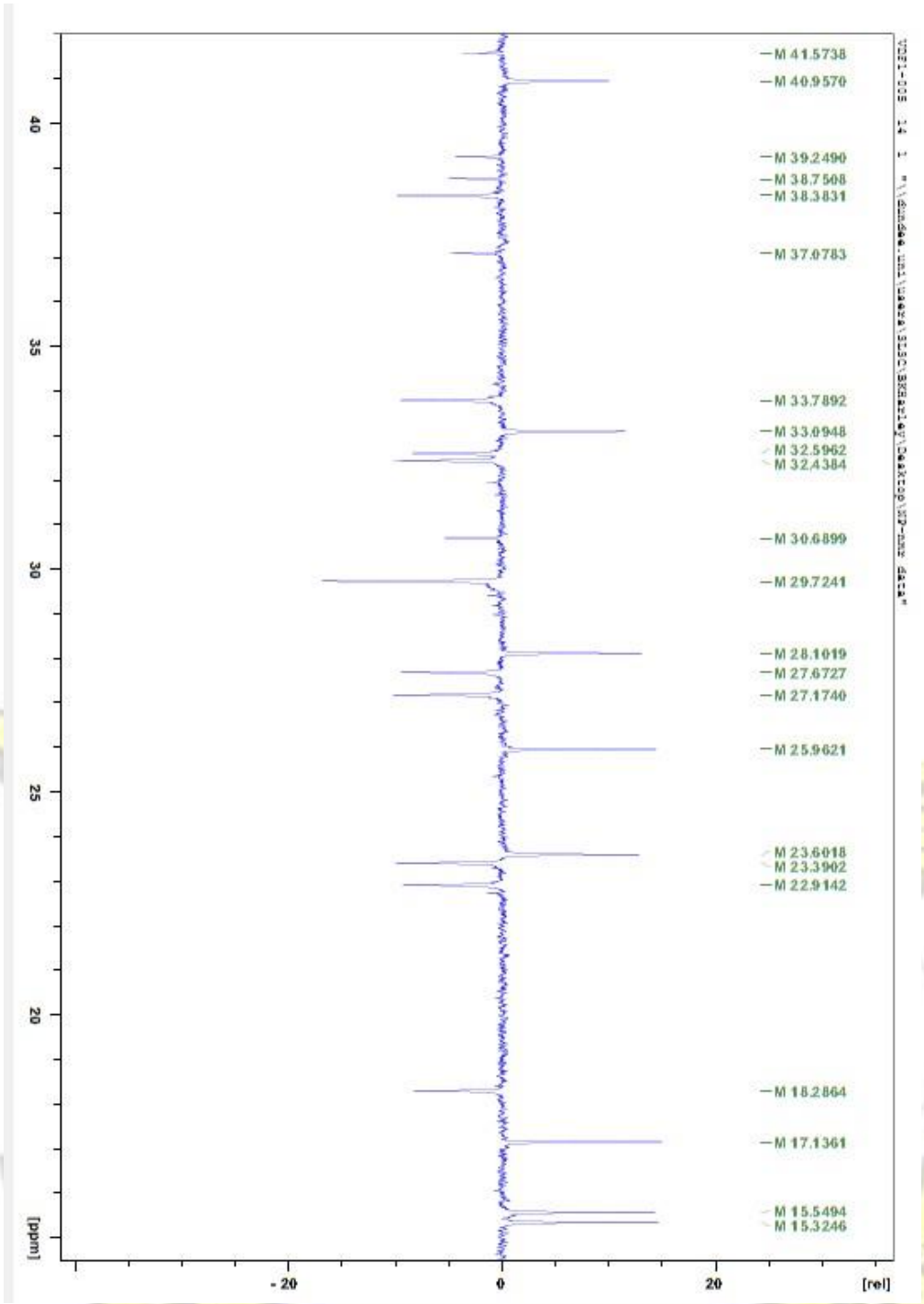



1.5078
4.8187
2.4722
4.9175
3.0000
3.1892
5.7680
5.3036
M 1.8901
M 1.8728
M 1.6176
M 1.6003
M 1.5527
M 1.2483
M 1.1284
M 0.9816
M 0.9220
M 0.9075
M 0.8994
M 0.7671
M 0.7451
[ppm]
[rel]

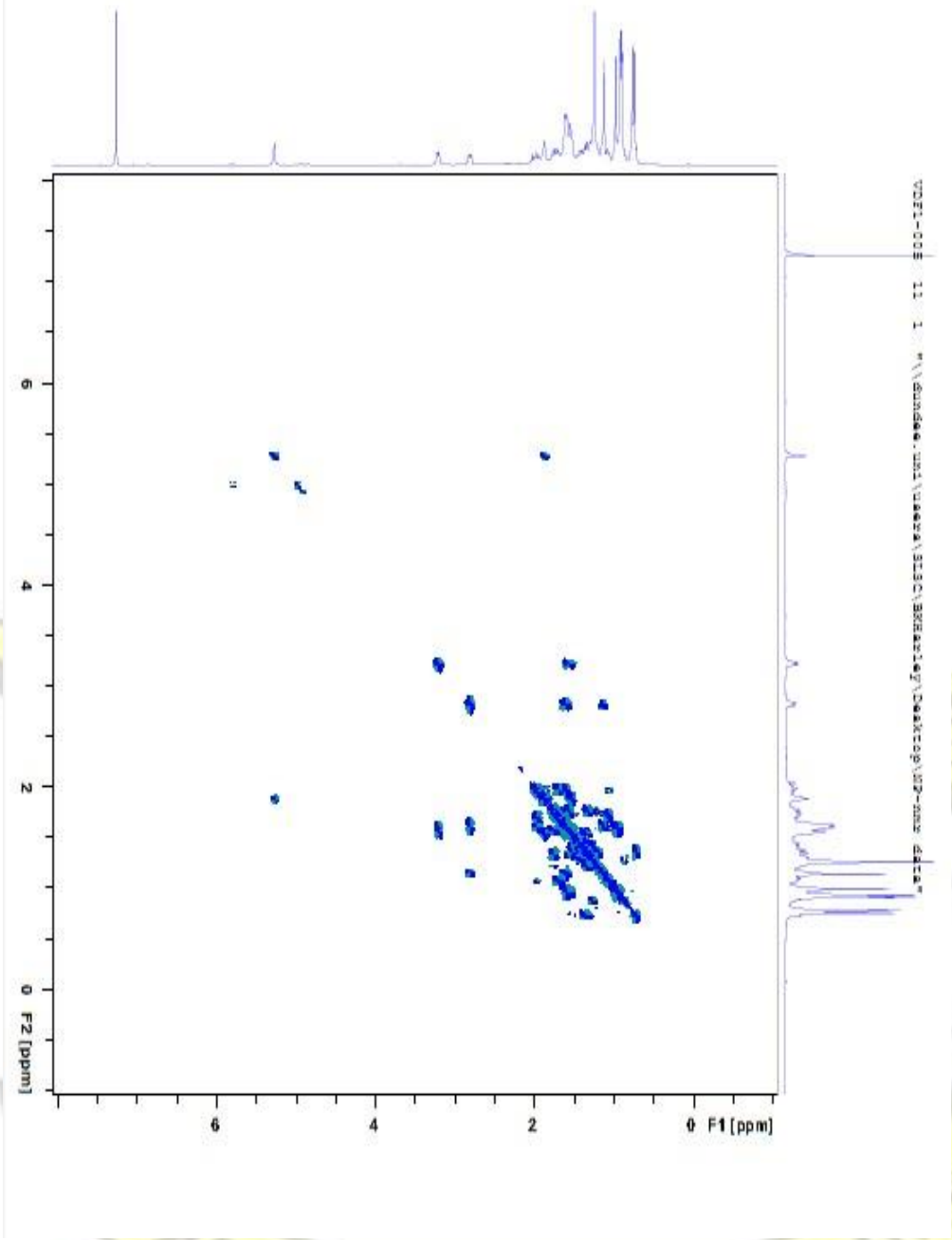
1B. DEPT spectra of VDF1 in $CDCl_3$ at 500MHz

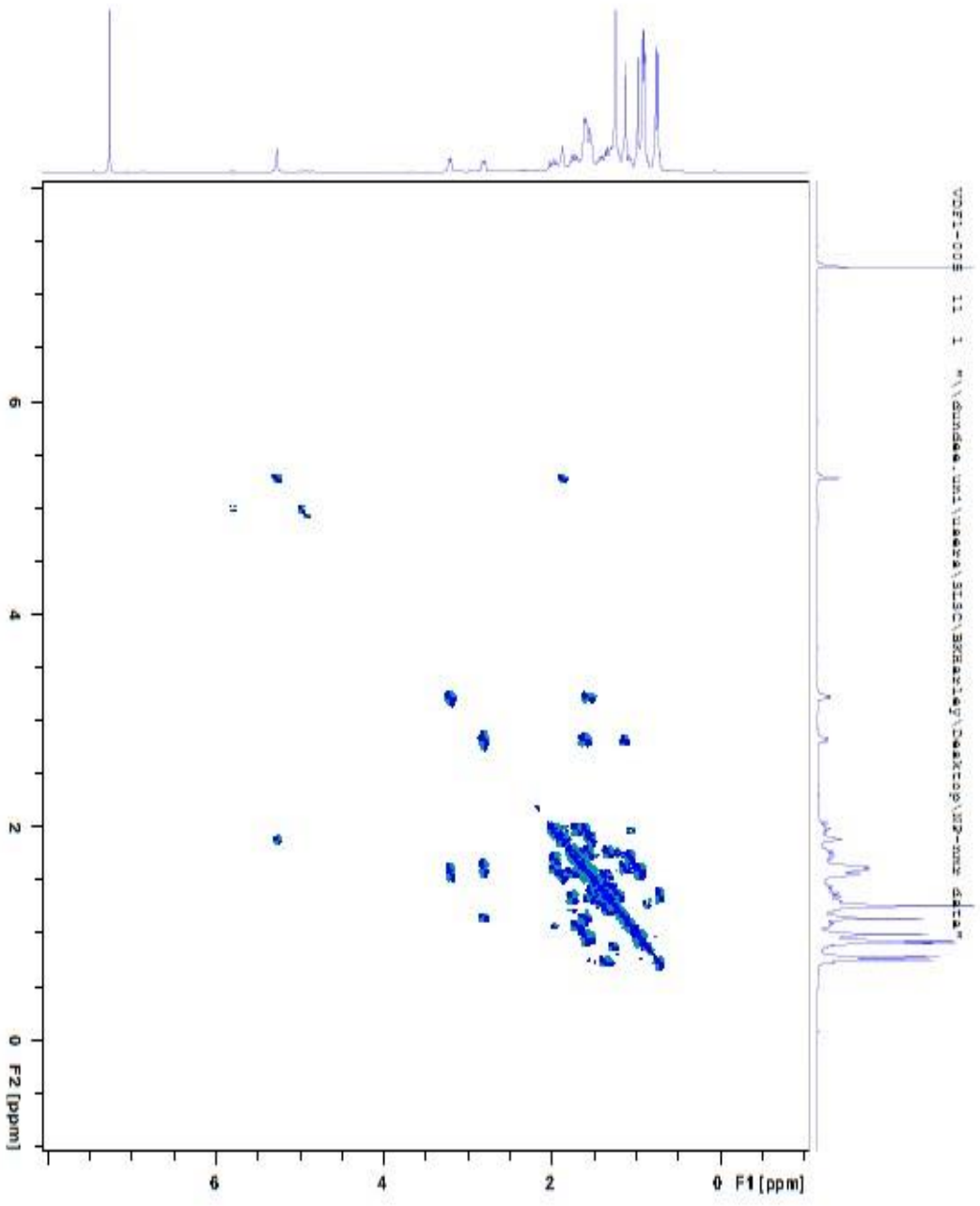


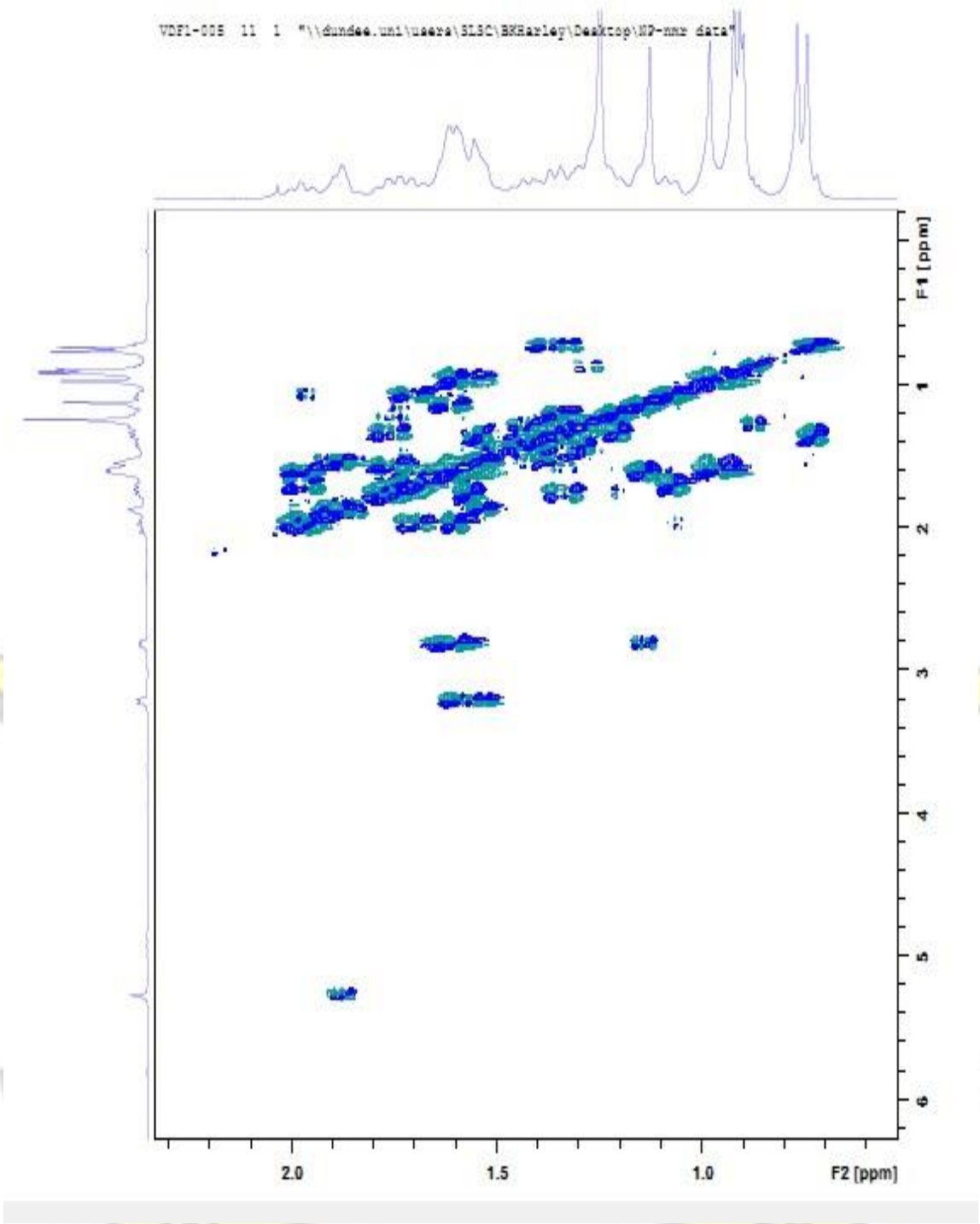

M 79.0433
M 77.2378
M 55.1905
M 47.6230
M 46.5318
M 45.8794
80
70
60
50
[ppm]
- 20
0
20
[rel]



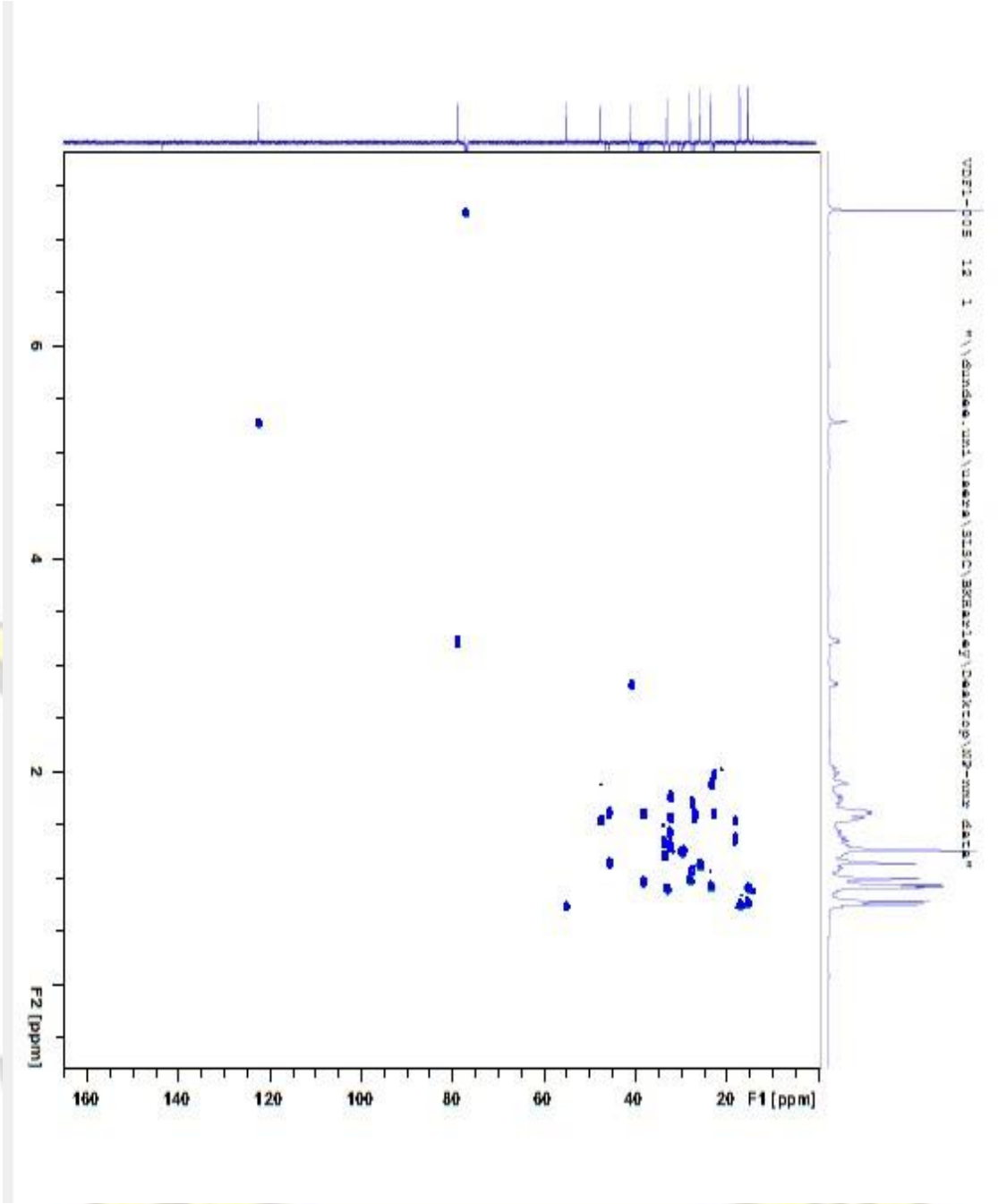
1C. COSY (^{1}H-^{1}H correlation) of VDF1 in $CDCl_3$ at 500MHz

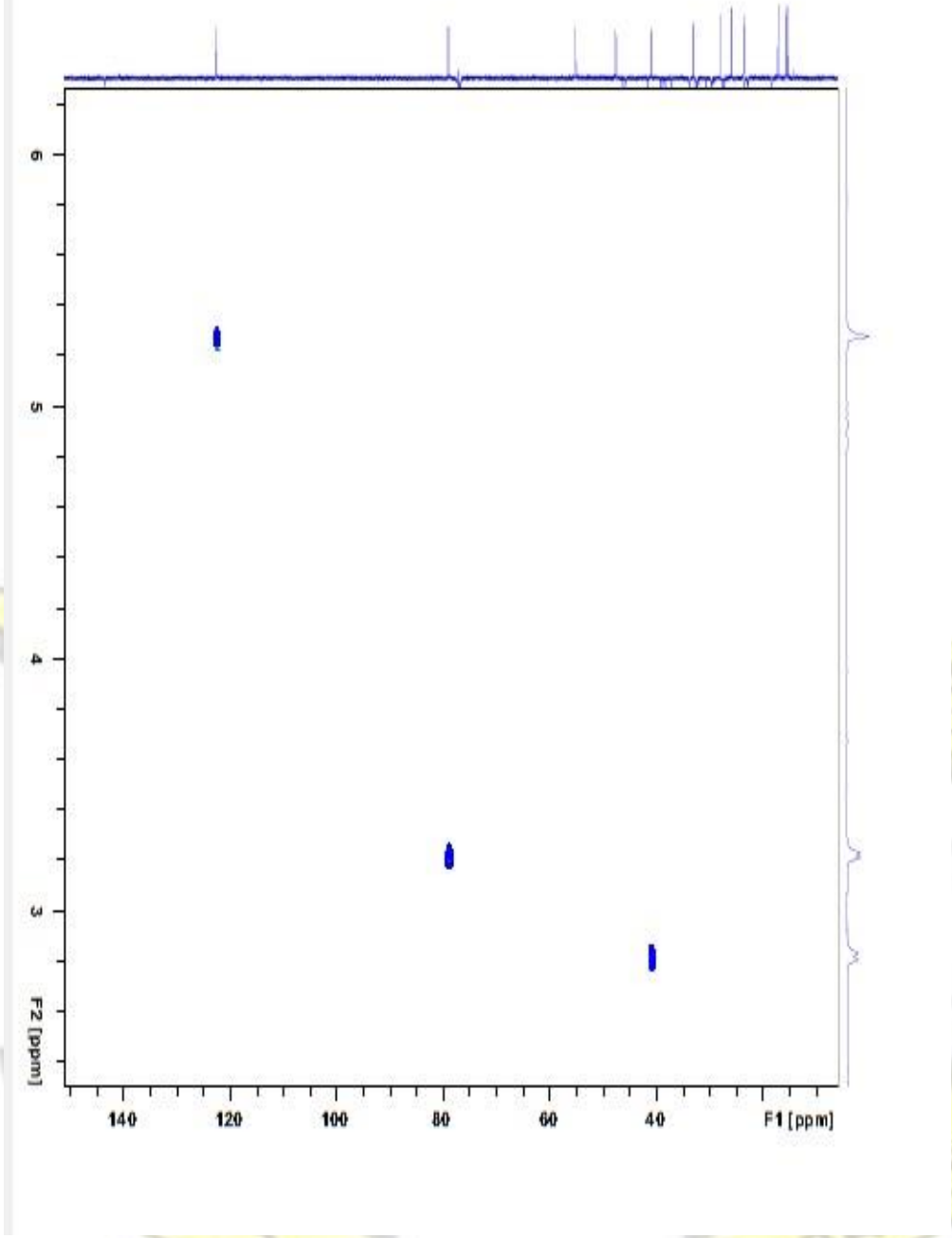

6
4
2
0 F2 [ppm]
6
4
2
0 F1 [ppm]

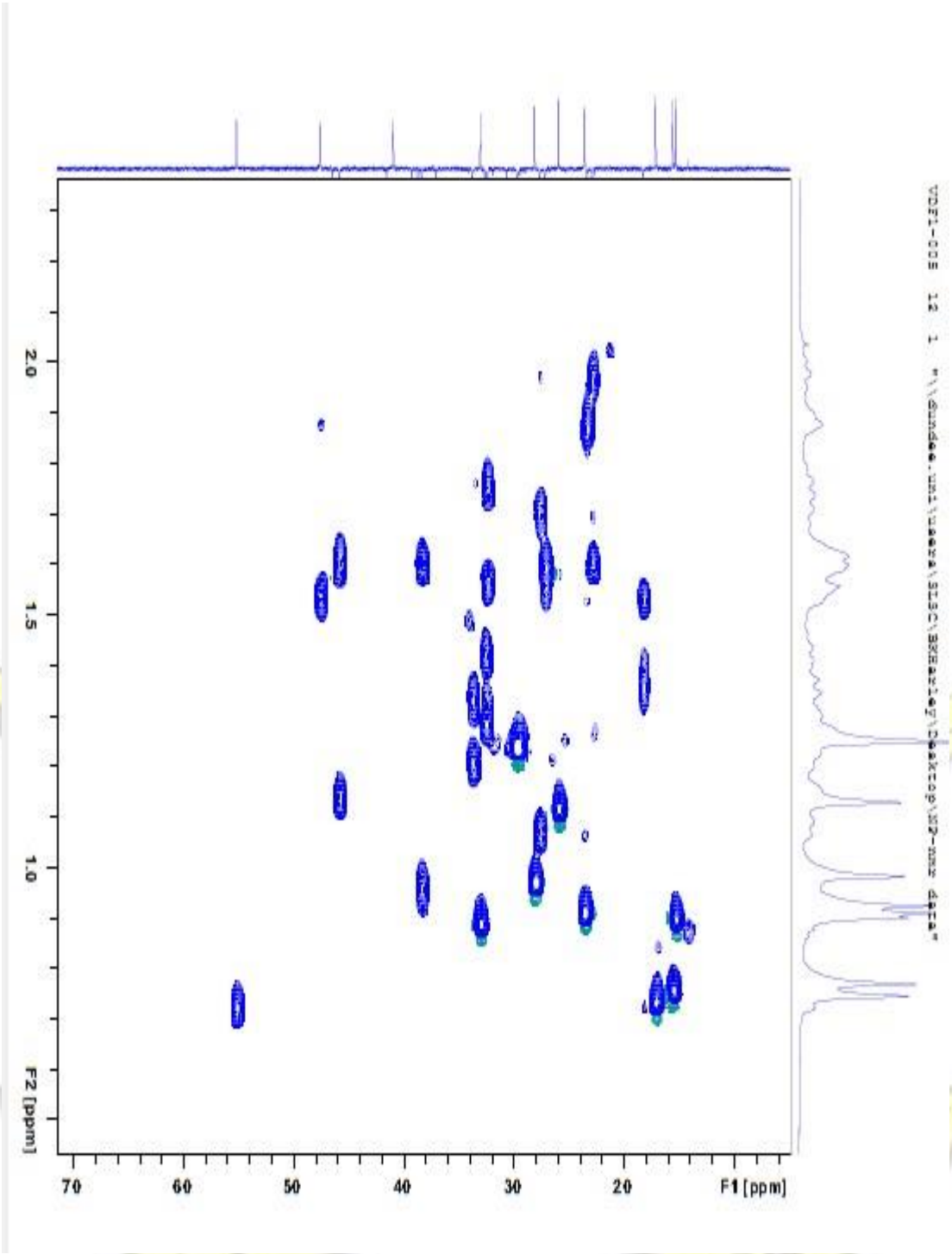


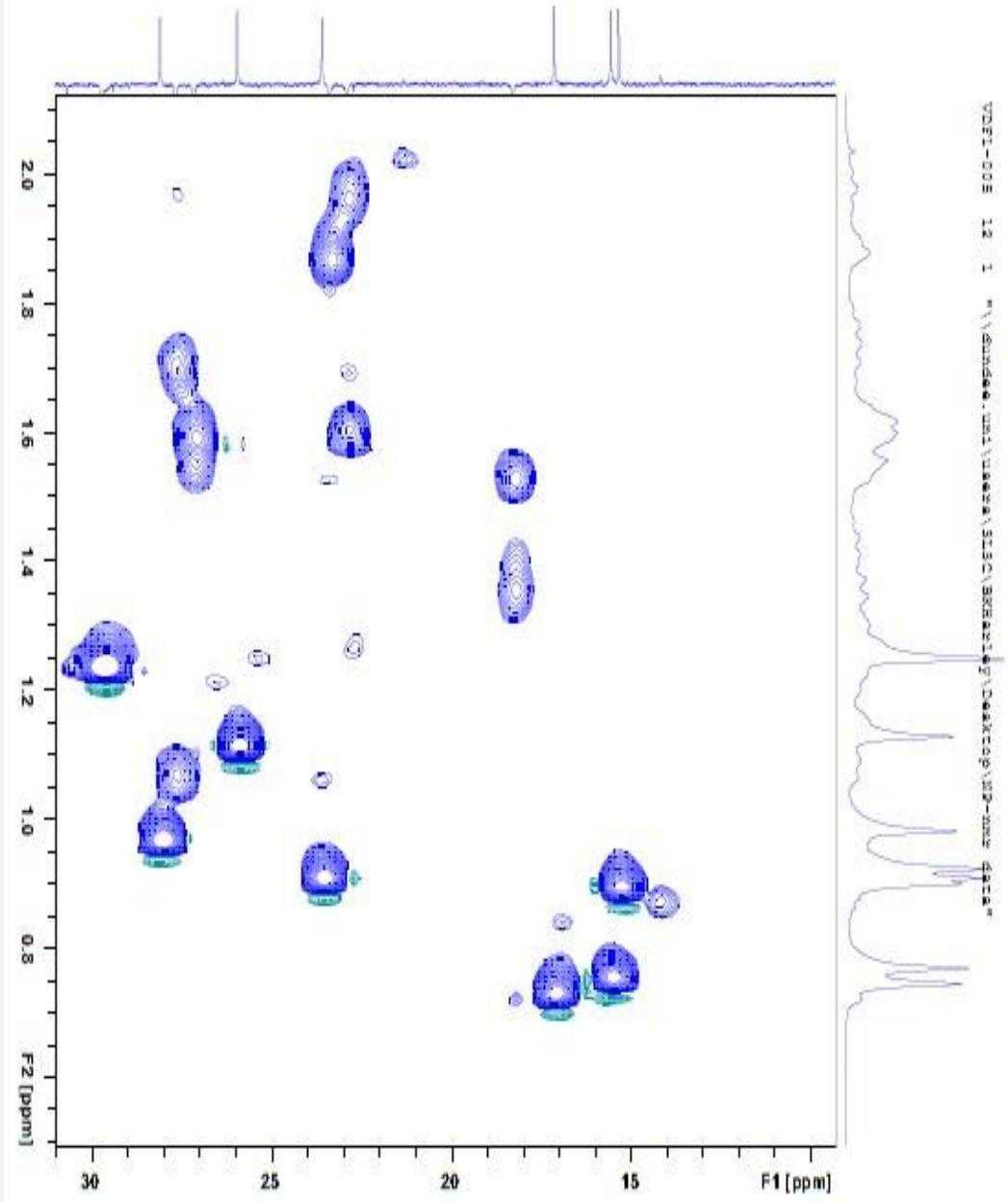

VDF1-005 11 1 "\\dundee.uni\users\SLSC\BKHarley\Desktop\NP-nmr data"
F1 [ppm]
1
2
3
4
5
6
2.0
1.5
1.0
F2 [ppm]

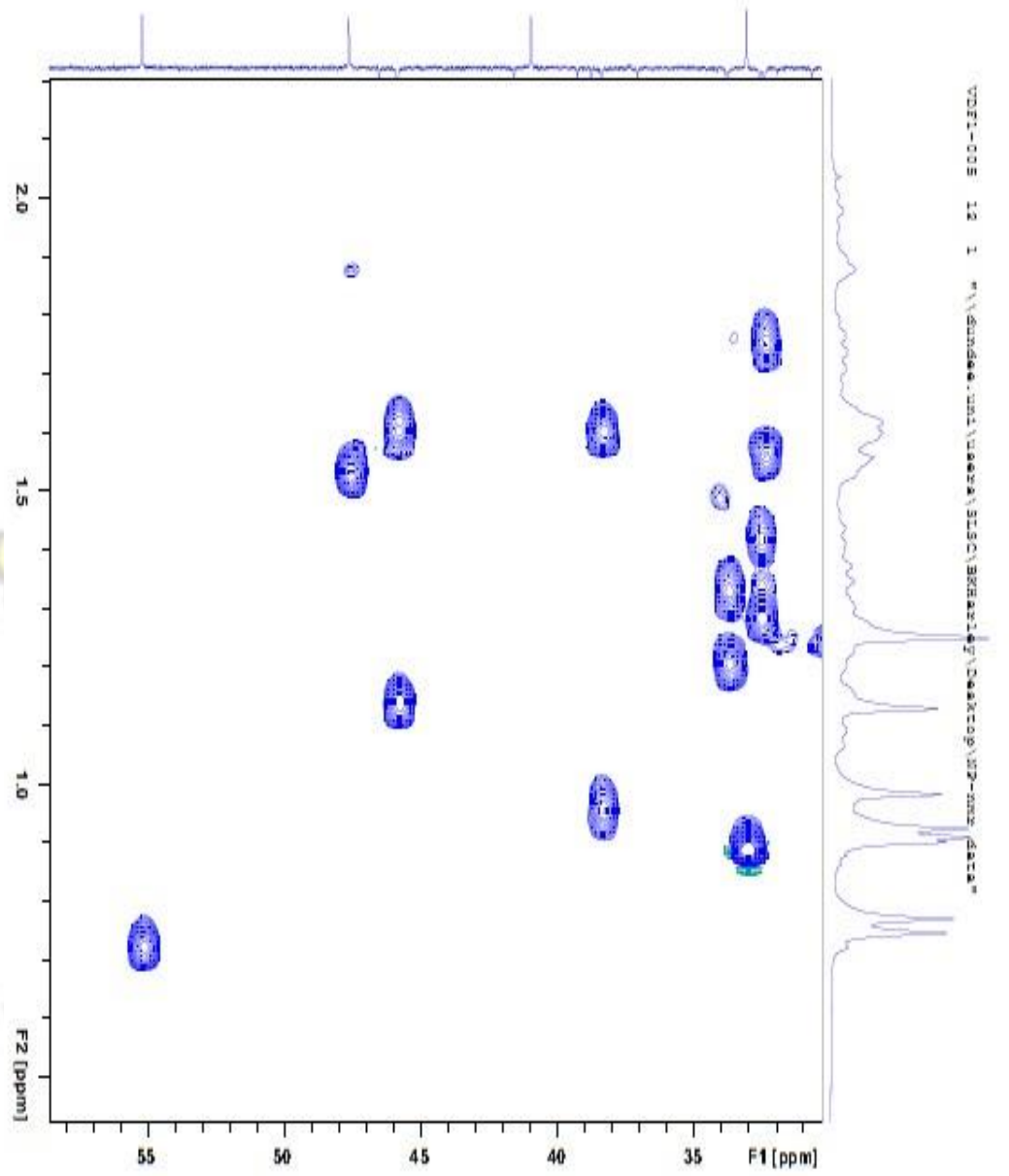
1D. HSQC (^{1}H-^{13}C correlation) of VDF1 in $CDCl_3$ at 500MHz



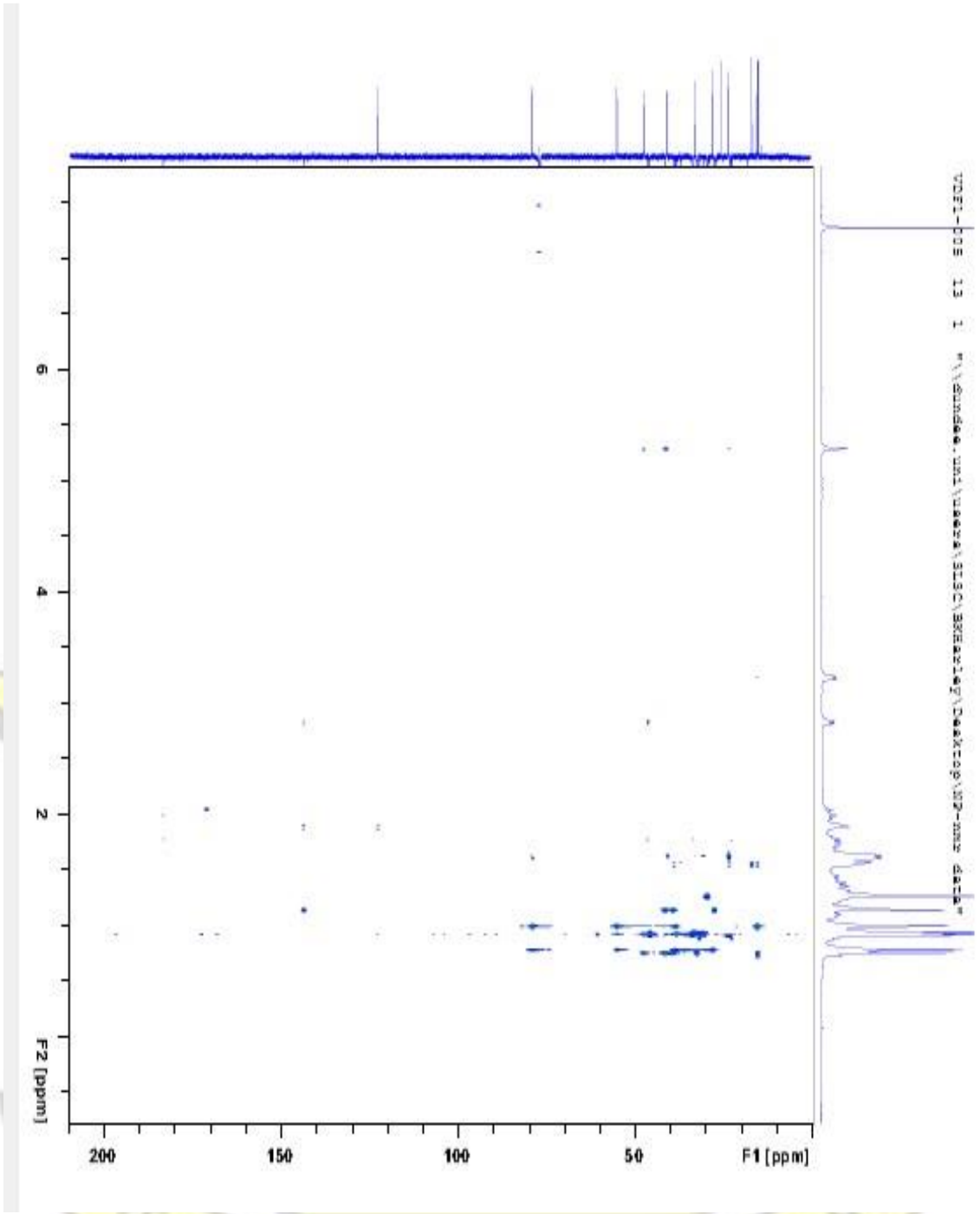

6
5
4
3
F2 [ppm]
140
120
100
80
60
40
F1 [ppm]

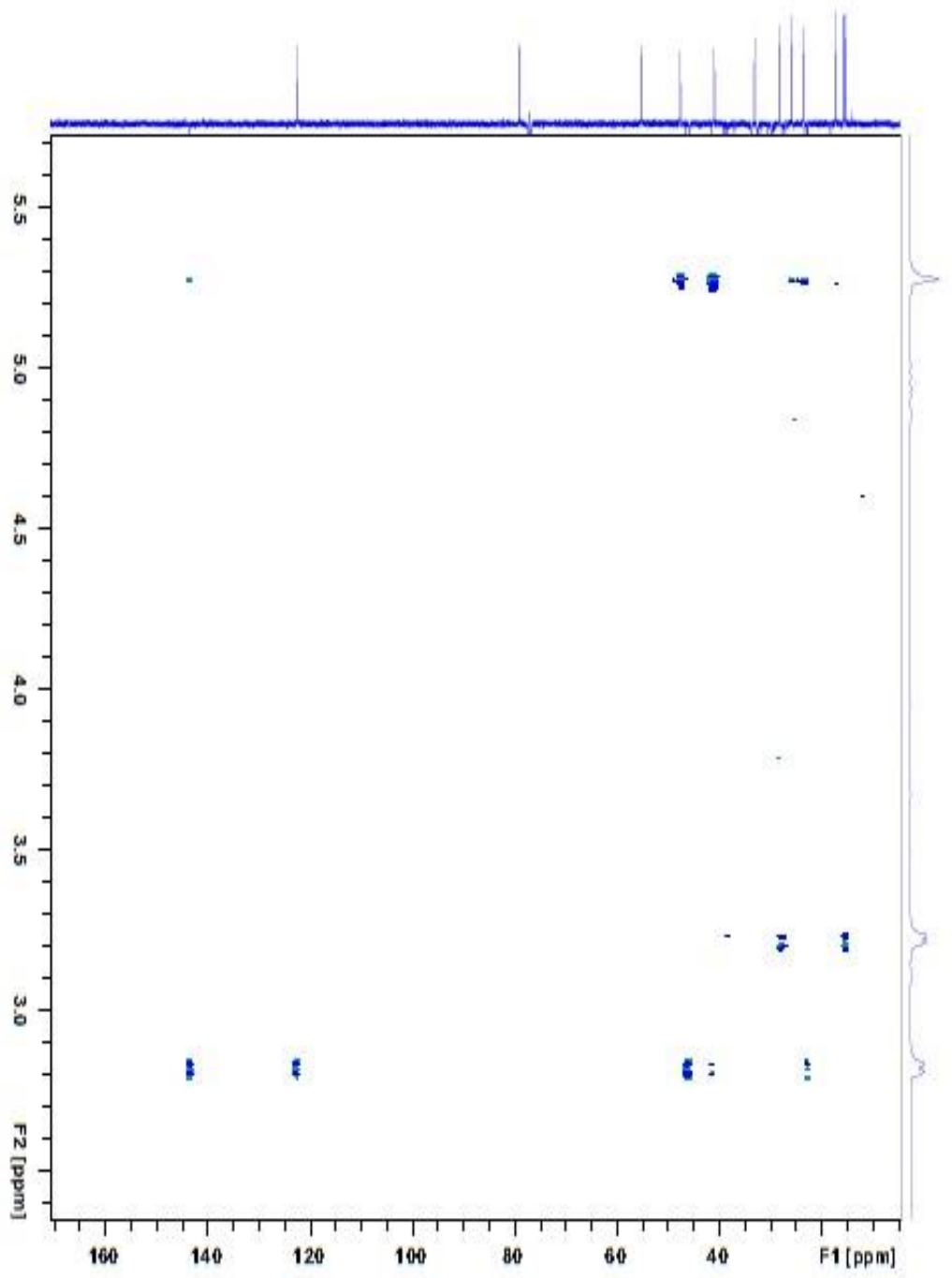

2.0
1.5
1.0
F2 [ppm]
70
60
50
40
30
20
F1 [ppm]

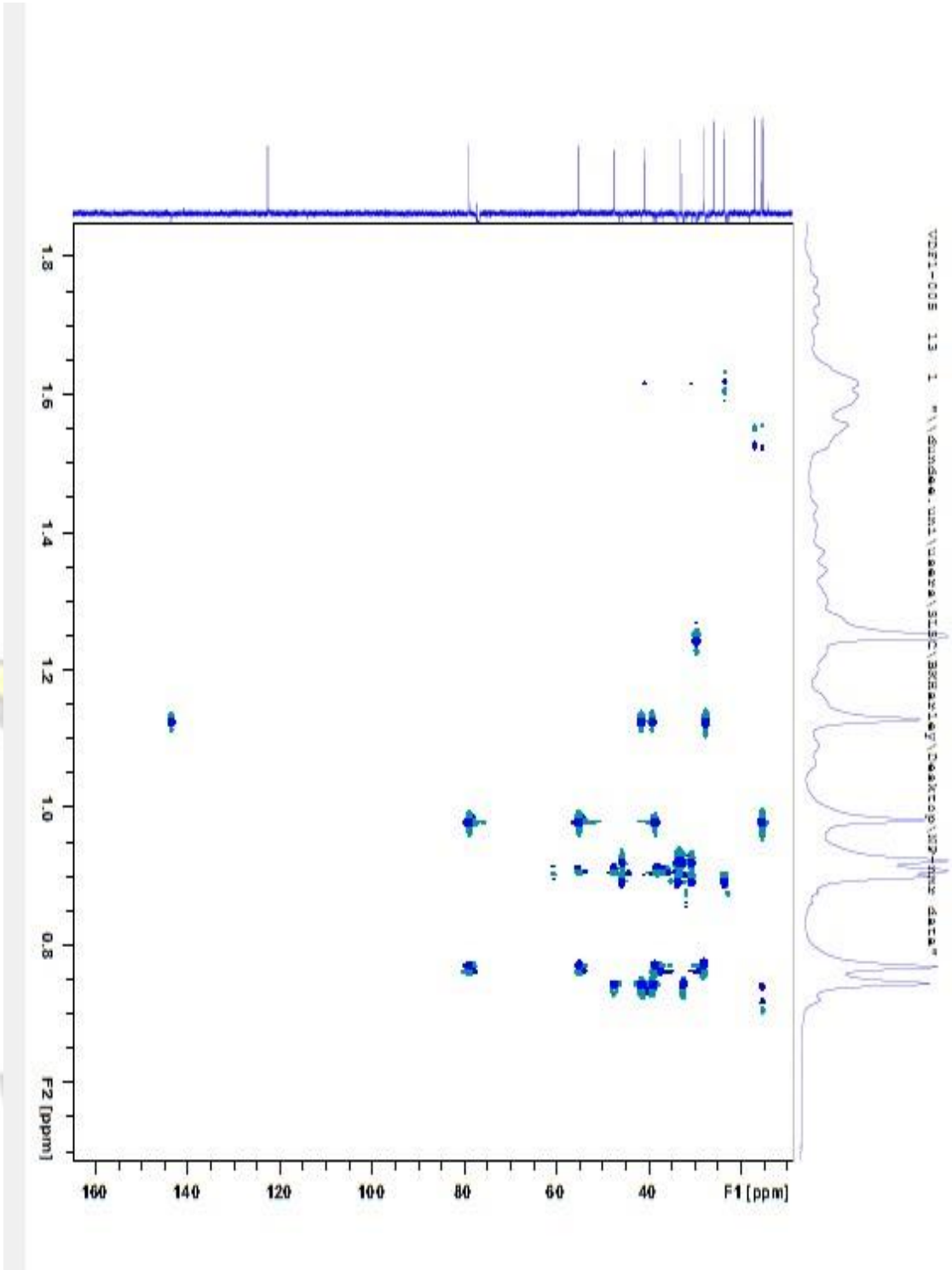

2.0
1.8
1.6
1.4
1.2
1.0
0.8
F2 [ppm]
30
25
20
15
F1 [ppm]



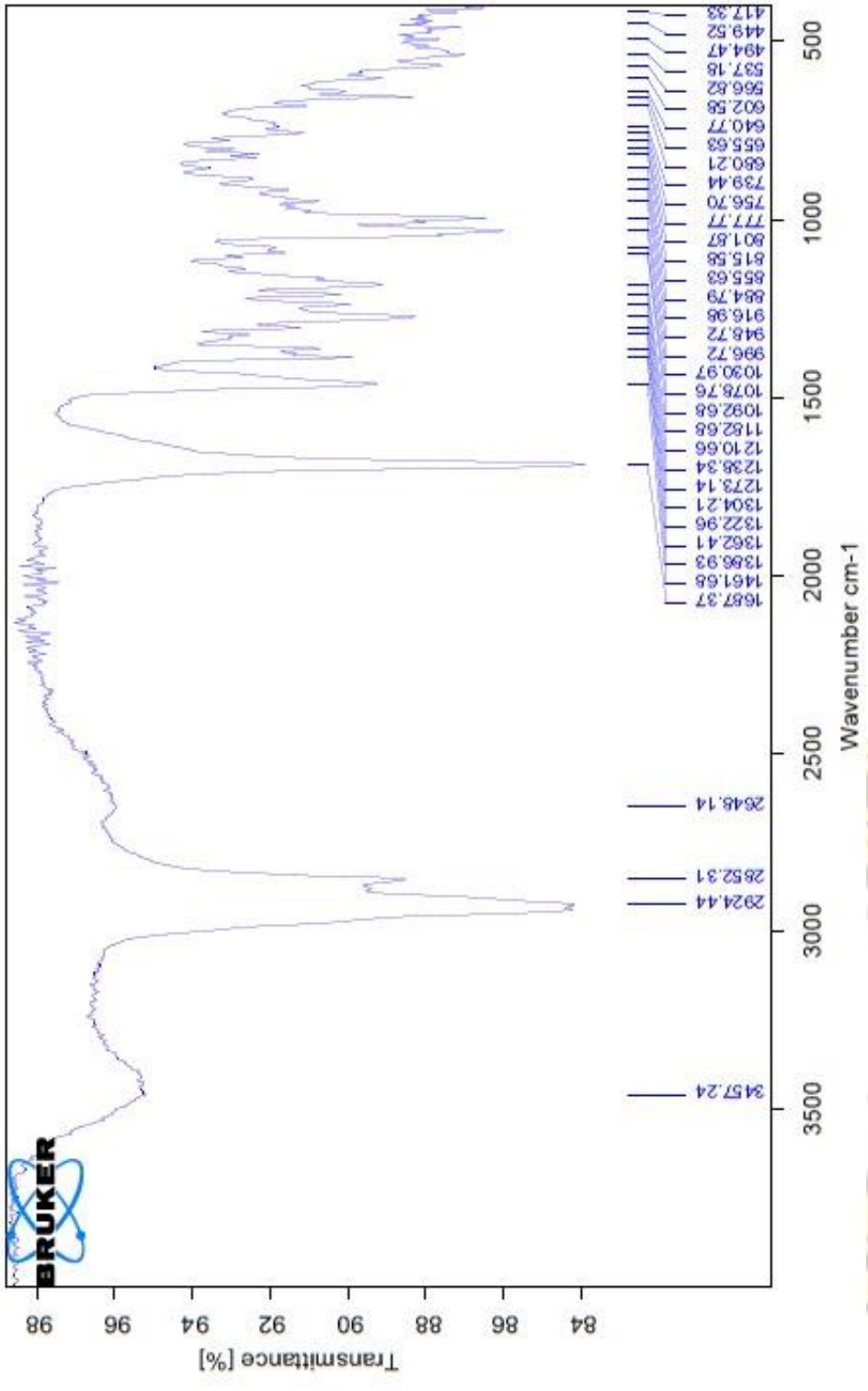
1E. HMBC (^{1}H-^{13}C correlation) of VDF1 in $CDCl_3$ at 500MHz







1F. IR spectra of VDF1


BRUKER
Transmittance [%]
98
96
94
92
90
88
86
84
Wavenumber cm-1
500
1000
1500
2000
2500
3000
3500
3457.24
2924.44
2852.31
2648.14
1687.37
1461.68
1386.93
1362.41
1322.96
1304.21
1273.14
1238.34
1210.66
1182.68
1092.68
1078.76
1030.97
996.72
948.72
916.96
864.79
855.63
815.58
801.87
777.77
756.70
739.44
680.21
655.63
640.77
602.58
566.82
537.18
494.47
449.52
417.33