Strathclyde Institute of Pharmacy and Biomedical Sciences

Activities of Extracts and Constituents of Two Libyan Ornamental Plants Relevant to Anti-inflammatory Activity

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[Submitted March 2011]

This thesis is the result of author's original research. It has been composed by the author and has not been previously submitted for examination which has led to the award of a degree.

Signed:

Date:

By name of Allah

Dedicated to my mother

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Acknowledgement

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Finally, I would like to express my deepest gratitude to my parents for their strong encouragement of my academic endeavours over the year. Thanks to my husband for supporting me at all times.

1.0 Introduction

1.1 Natural Medicines

Natural medicine is a system which we encounter almost every day and many natural products play a vital role in treatment of malignant diseases. They have been and are a major source in development and discovery of new pharmaceuticals all over the world (Harborne, 1999). There are many plant families that have made an important contribution in treatment of disease, such as *Apocynaceae, Caesalpinaceae, Piperaceae, Liliaceae, Rutaceae, Sapotaceae* and *Solanaceae.* In the 1900s, 80% of all medicines were obtained from different plant parts and thus, natural products are considered a significant source for new drugs. McChesney (2007) reported that approximately 60% of anticancer compounds and 75% of medicines for infectious diseases are either natural products or their derivatives. Recently, the demand for natural products and their popularity has increased due to side effects associated with synthetic medicines (Verma *et al*., 2009). In the last 20 years in the United States, increasing public dissatisfaction with the cost of prescription medication, combined with an interest in returning to natural or organic remedies, has led to an increase in the use of herbal medicines. In Germany, roughly 600 - 700 plant-based medicines are available and are prescribed by approximately 70% of German physicians (Heinrich, 2004).

1.2 Plants with Anti-inflammatory Properties

Anti-inflammatory agents are produced by many plants, such as *Lactuca scariola*, also known as Prickly lettuce, Wild lettuce, China lettuce and Compass plant and *Artemisia*

absinthium (Absinth wormwood, Common wormwood and Wormwood sage) (Ahmad, 1992). One well known family of plants that possesses anti-inflammatory activity is the Asclepiadaceae. These plants have been used as folk medicine in Southeast Asia and in Thailand. For example, the stems of *Cryptolepis buchanani* and *Derris scandens* are used for the treatment of inflammation in arthritis (Laupattarakasem *et al*., 2003). Laupattarakasem *et al*. (2006) demonstrated the anti-inflammatory effects of an ethanol extract of *Cryptolepis buchanani* (Asclepiadaceae) using various experimental methods; 1) In the carrageenan-induced rat paw oedema model, a significant dose-related reduction in oedema was shown in the animals that were pre-treated with an intraperitoneal injection of the extract. 2) On eicosanoid generation by ionophore-stimulated rat peritoneal leukocytes, *C. buchanani* showed similar inhibitory effects on both cyclooxygenase (COX) and 5-lipooxygenase (5-LO) pathways. 3) The reduction of tumor necrosis factor-α (TNF-α) release from LPS stimulated human monocytic cells (THP-1) was also established in cells that were pre-incubated with the ethanolic extract of *C. buchanani*. 4) Finally, the 50% ethanolic extract of *Cryptolepis buchanani* examined at a dose of 300 mg/kg administered intraperitoneally and 1000 mg/kg administered orally and showed significant anti-inflammatory activity in a cotton thread-induced inflammation model. After one week, the mean weight of dry granulomatous tissue surrounding the threads was significantly lower for the group treated with the plant extract compared with the control group. Another plant that has been shown to have immunomodulatory effects is *Sclerocarya birrea* (Anacardiaceae). According to Ojewole (2003), *S. birrea* stem-bark aqueous and methanol extracts (500 mg/kg) were examined by inducing acute inflammation in Wistar rats by injecting 0.5 ml/kg of fresh

egg albumin into the subplantar surface of the right hind paw. Moderate reduction in rat paw oedema was observed compared with Acetylsalicylic acid (ASA, 100 mg/kg). This supports the folkloric use of the plant in the treatment of inflammatory conditions such as arthritis, in certain communities of South Africa (Ojewole, 2003). Napolitano *et al.* (2005) reported that some plant extracts of the Sapindaceae family interfere in the production of nitric oxide (NO), an important inflammatory mediator (Figure 1). In the same study, it was reported that *Serjania lethalis* stem extracts and *Cupania vernalis* leaf extracts significantly inhibited NO production and that indicates the presence of active compounds that might be used as anti-inflammatory agents.

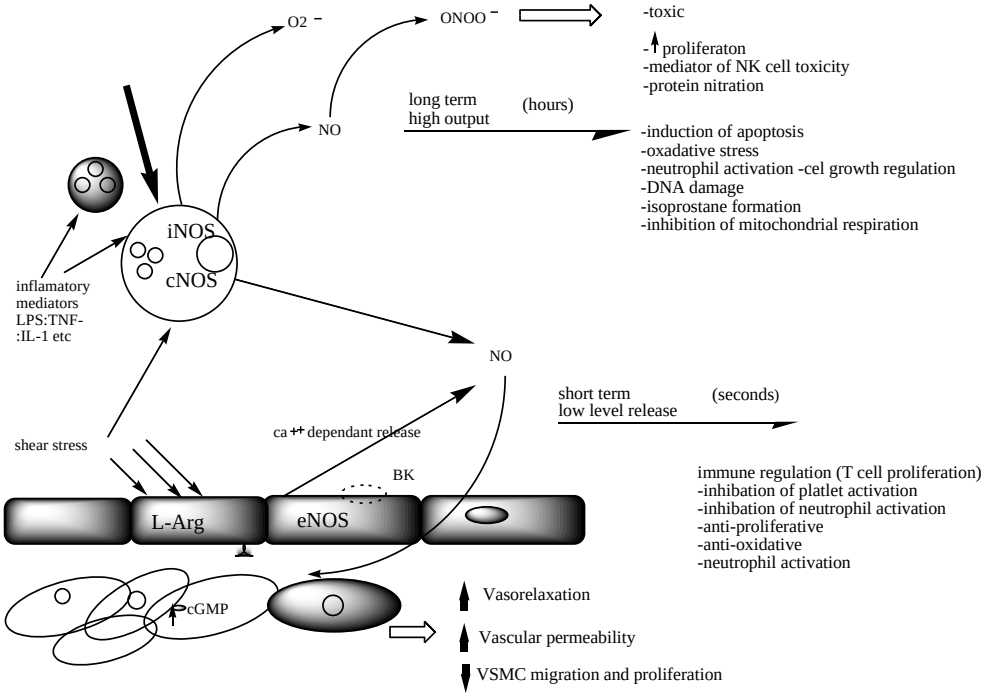


Figure 1: Involvement of NO in the inflammation process
http://www.jpp.krakow.pl/journal/archive/1203/articles/01_article.html

The Asparagaceae family is another group of plants that has been shown to have anti-inflammatory properties as demonstrated by Lee *et al.* (2008) using the ethanolic extract of *Asparagus cochinchinensis* (Chinese Asparagus). An intraperitoneal injection of 200mg/kg of *A. cochinchinensis* showed inhibition of topical oedema in the mouse ear model in which significant reductions in skin thickness, tissue weight, and inflammatory cytokine production were observed. Simpson *et al.* (2010) investigated anti-inflammatory activity of leaf extracts of *D. polyandra* on mouse ear oedema induced by croton oil and 12-o-tetradecanoylphorbol-13-acetate (TPA). This study demonstrated significant inhibitory potential of the extracts (methylene chloride/methanol and *n*-hexane) tested reducing oedema by 72.12 and 79.81%, respectively, after 24 h topical application of 0.4 mg/ear. This study suggests that the prostaglandin pathway of the inflammation process may be a potential target of the active components. Therefore, these studies emphasize the importance of both Asparagaceae and Sapindaceae families in discovery of new therapeutic compounds, which have been chosen for study in this project.

1.3 Anti-Inflammatory Drugs

The inflammation process includes multiple factors and cascades. At an inflamed site, inflammatory cells are activated to release inflammatory mediators (pro-inflammatory cytokines) which cause a change from the acute phase to the chronic phase of inflammation. In addition, inflammation is thought to be the most important component of the damage caused by autoimmune diseases, and is the main contributor to diseases such as diabetes, cancer and cardiovascular disease (Lucas *et al*., 2006). Dinarello

(2000) defined cytokines as "regulators of host responses to infection, immune responses, inflammation, and trauma". There are two kinds of cytokines: the first serves to make the disease worse and are denoted as pro-inflammatory such as interleukin I (IL-1) and tumour necrosis factor (TNF). The second type work to reduce inflammation and support healing and they are termed anti-inflammatory. Figure 2 shows the role of anti-inflammatory cytokines in inflammation process where T helper cells produce some cytokine that activate cytotoxic cells which destroy antigens and reduce inflammation. An inhibition of TNF-alpha (TNF-α) released from macrophages *in vitro* is commonly used as an indicator for screening anti-inflammatory activity. For *in vivo* tests, inflammation can be induced by many substances in animals and carrageenan-induced rat paw oedema in rats is the most commonly used model to examine anti-inflammatory action (Shinde *et al*., 1999).

Anti-inflammatory drugs can be classified according to mechanism of action into COX-1, COX-2 and 5-lipoxygenase (5-LOX) inhibitors, and NO synthetase inducible. Most non-steroidal anti-inflammatory drugs (NSAIDs) act as nonselective inhibitors of the enzyme cyclooxygenase (COX), inhibiting both the cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) isoenzymes. This enzyme catalyzes the formation of prostaglandins (messenger molecules in the process of inflammation) and thromboxane from arachidonic acid.

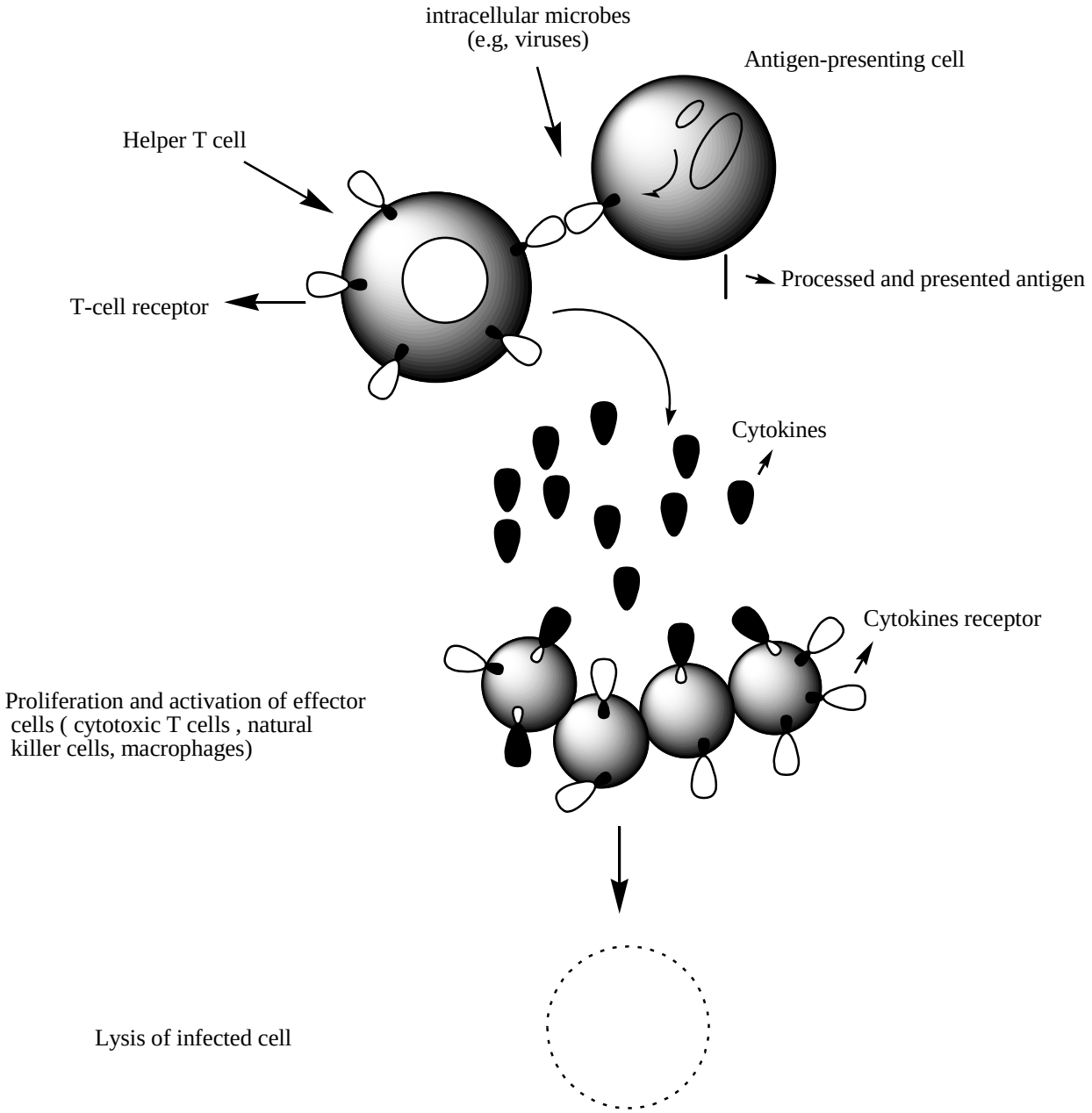


Figure 2: The role of cytokines in inflammation.
http://arapaho.nsuok.edu/~castillo/ImmuneResponse.html.

The problem with NSAIDs is that they are not specific enough and act by blocking the COX-1 and 2 enzymes. Blockade of COX-2 enzyme activity is successful in treating inflammation, as this enzyme is responsible for the formation of prostaglandins. However, by also blocking COX-1, which does not have a major role in inflammation, potentially protective responses can be suppressed and this results in side effects. For example, aspirin and non-selective NSAIDs cause platelet function inhibition due to their inhibition of COX-1. In the case of aspirin, the inhibition is irreversible therefore the effect on platelet function continues for the life-span of the affected platelet (up to10 days). On the other hand, non-selective NSAIDs produce a reversible inhibition of COX-1. Because COX-2 inhibitors have little effect on COX-1, they are less likely than non-selective NSAIDs to cause clinically significant actions on platelet function (Dugowson and Gnanashanmugam, 2006).

COX-2 selective inhibitors directly targets COX-2, an enzyme responsible for inflammation and pain. Selectivity for COX-2 reduces the risk of peptic ulceration, and is the main feature of celecoxib and rofecoxib. In addition, most of steroidal anti-inflammatory drugs work as inhibitors of gene transcription for COX-2, cytokines, cell adhesion molecules, and inducible NO synthetase such as prednisone and dexamethasone. They suppress infammation in a wide variety of diseases, including allergic diseases, rheumatoid arthritis, infammatory bowel disease and autoimmune diseases.

The use of anti-inflammatory drugs (NSAIDs and steroids) is associated with serious complications such as acute renal failure, nephrotic syndrome, electrolyte complications, heart disease and stroke, dyspepsia, bleeding from gastric and duodenal ulcers and

inflammation with blood and protein loss called NSAID enteropathy (Chuan and Wolfe, 2000). This gives impetus to the importance of development of new anti-inflammatory treatments with fewer side effects. For example, natural products reduce inflammation effectively with few adverse effects. Burnett *et al.* (2009) demonstrated that specific flavonoid mixtures, composed of baicalin and catechin, act to inhibit COX-1 and COX-2 in a balanced manner with additional 5-lipoxygenase (5-LOX) inhibitory activity with less side effects compared with synthetic drugs.

A study carried out by Lo´pez-Posadas *et al*, (2008) looked at the effect of flavonoids on lymphocytes stimulated with Concanavalin A. They assayed the effects on proliferation, COX-2 expression, iNOS synthase expression and proinflammatory cytokines such as TNF-α, IFN-γ and IL-2. They found that quercetin acted as a powerful antiproliferative and down regulated the expression of the enzymes COX-2 and iNOS synthase. These enzymes are important in the pathways of the inflammatory mediators prostaglandins and nitric oxide, and therefore antagonism of them causes a potent anti-inflammatory effect. They also found that quercetin caused a significant inhibitory effect on the production of the pro-inflammatory cytokines.

1.4. *Dodonaea viscosa* دودينيا

D. viscosa was selected for discovering of compounds with potential anti-inflammatory activity because of the fact that the plant family has been reported to have therapeutic value in these areas (Simpson *et al.*, 2010). It is known as Desert hopbush, Canyon hopbush, Hopseed and Hopseed bush. It belongs to the Sapindaceae family and is indigenous and wide spread throughout tropical, subtropical and Mediterranean regions. It is also distributed in temperate regions of Africa, Arizona, Australia, Florida, India, Mexico, New Zealand, Northern Mariana Islands, South America, and Virgin Islands (Selvam, 2007).

Figure 3: Shows the aerial parts of *Dodonaea viscosa* (http://www.nzplants.co.uk).

In addition, it is widely distributed and used in urban regions of some Arabic countries including Libya. For example, this plant was used for preventing of environmental pollution in many Libyan rural areas. Furthermore, it is used as an ornamental plant in many Libyan cities. It is a small tree growing up to 9 m (30 ft) tall. The leaves are a simple elliptical shape, 4–7.5 cm (1.6–3.0 in) long, 1–1.5 cm (0.39–0.59 in) broad, and alternate in arrangement (Figure 3). The flowers are yellow to orange-red and that produces a highly ornamental effect. The fruit is a capsule 1.5 cm (0.59 in) broad, red ripening to brown, with two to four wings (Selvam, 2007).

1.4.1. Historical Uses of *Dodonaea viscosa*

In traditional medicine, indigenous people from various countries around the world use different parts of this plant such as the stem, leaves, seeds, roots, and bark as antimicrobial, antispasmodic, analgesic, antiviral, anti-inflammatory, antiulcer and antioxidant treatments (Venkatesh *et al*., 2008). Additionally, a fresh leaf infusion has been used traditionally to treat malaria and as an anti-fever treatment in Australia, Gabon, Madagascar and Malawi (Burkill, 2005; Manfron, 2010).

1.4.2. Therapeutic Activities of *Dodonaea viscosa*

a) Antimicrobial Properties

Khurram *et al*. (2009) found that aqueous, methanol and ethanol extracts of *D. viscosa* leaf have antimicrobial activity against various pathogenic microbes such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Candida albicans* and *Mycobacterium smegmatis*. Thring *et al*. (2007) reported inhibition against *S. aureus* and *M. smegmatis* of methanolic and ethanolic extracts; showing minimum inhibitory

concentrations (MIC) of 2.5 mg/ml and 1.25mg/ml, respectively. Additionally, the ethanolic extract of *D. viscosa* was found to inhibit *C. albicans* in a disc diffusion assay with MIC of 2.5 mg/ml. Patel and Coogan (2005) found that the ethanolic, n-hexane, dichloromethane, ethyl acetate, n-butanol and aqueous extracts of *D. viscosa* have antibacterial activity against three Gram negative bacteria: *Escherichia coli*, *Salmonella typhi*, and *P. aeruginosa* and four Gram positive bacteria: *Bacillus subtilis*, *B. aureus*, *Micrococcus luteus* and *S. aureus*. Additionally, it has been found to display anti-fungal activity on *C. albicans* from HIV-infected patients (Patel *et al.*, 2009).

b) Wound Healing Properties

In excised and incised wound models in rats, significant wound healing activity resulted from an ethanolic extract of the plant leaves. Moreover, a significant response was observed from a suspension and ointment of ethanol extract which were also found to prevent the anti-healing properties of dexamethasone (Prassana *et al.*, 2007).

c) Antiulcer Properties

Arun and Asha (2008) found that the water and ethyl acetate extracts of *D.viscosa* leaves have moderate gastroprotective activity compared to the hexane extract in two different models (ethanol and indomethacin induced gastric ulcer) in Wistar rats. In other words, oral dose of 500mg/kg of hexane extract suppressed acid secretion to avoid ulcer aggravation and exhibited 90% and 92% protection against ethanol-induced and indomethacin-induced gastric lesions, respectively. Additionally, 77% and 52% protection was demonstrated at 250 mg/kg and 125 mg/kg of hexane extract, respectively.

d) Anti-inflammatory Properties

D. viscosa extracts have anti-inflammatory activity and have been found to inhibit carrageenan-induced oedema in the hind paw of rats. Oral dose of 300mg/kg of hydroalcoholic extract showed 56.67% anti-inflammatory activity equal to inhibition by 100mg/ml phenylbutazone given intra-peritoneally (Khalil *et al.,* 2006). In contrast, anti-inflammatory activities of the plant extracts were tested, the crude extracts of plant leaves and roots did not show any effects on the synthesis of prostaglandin (PG) (Getie *et al.,* 2003).

e) Antioxidant Properties

According to Ramzi *et al*. (2008) all *D. viscosa* extracts have the ability to decrease peroxidation. Nevertheless, in both spectrophotometer and micro-plate methods, the most potent antioxidant activity was identified in hot water extracts of *D. viscosa*. In the spectrophotometer assay, antioxidant activities were calculated as percentage inhibition of control rate of oxidation and extracts of hot water and ethanol exhibited 95% and 92% inhibition, respectively. The highest inhibition rate was observed at 5mg/ml of hot water extracts in a micro-plate assay. At a concentration of 50µg/ml, the methanolic extract of *D. viscosa* showed a highly effective free-radical scavenging ability and exhibited a remarkable antioxidant effects. This extract showed 94.29% and 92.45% radical scavenging activity at concentrations of 500µg and 1000µg/ml, respectively. Thus, researchers have reported that this indicates potential prophylaxis against diseases such as heart disease, arteriosclerosis and cancers, which benefit from anti-oxidant therapy (Ramzi *et al.,* 2008; Brand *et al.,* 1995).

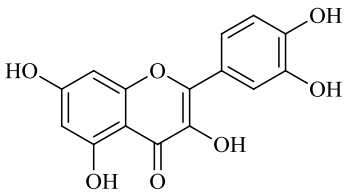
f) Other Activities

Krupanidhi *et al.* (2009) observed significant antidiabetic and antihyperlipidemic activities of ethyl acetate and methanol fractions of *D. viscosa* seeds in an alloxan-induced diabetic rat model and in a triton-induced hyperlipidemic rat model, respectively. In addition, *D. viscosa* leaves are used as a mouthwash in Pakistan and Madagascar (Quershi *et al*., 2008). Furthermore, different extracts of *D. viscosa* seed have been shown to display insecticidal activity against the cotton leaf worm *Spodoptera littoralis* (Rani *et al.*, 2009).

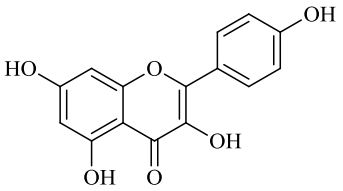
1.4.3. Active Ingredients of *Dodonaea viscosa*

In general, di- and triterpenes, saponins, flavonoids and a complex mixture of other phenolic compounds are the main compounds in *Dodonaea* species. There are 23 flavones **(1-17)** identified from seeds, bark, flowers and leaves that are characterized by oxygenation at C-3 and in almost 50% of cases, methoxylation at C-6 (Ghisalberti, 1998). Also, an extract of *D.viscosa* leaves has shown large concentrations of quercetin **(1)**, kaempferol **(2)** isorhamnetin **(3),** and coumarin fraxetin **(18)** and they were isolated from the the chloroform fraction of alcoholic extract and afforded to chromatographic separation using silica gel column chromatography and Sephadex LH-20 using different solvent systems. Furthermore, an older chemical composition study has shown the isolation and characterization of several flavonoids, diterpenoid acids, some biologically active saponins and plant acids, a novel P-coumaric acid ester, essential oils, sterols, and tannins, from the aerial parts of *D. viscosa* and saponin esters from the seeds and they were separated using preparative TLC (Sachdev and Kulshreshtha, 1983). A saponin mixture from the seeds has been shown to have phagocytosis-enhancing, analgesic and

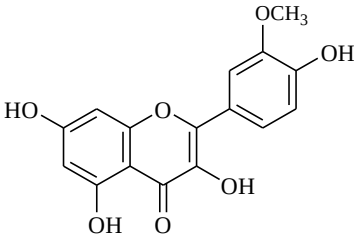
molluscicidal properties. In addition, the seeds are used as a fish poison due to the presence of saponins and triterpenes (Cao *et al.*, 2009). Moreover, significant antiviral activity is observed as a result of 3-methoxy flavones derived from quercetin and kaempferol in the seeds, bark, and leaves. These compounds have been shown to be active in tissue culture against polio-, rhino- and picorna-viruses. In the seeds, bark, and leaves of this plant, some diterpenes and the flavonoids sakuranetin **(8)**, quercetin **(1)** and rutin **(17)** were found to have marked spasmolytic activity. The spontaneous contraction of the intestinal smooth muscle of isolated rat and guinea-pig ileum was notable in a chloroform-methanol extract from aerial parts which supports the use of *D. viscosa* preparations as a cure for gastrointestinal disorders (Arun and Asha, 2008).



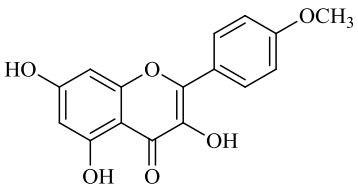
1: Quercetin (QU)



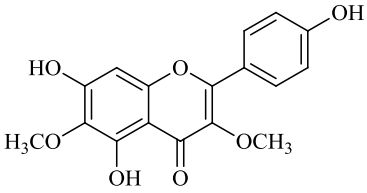
2:Kaempferol(KA)



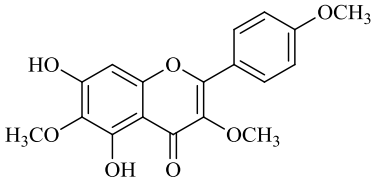
3:Isorhamnetin (IS)



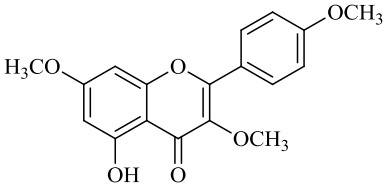
4: 4'-O-methylkaempferol (3, 5, 7-trihydroxy-4'-methoxyflavone)



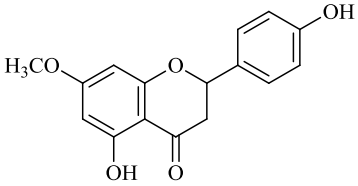
5: 5, 7, 4'-Trihydroxy-3, 6-dimethoxyflavone



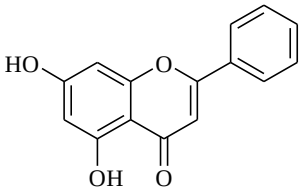
6: 5, 7-dihydroxy-3, 6, 4'-trimethoxyflavone (santin)



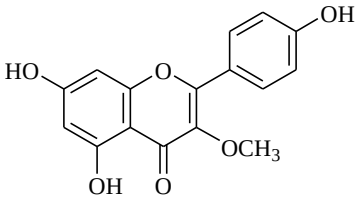
7: 5-Hydroxyl-3, 7, 4'-trimethoxyflavone



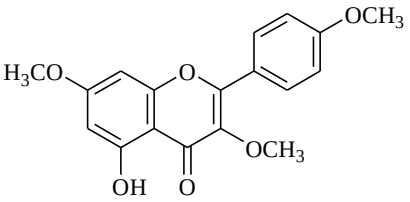
8: Sakuranetin



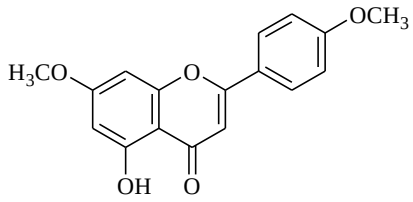
9: 5, 7-Dihydroxyflavanone (pinocembrin)



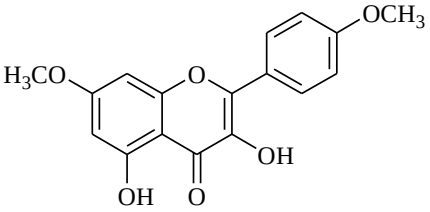
10: 5, 7, 4`Trihydroxy-3-methoxyflavone



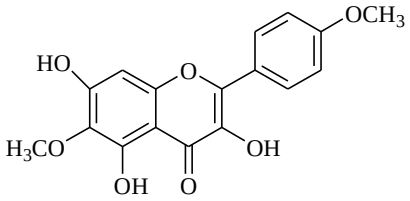
11: 5-Hydroxy-3, 7, 4`-trimethoxyflavone
(Kaempferol3, 7, 4`-Trimethylether)



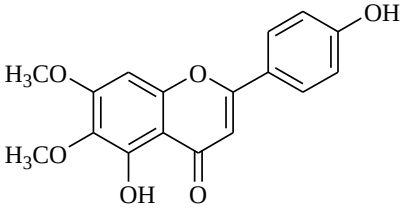
12: 5-Hydroxy -7, 4`-dimethoxyflavone



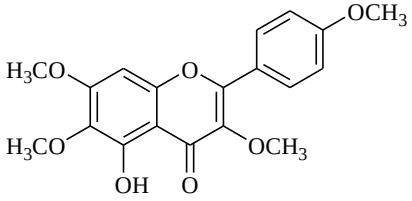
13: 3, 5-Dihydroxy-7, 4`-dimethoxyflavone
(Kaempferol 7, 4`-dimethylether)



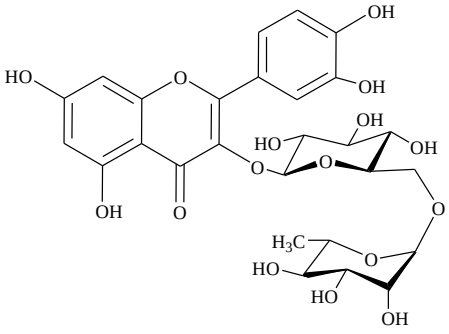
14: 5, 7 -Dihydroxy-6, 4'-dimethoxyflavone
Pectolinarigenin (Scutellarein 6,4'-Dimethyoxy flavone)



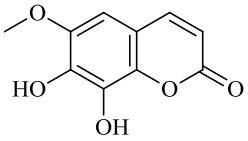
15: 5, 4'-Dihydroxy-6,7-dimethoxyflavone
(Scutellarein 6,7-Dimethyoxyflavone)



16: 5-Hydroxy-3,6,7,4`-tetramethoxyflavone



17: Rutin



18: Fraxetin
(7,8-Dihydroxy-6-methoxycoumarin; 7,8-Dihydroxy-6-methoxy-2-benzopyrone)

1.5. *Asparagus setaceus* هليون ريشي

Asparagus setaceus (Figure 4) is known as Common Asparagus Fern, Lace Fern, Climbing Asparagus or Ferny Asparagus and is grown as an ornamental plant. It belongs to the family Asparagaceae which is reported to have anti-inflammatory activity (Gautam *et al*., 2009). This plant is widely distributed especially in South Africa and Mediterranean regions. There are some kinds of *Asparagus* used as a food. For example, *A. officinalis* shoots are used in salads and soups and contain many essential nutrients such as folic acid and potassium. It can be eaten as a green vegetable in salad or lightly cooked. In addition, it can be made into an infusion and drunk as a tea or juice (Sun *et al*., 2007).

Figure 4: Shows the aerial parts of *Asparagus setaceus* .*0*(http://www.puutarha.net/suorakanava/kasvikortti.asp?id=343).

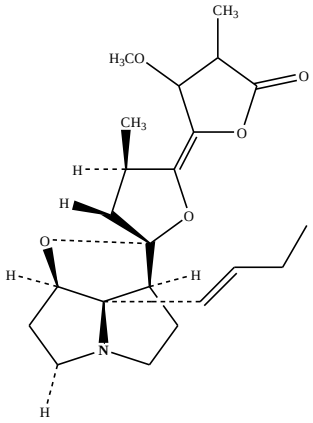
1.5.1. Historical Uses of *Asparagus* Spp

Plants from the *Asparagus* family have been used for a long time in traditional medicine. For example, in India, *A. racemosus* roots are used as an anti-inflammatory agent, as well as in Ayurveda, Siddha and Unani traditional systems to treat conditions such as ulcer and dyspepsia (Gautam *et al*., 2009). In Chinese traditional medicine, *A. officinalis* has been used as a tonic, antitussive, antifebrile, hair growth stimulator, and as a diuretic agent (Shao *et al*., 1996).

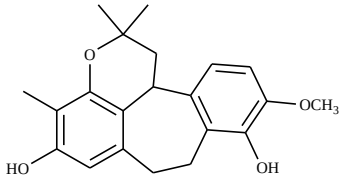
1.5.2. Active Ingredients and Therapeutic Properties of *Asparagus* Spp

There are no reported studies on any part of *A. setaceus*. On the other hand, there are many studies on other plants of the family. For example, crude saponins obtained from *A. officinalis* shoots have been shown to have antitumor activity (Shao *et al.*, 1996). The main compounds isolated from *A. officinalis* are flavonoids, oligosaccharides, amino acid derivatives and steroidal saponins. The steroidal saponins are considered the major constituents in several *Asparagus* species. In addition, *A. racemosus* roots contain steroidal saponins known as shatavarins I-IV, iso-flavones and alkaloids including asparagamine **(19)** and racemosol **(20)** as their main constituents (Wiboonpun, 2004; Sekine *et al*., 1995). Asparagamine was separated from the 75% EtOH extracts of air-dried roots of *A. racernosus* and it was isolated as a major compound (0.13% of dry wt.) by chromatographic (silica gel) separation and has been elucidated by spectroscopic, chemical and single-crystal X-ray analyses (Sekine *et al*., 1995). Hayes *et al*. (2006) have reported several known saponins: asparinins A and B; asparosides A and B; curillin H; curillosides G and H and shavatarins I and IV (**21-25)** from *A. racemosus* roots. A

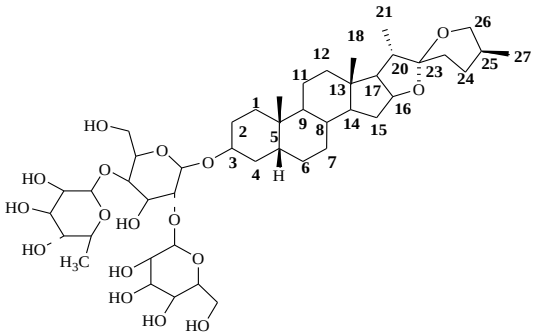
recent study (Gautam *et al.*, 2009) performed on *A. racemosus* extract showed immunoadjuvant activity. An aqueous root extract was evaluated in Swiss albino mice by co-administration of aqueous *A. racemosus* extract with lower immunogenic doses of diphtheria, tetanus, pertussis (DTP) vaccine; the animals showed higher anti-pertussis antibody titres (P= 0.0052) and immune-protection against lethal pertussis challenge compared to untreated (control) animals (Gautam *et al*., 2004; Gautam *et al.*, 2009). Sautour *et al*. (2007) reported six new steroidal saponins from the root of *A. acutifolius*. Four of them demonstrated antifungal activity against the human pathogenic yeasts *C. albicans, C. glabrata* and *C. tropicalis* with MIC values between 12.5 and 100 μg/ml. Sakaguchi *et al*. (2008) reported two anthocyanins with high antioxidant activities. They were isolated from *A. officinalis* and purified by column, paper and high-performance liquid chromatographic separations, and their structures were elucidated by high-resolution Fourier transform ion cyclotron resonance mass spectrometry (HR-FT-ICR MS), ^{1}H, ^{13}C and two-dimensional NMR spectroscopic analyses. They were identified as cyanidin-3-[3``-(*O*-β-D-glucopyranosyl)-6``-(*O*-α-L-rhamnopyranosyl)-*O*-β-D-glucopyranoside] (**26**) and cyanidin-3-rutinoside (**27**).



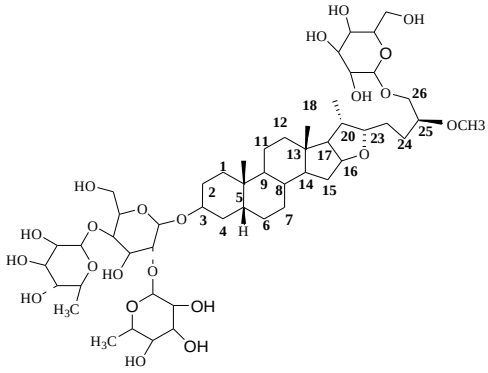
19: Asparagamine



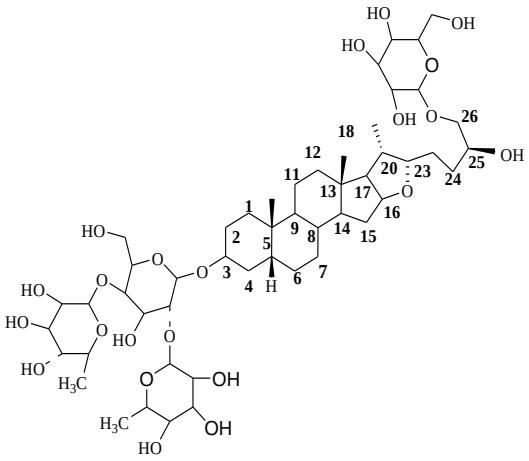
20: Racemosol



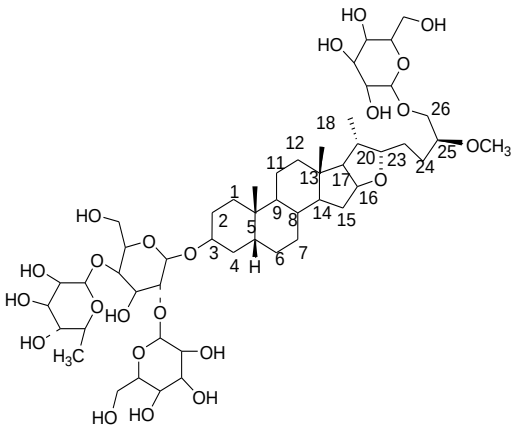
21: AsparaninB; Curillin H; Shatavarin IV



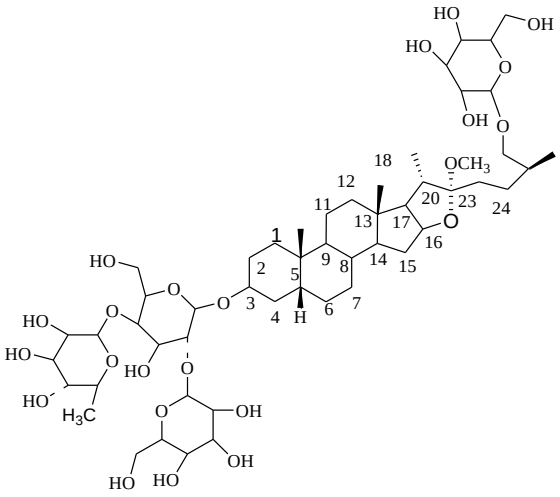
22: Curilloside G



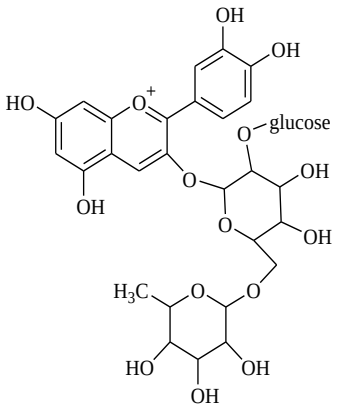
23: Curilloside H



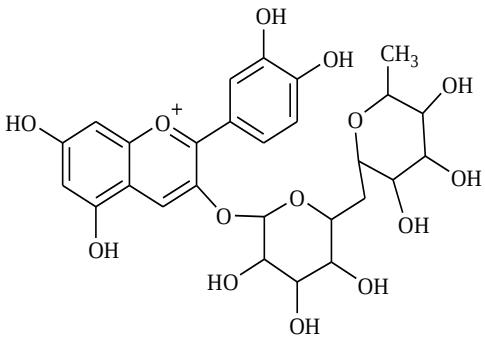
24: Asparoside B



25: Asparoside



26: Cyanidin-3-[3``-(*O*-β-D-glucopyranosyl)-6``-(*O*- α-L-rhamnopyranosyl)-*O*-β-D-glucopyranoside]



27: Cyanidin-3-rutinoside

1.6 Project Aims

This study will examine the potential anti-inflammatory properties of two plants from Libya belonging to the Sapindaceae and Asparagaceae families. The experimental aims were as follows:

- To provide information about the total extractable constituents and preliminary data on the cytotoxicity and anti-TNF-α activities using an ethanol extract prepared by maceration.
- To obtain extracts from the leaves using a Soxhlet apparatus with solvents in order of polarity; hexane, ethyl acetate and methanol to obtain further crude extracts.
- To evaluate the crude extracts for cytotoxicity effects in an *in vitro* bioassay, using THP-1 cells (a human monocytic cell line).
- To test the ability of the extracts to inhibit the production of the pro-inflammatory cytokine TNF-α produced by THP-1 cells stimulated with lipopolysaccharide (LPS). These cells are widely used in testing for potential anti-inflammatory activity *in vitro* because they have the ability to secrete mediators such as tumour necrosis factor-alpha (TNF-α), interleukin-1α (IL-1α) and interleukin-1β (IL-1β) when stimulated with LPS (Auwerx, 1991). The plant extracts that exhibited the most potent anti-inflammatory effects would then be selected for further chromatographic fractionation.

- To pool similar fractions as judged from their thin layer chromatography profile and to carry out structure elucidation using nuclear magnetic resonance and other spectroscopic methods.
- To re-test fractions and pure compounds for potential anti-inflammatory actions (anti- TNF-α and IL-1β activities).

2.0 Materials and Methods

2.1 Materials and Equipment

- 0.22μm filter (Millipore, UK)
- 24-well plates (TTP, Switzerland)
- 96-well plates (TPP, Switzerland)
- β-sitosterol (Sigma Aldrich, UK)
- $75cm^2$ cell culture flasks (Corning Incorporated, USA)
- Anisaldehyde (FSA laboratory, UK)
- Anti-bumping granules (BDH, UK)
- *Asparagus setaceus* leaves (Zawia, Libya)
- Avance DRX500 MHz NMR (Bruker, UK)
- Bulk Isolute sorbent Isolute® HM-N (Intl. Sorbent Tech. Ltd, UK)
- $CDCl_3$ (Sigma Aldrich, UK)
- Chloroform (Sigma Aldrich, UK)
- Dimethylsulphoxide (DMSO, Sigma Aldrich, UK)
- *Dodonaea viscosa* leaves (Zawia, Libya)
- Ethanol (Sigma Aldrich, UK)
- Flash Master Personal (Jones Chromatography, UK)
- Foetal calf serum (Sigma, UK)
- Jeol Eclipse 400 NMR spectrometer.
- Hexane (Sigma Aldrich, UK)

- Liquid Chromatography-Mass Spectrometry (LC-MS) (Thermo Fisher , Hemel Hempstead, UK)
- L-glutamine (Sigma, UK)
- Lipophilic Sephadex LH-20 (Sigma Aldrich, UK)
- Lipopolysaccharide (LPS) - *Salmonella abortus equi*, (Sigma, UK)
- Mass spectroscopy (Exactive Thermo Scientific, Germany)
- Methanol (Sigma, UK)
- Microcentrifuge (Centaur, SANYO, Japan)
- Microscope (Olympus, Japan)
- Neubauer-Improved Haemocytometer (Marienfeld, Germany)
- NMR tubes ((5mm x178 mm, Sigma-Aldrich, UK Ltd)
- Nunc-Immuno™ modules (Thermo fisher scientific, Denmark)
- Reverse Phase (RP) TLC Aluminium Sheets, Silica gel 60 F_{254} (Merck, Germany)
- Rotary evaporator (Büchi, Switzerland)
- RPMI 1640 medium (Sigma, UK)
- Safety Cabinet (Walker Safety Cabinets Ltd, UK)
- Sephadex LH-20 (Sigma-Aldrich)
- Silica gel 60H for thin layer chromatography (Merck, Germany)
- Silica gel 60 0.063-0.200 mm for column chromatography (Merck, Germany)
- SpectraMax M5 Microplate Reader (Molecular Devices Corporation, USA)
- Soxhlet apparatus (Quickfit, UK)

- Strata® SI-1 Silica (55µm, 70A) reverse phase flash chromatography column (Phenomenex®, USA)
- Streptomycin/ Penicillin (Cambrex, UK)
- THP-1 cells (European Collection Animal Cell Culture, UK)
- TNF-α ELISA Ready-Set-GO! Kit (eBioscience, UK)
- Trypan blue (Sigma Aldrich, UK)
- IL-1-β ELISA Ready-Set-GO! Kit (eBioscience, UK)
- Tween 20 - Polyoxyethylenesorbitanmonolaurate (Sigma, UK)
- UV-detector 254nm and 364nm UVGL-58 (UVP, USA)

2.2 Extraction of the Plant Material

Two plants were collected from M. Benzaed`s garden in Zawia, Libya in December 2009. The leaves were air dried and ground using a blender. The ground materials (4g) were extracted by maceration in ethanol as this was carried out in the literature (Thring *et al*., 2007; Prassana *et al*., 2007; Ramzi *et al*., 2008; Khurram *et al*., 2009). A Soxhlet apparatus was also used to extract larger amounts using increasing polarity of solvents (hexane, ethyl acetate, and methanol) for 2-4 days with each solvent (Appendix I and II) as this is the established methodology in the Phytochemistry Group Lab. The extracts were then evaporated to dryness at 40˚C under vacuum using a rotary evaporator.

2.3 Chromatographic Techniques

A range of traditional and modern chromatographic methods were applied in this work**.** They are useful techniques in the isolation and purification of compounds from plant extracts.

2.3.1 Thin Layer Chromatography (TLC)

Pre-coated silica gel 60H aluminum plates (Merck), normal phase, were used for the initial screening of different plant extracts and fractions. This technique is essential for choosing suitable mobile phases for different separation methods such as column chromatography (CC), flash chromatography (FC), and vacuum liquid chromatography (VLC). TLC was also used to examine the purity of isolated compounds. Plant extracts, fractions and pure compounds were dissolved in a suitable solvent and spotted 1 cm above the bottom edge of TLC plate. Based on the expected polarity of the extract or compound, a suitable solvent mixture was added to the TLC tank and left to saturate the

tank environment. Filter paper was put inside the tank to help the saturation. The spotted TLC plate was then placed in the tank to separate the compounds in the extract. When the mobile phase had moved from the baseline of the plate, the plate was removed, the solvent front marked and the plate dried immediately. It was then observed, under UV light at 254nm and 366nm; visible bands were marked using a pencil. Finally it was sprayed with anisaldehyde-H_2SO_4 spray (5ml sulphuric acid, 85ml methanol, 10ml glacial acetic acid and 0.5ml anisaldhyde) and heated at 110°C for a minute. The R_f values for each spot were calculated by dividing the distance the spot travelled, by the distance of the solvent front. This was used to pool similar fractions together, which were then dried and further analyzed by nuclear magnetic resonance (NMR) to elucidate the structure of the compounds (Stahl and Mangold, 1975).

2.3.2 Column Chromatography (CC)

This technique was applied to fractionate polar and non-polar components, using an open glass column plugged with cotton wool. The glass column 55×3 cm was packed with 50g silica gel 60 and 13.20g, 12g of hexane extracts and 9g of ethyl acetate extract (Section 3.4, Appendix I and Π) were eluted beginning with hexane after preadsorbing the extract on to silica gel introduced to the top of the column. The eluent was passed through the column with gradient elution. Fractions in vials of 30ml were analysed by TLC and fractions with similar bands were combined and evaporated to dryness.

2.3.3 Sephadex Column Chromatography (SCC)

This is a useful technique in separating compounds of different molecular size, and especially in the separation of chlorophylls from mixtures containing other small-molecule secondary metabolites. A slurry of sephadex LH-20 (40g) was added to a glass column of approximately 20.5cm height and 1.5cm diameter. The ethyl acetate extract (22.37g) was applied in a small quantity of chloroform to the top of the column (Appendix I). Elution was commenced with 100% chloroform. The polarity was subsequently changed to 5% methanol in chloroform to collect more polar compounds and 300 ml of solvent system was used each time. Finally, the column was washed with pure methanol and vials of 15 ml were used to collect different fractions.

2.3.4 Vacuum Liquid Chromatography (VLC)

A column was dry-packed with Silica gel 60H (300g) under vacuum applied via a water vacuum pump. The methanol extract (20.57g) was adsorbed on to a small amount of silica, allowed to dry and applied to the top of the VLC column (Appendix I). Elution was carried out with 400ml amount of different solvent systems according to the polarity and as determined by TLC.

2.3.5 Flash Chromatography (FC)

A methanol extract (38g) was dissolved in methanol and added to sorbent silica granules (Appendix II). This was left in a fume cupboard until the solvent had evaporated off. These dried granules were then packed into a normal phase flash column, closed using a filter cap and then attached to the flash apparatus. Elution was performed using various solvent systems (400 ml each) passed through the column under pressure starting with

ethyl acetate (Table 1). The eluates were collected in 15ml glass vials and similar fractions were pooled together using TLC to help with the selection process. The vials were then left in a fume cupboard for the solvent to evaporate.

Table 1: Sequence of Flash Chromatography Solvent Systems

Solvent Mixture
100% Ethyl acetate
10% Methanol in ethyl acetate
20% Methanol in ethyl acetate
30% Methanol in ethyl acetate
40% Methanol in ethyl acetate
50% Methanol in ethyl acetate
60% Methanol in ethyl acetate
70% Methanol in ethyl acetate
80% Methanol in ethyl acetate
90% Methanol in ethyl acetate
100% Methanol

2.4 Spectroscopic Examination

2.4.1 Nuclear Magnetic Resonance (NMR)

Nuclear magnetic resonance spectra were obtained on a Jeol Eclipse 400 spectrometer operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C and a Bruker Avance DRX-500 (500MHz) spectrometer for heteronuclear multiple quantum coherence (HMQC) and heteronuclear multiple bond connectivity (HMBC). Each pooled fraction (15mg) was dissolved in 500µl of deuterated solvent ($CDCl_3$ or DMSO-d_6), depending on the solubility of the compound and the extent of resolution obtained. The sample was then transferred to a 5mm internal diameter NMR tube and from the resulting spectra; the structures of the compounds were elucidated. ChemBioDraw Ultra, Version 12, was used to draw compound structures.

2.4.1.1 One-Dimensional NMR (1D)

This is the simplest technique used in structure elucidation. ^{1}H was used for determination of the types of protons in the compounds and ^{13}C for providing data on the number and kinds of carbon atoms in the compounds. Both ^{1}H and ^{13}C 1D spectra can be less informative than two-dimensional (2D) NMR analysis especially in the case of some of the more complex organic molecules.

2.4.1.2 Two-Dimensional NMR (2D)

2D NMR includes ^{1}H-^{1}H proton-proton *J*-correlations (COSY), HMQC was used to identify the correlation between protons and carbons atoms in samples through the ^{1}J

coupling between them. HMBC provided the correlation between the chemical shift of the protons in the samples and the heteronucleus ^{13}C through ^{2}J and ^{3}J coupling interaction between the nuclei (long-range H-X-C-C-C correlations).

2.4.2 Liquid Chromatography-Mass Spectrometry (LC-MS)

LC-MS is an analytical technique used for measuring the molecular mass of a sample and for elucidating the chemical structures of different molecules. This technique is associated with high-performance liquid chromatography (HPLC) and the detection was carried out with mass spectrometry. LC-MS was carried out to confirm the molecular weight of isolated compounds.

2.5 Bio-activity Examination

2.5.1 Tissue Culture and Cytotoxicity Assessment

2.5.1.1 Preparation of Complete Medium (RPMI 1640)

To 500ml RPMI 1640 medium, 50ml foetal calf serum, 5ml penicillin/streptomycin and 5ml glutamine were added. The medium was prepared in a sterile flow hood and was then stored at 4°C until required.

2.5.1.2 Maintenance of THP-1 Cells

THP-1 cells (European Collection Animal Cell Culture, UK) were maintained at 37°C, 5% CO_2, and 100% humidity in 75 cm^2 sterile flasks containing 50ml complete medium. Forty ml cell suspension from the flask was used or discarded every 4 days and replaced with fresh 40ml complete medium to keep the cells in log phase of growth. In each

bioassay, the cell numbers were counted in a haemocytometer and adjusted to $1x10^6$cells/ml.

2.5.1.3 Cytotoxicity Assay

Twenty mg of plant extract was dissolved in 1ml dimethyl sulfuxide (DMSO) and diluted with complete medium to give 2mg/ml of plant extract in 10% (w/v) DMSO. Extract (100µl) was then added to the first well of rows A, B, C and G of a 96-well tissue culture plate. Row G contained extract and medium only and acted as a background control for coloured material. DMSO (100µl) was added to the first wells of rows D and E (solvent control). 100µl of medium was then added to all the remaining wells, and a 1:2 serial dilution was carried out across the plate by transferring 100µl from one column to the next and the last 100µl discarded. Next, 100µl of cells ($1x10^6$cells/ml suspended in complete medium containing 10% (v/v) resazurin solution; 50mg in 500 ml deionised H_2O) was added to all the wells except row G. Medium containing 10% (v/v) resazurin solution (100µl/well) was then added to row F which contained cells only. After that, medium in row H was carefully removed, and 200µl/well of NaCl solution (300mg/ml) containing 10% (v/v) resazurin solution was added (negative control). The plate was placed in an incubator overnight at 37°C, 5% CO_2 and 100% humidity. On the following day, 100µl supernatant from each well was carefully transferred to a clean 96-well plate. The plate was read using a SpectraMax M5 micro-plate reader at 570nm and 600nm, to give day 1 readings. All residual medium in the wells of the original plate was carefully removed and replaced with 100µl of fresh medium plus 10% (v/v) resazurin solution. Finally, the original plate was returned to the

incubator overnight and then the plate read the next day to give day 2 readings. The second day reading was carried out in the absence of test agent to see if any of the cells would recover from toxic effects of the test agent. The % proliferation for each day was calculated using the following equation:

$$\% \text{ Proliferation} = \frac{\text{Mean (OD570nm-OD600nm) test agent}}{\text{Mean (OD570nm-OD600nm) positive control}} \times 100$$

2.5.1.4 Trypan Blue Dye Exclusion Testing

This method was chosen due to the low yield of the plant fractions. The cell cytotoxicity of the plant fractions obtained from the VLC and SC were determined using a trypan blue dye exclusion test. Only dead cells with permeable membranes take up this dye, therefore the viable cells do not appear blue. It was carried out after cells had been treated with the plant extract for a minimum of 20 hours (37°C, 5% CO2 and 100% humidity). Cells suspensions (100µl) were mixed with an equal volume of trypan blue solution (0.4%) and counted in a Neubaur haemocytometer. The percentage of viable cells was calculated. If the cell viability was over 50%, then the plant material was considered to be non-cytotoxic at that particular concentration and a percentage of 50% or fewer was considered to be cytotoxic.

2.5.2 TNF-α and IL-1β Assessment

Both TNF-α and IL-1β were evaluated using separate kits. One ml of THP-1 cells ($1x10^6$cells/ml) was added to all wells of a 24-well plate. Plant extracts at different non-toxic concentrations based on the cytotoxicity assay were added, with the controls containing cells only and medium added to ensure all wells contained the same final volume. Lipopolysaccharide (LPS, 0.05mg/ml) was added to half of the wells. The plate was left in an incubator at 37°C, 5% CO_2 and 100% humidity overnight. After at least 20 hours, the medium from each well was placed in 1.5ml microfuge tubes, which were then spun down at 800g for 5 minutes. The supernatant was then carefully removed and transferred into clean 1.5ml microfuge tubes. The supernatants were stored at -20°C until assessed by ELISA. The ELISA was carried out using an ELISA Ready-Set-GO! Kit (eBioscience) and following the manufacturer's instructions. Briefly, the wells were coated with 100µl capture antibody, diluted 1:250 in coating buffer and left for 1 hour at 37°C. The wells were then washed three times in wash buffer (8g NaCl, 0.2g KCl, 1.15g Na2HPO4 and 0.02g KH2PO4 per liter of distilled water, pH 7.2 and 0.01% (v/v) Tween), then blocked using 200µl assay diluent. This was then left for 1 hour at room temperature and washed as before. 100µl supernatant was added in triplicate wells. For the standards, a 1:2 serial dilution was carried out using a top concentration of 500pg/ml. The plate was then incubated overnight at 4°C, and the following day washed five times. Detection antibody (100µl /well, 1:250 dilution) was added and left for 1 hour at room temperature, followed by five washes. Avidin-HRP (100µl /well) was added for 30 minutes, followed by 7 washes. Tetramethylbenzidine (TMB, 100µl /well) solution was added and the reaction stopped after 15 minutes by the addition of 50µl of 10% (v/v) sulphuric acid. The plate was then read at 450nm in a SpectraMax M5 microplate reader.

From the standard curve the concentration of cytokine produced by the cells could be quantified.

2.5.3 Software Used for Data Presentation and Analysis

All graphs were produced using Microsoft Excel Version 9. Minitab Statistical Software, Version 15, was used to perform 2 sample unpaired t tests to calculate the p-values for the data obtained. For these experiments, p-values less than 0.01 were considered to be significant. ChemBioDraw Ultra, Version 12, was used to draw compound structures, and also to predict 1H-NMRs.

3.0 Results

3.1. Solvent Extraction and Yield

Preliminary cold maceration of 4g of each plant in 25ml ethanol was performed in order to obtain some idea of cytotoxicity and anti-TNF-α activities. Hot solvent extraction was then carried out using a Soxhlet apparatus to obtain crude extracts (hexane, ethyl acetate and methanol) from the leaves of *D. viscosa* and *A. setaceus* for further and detailed evaluation (Table 2).

Table 2: Plant yields for the species investigated in the present study.

Plant Name	Plant Code	Amount of Plant Material (g)	Amount Extracted (g)	% Yield (w/w)
Asparagus setaceus	AS	450	Hexane (12) Ethyl acetate (9) Methanol (38)	2.7 2.0 8.4
Dodonaea viscosa	Dod	300	Hexane (13.20) Ethyl acetate (22.37) Methanol (20.57)	4.4 7.4 6.8

3.2 Crude Ethanolic Extract Cytotoxicity

Cytotoxicity is defined as ≤ 50% cell growth for a particular plant extract concentration. The cytotoxicity of the plant extracts was tested in order to calculate the highest concentration that could be used in the cell stimulation assay without killing the cells. Day 1 and day 2 readings of the *Asparagus setaceus* ethanol extract showed no toxicity to the THP-1 cells at the highest concentrations tested 2mg/ml, (Figure 5). Similarly, the crude ethanol extract of *D.viscosa* did not show any cytotoxic effects at the maximum concentration of 2mg/ml over the two days (Figure 6).

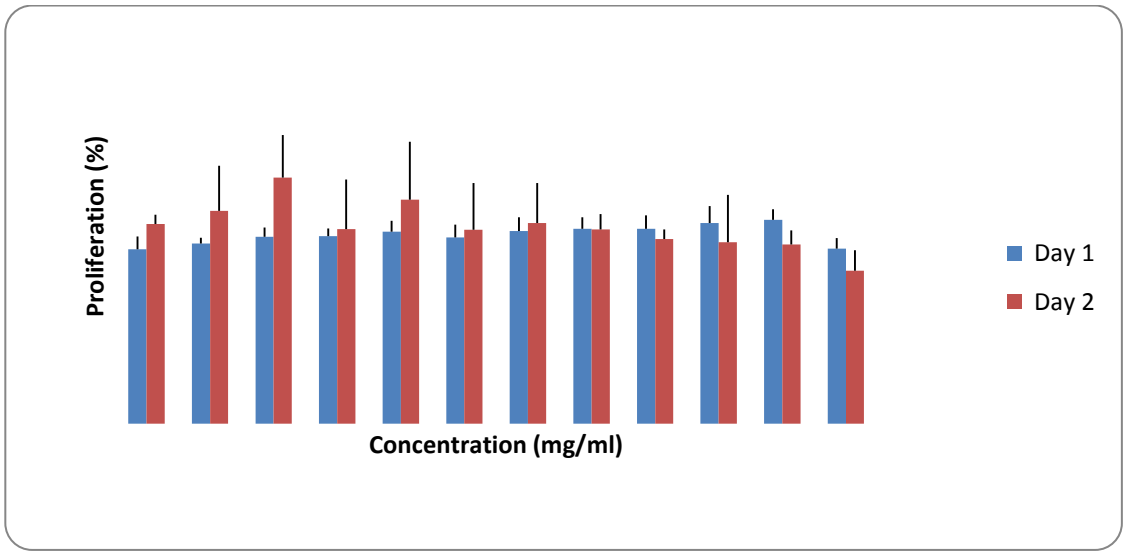


Figure 5: Percentage cell proliferation of THP-1 cells treated with crude *Asparagus setaceus* ethanol extract in 1ml DMSO incubated with 10% (v/v) resazurin solution.

Each bar shows triplicate readings ±SD and the figure is representative of 2 separate experiments.

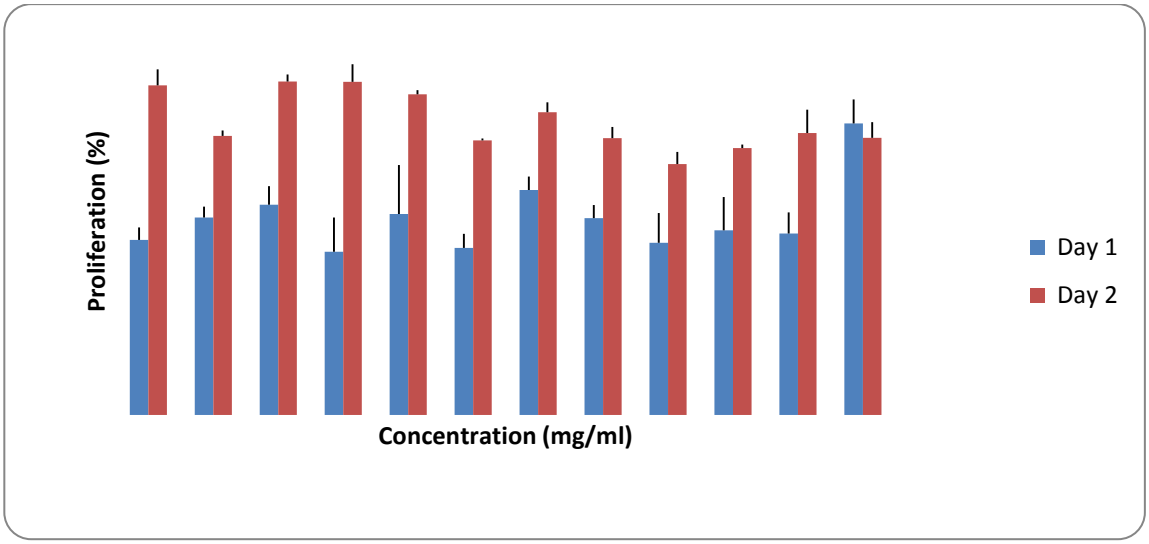


Figure 6: Percentage cell proliferation of THP-1 cells treated with crude *Dodonaea viscosa* ethanol extract in 1ml DMSO incubated with 10% (v/v) resazurin solution. Each bar shows triplicate readings ±SD and the figure is representative of 2 separate experiments.

3.3 TNF-α ELISA Results for Crude Ethanolic *Dodonaea viscosa* and *Asparagus setaceus* Extracts

A cell stimulation of the THP-1 cells with LPS was carried out with 2mg/ml crude ethanolic extract of the two plants. The levels of TNF-α were quantified using an ELISA. Any reduction of TNF-α levels produced by the LPS stimulated cells in the presence of the plant extract meant that the extract had an anti-inflammatory effect at that concentration, while an increase of TNF-α levels in the absence of LPS was indicative of pro-inflammatory activity of the extract. These preliminary results indicated some activity and therefore, it was decided to proceed with a full Soxhlet extraction.

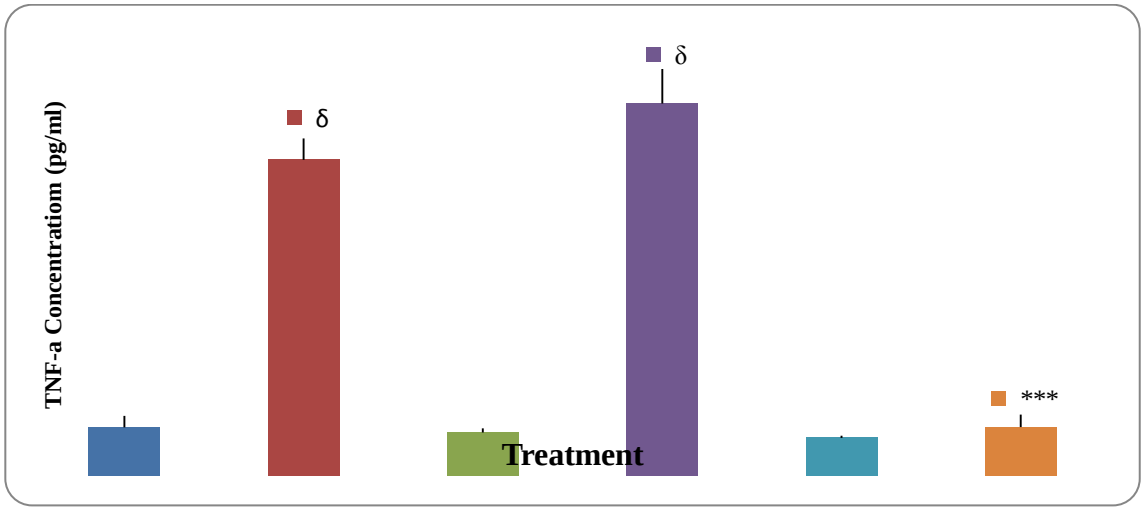
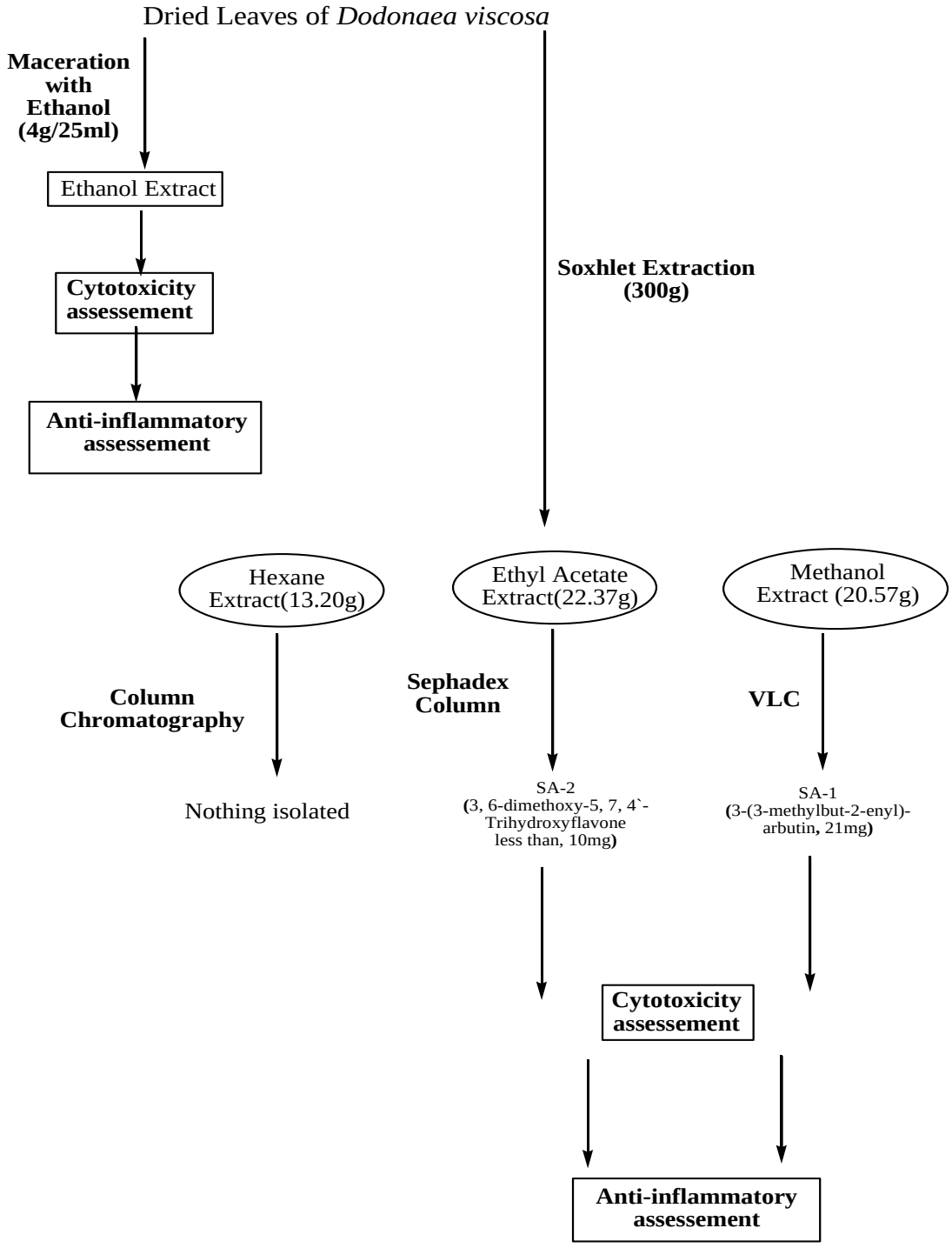


Figure 7: Levels of TNF-α produced by the THP-1 cells stimulated with LPS (0.05mg/ml) and without LPS in the presence and absence of 2mg/ml ethanol crude extract of two plants. Each bar is representive of the mean of four readings ±SD; the experiment was carried out at least three times. $^{\delta}$ Significant ($p \leq 0.032$) increase in TNF-α compared with cells only control.***Significant ($p \leq 0.0001$) decrease in TNF-α compared with cells + LPS control. E1= *Asparagus setaceus* ethanol extract, E2= *Dodonaea viscosa* ethanolic extract.

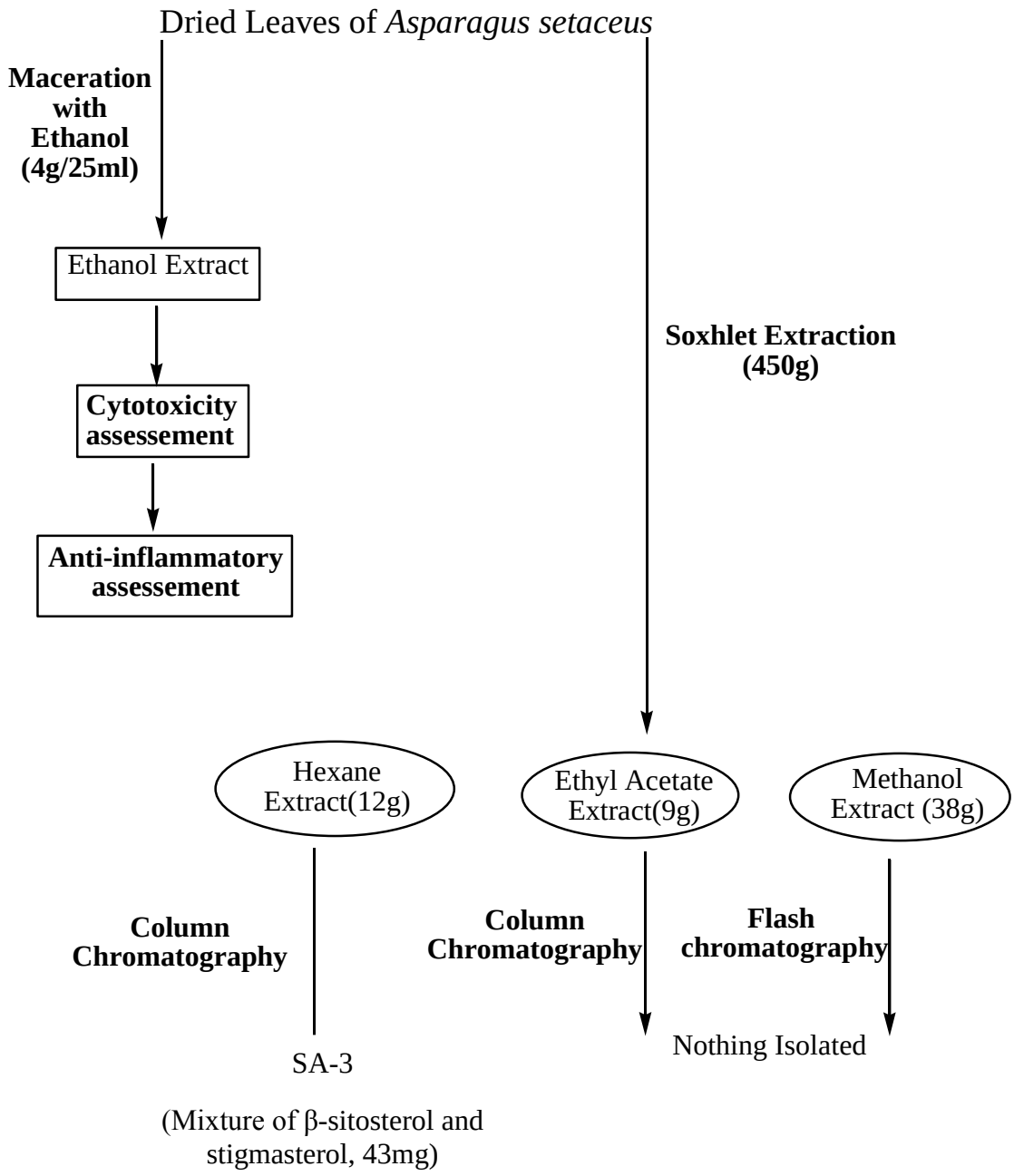
3.4 Fractionation of Crude *Dodonaea viscosa* and *Asparagus setaceus* Extracts

Vacuum liquid Chromatography (see section 2.3.4 and Appendix I) was applied to fractionate *D. viscosa* methanolic extract. The volume for each solvent phase was 400ml and 16 vials were collected from 2 sections of solvent sequences; ethyl acetate and methanol. After the vials were collected, they were left in a fume cupboard to evaporate. During this, a compound in vials 5 and 6 became concentrated and precipitated out of solution in the form of a green solid. The contents of these vials were assessed using NMR to elucidate structure of compound, which was named SA-1 (21mg). A Sephadex column (see section 2.3.3 and Appendix I) was used to fractionate *D. viscosa* using ethyl acetate. The polarity was subsequently changed from 100% chloroform to 5% methanol in chloroform until 100% methanol to collect more polar compounds. The volume for each solvent phase was 300ml and 56 vials were collected. A compound present in vial 12 evaporated to give light green crystals to give SA-2 (less than 10mg). No compounds were separated from the fractionation of *D. viscosa* hexane extract using column chromatography. White crystals of SA-3 (43mg) were isolated from *A. setaceus* hexane extract and the early fractions of ethyl acetate extract using column chromatography (see section 2.3.2 and Appendix Π). Flash chromatography (section 2.3.5) was applied to fractionate the methanolic extract of *A. setaceus* but no compounds were isolated.

Appendix I. Isolation scheme for *Dodonaea viscosa*



Appendix II. Isolation scheme for *Asparagus setaceus*



3.5 Characterization of Isolated Compound SA-1 as 3-(3-methylbut-2-enyl)-arbutin

The compound (SA-1) was obtained as a green solid with some impurities from the methanol extract of *Dodonaea viscosa* leaves using vacuum liquid chromatography (VLC). Using 100% EtOAc as the mobile phase on TLC, the compound appeared as a brown spot ($R_{f=}$ 0.4) after spraying with anisaldehyde reagent and heating. LC-MS **[**M-H**]** $^{-}$**=** 339≡ ($C_{17}H_{23}O_7$) showed the molecular formula to be C_{17} $H_{24}O_7$. The ^{1}H NMR spectrum (400MHz, DMSO-d_6, Figure 15) revealed the presence of an aromatic ring with protons at 6.71 (*d*, J = 2 Hz ,C =118.3), 6.65 (*d*, J =8.5Hz ,C=115.4), and δ 6.69 (*dd*, J= 2, 8.5Hz, C=114.8) ppm and a phenolic hydroxyl at δ 8.89 with HMBC correlation to three aromatic ring carbons including one at 150.1ppm to which the OH group is attached and two ortho carbons , a quaternary carbon at 128.5ppm and a CH at 115.4 (δ_H 6.65)ppm. The splitting pattern observed (Figure 8) indicates the phenyl ring is trisubtituted at positions 1, 3 and 4 (A) at (δ_c 150.8, 128.5 and 150.1). The ^{1}H NMR also showed two geminal methyl groups at δ 1.65 and δ 1.68 ppm and one double bond methine (═══CH) at 5.25 and a saturated methylene (-CH_2) at δ 3.16 ppm. This was indicative of a 3-methylbut-2-enyl side chain (Figure 8, B).

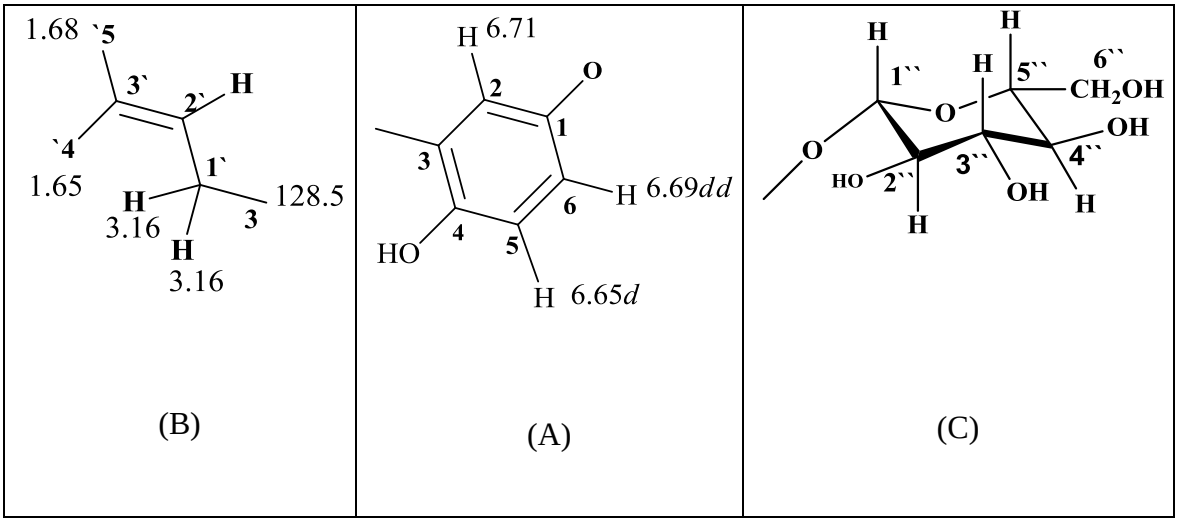


Figure 8: Partial structures for three parts of the compound SA-1 based on NMR evidence, especially the HMBC experiment (See Table 3).

The proton at δ 4.60 ppm indicated the presence of a sugar moiety, probably glucose based on the splitting and coupling constants observed in the ^{1}H NMR spectrum, attached as a glycoside (Figure 12, C). The ^{13}C *J* modulated spectrum (Figure 16) showed 17 carbons made up of two methyl groups at 18.1ppm and 26.0 ppm, two methylene groups at 28.4 and 61.2 ppm ; nine methine carbons at 123.1, 118.3, 115.4 , 114.8, ,102.1, 70.6, 73.8, 77.2 and 77.4ppm, and four quaternary carbons at 150.8 , 128.5, 131.9 and 150.1 ppm. Two phenolic carbons were observed at 150.1 and 150.8 ppm. Hence the glycoside link was on one of the phenolic carbons while the other attachment was an –OH group. This hydroxyl proton appeared at δ 8.89 in the proton spectrum. The quaternary aromatic carbon at 128.5ppm showed a strong correlation to the methylene protons (δ_H 3.16) of the prenyl side chain indicating the attachment of the prenyl group to the quaternary aromatic carbon at 128.5 and ortho to the hydroxyl group (δ_H 8.89, δ_c 150.1) and the aromatic CH (δ_c 118.3, δ_H 6.71).

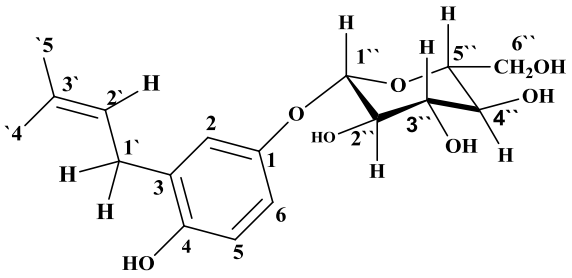


Figure 9: Structure of SA-1

Table 3: ^{1}H (400MHz), ^{13}C (100MHz), HMBC and COSY data of SA-1 in DMSO-$d_{6.}$

Position	$^{1}H^{*}$	^{13}C*	HMBC	COSY
1	-	150.8	-	-
2	6.71*d (* J=2Hz)	118.3	28.4/114.8/150.1	6.69
3	-	128.5	-	-
4	-	150.1	-	-
5	6.65*d* (J=8.5Hz)	115.4	128.5/150.8	6.69
6	6.69 *dd (*J=2Hz, 8.5Hz)	114.8	118.3/150.1/128/116/113	6.65,6.71
4-OH	8.89 *s*	-	150.1/128.5/115.4	-
1′	3.16 *dm*	28.4	150.1/118.3/131.9	5.25
2′	5.25 *tm*	123.1	28.4/18.1/26.0/128.5	3.16
3′	-	131.9	-	-
4′	1.65 *brs*	18.1	123.1/ 26.0	-
5′	1.68 *brs*	26.0	123.1/ 18.1	-
1″	4.60*d* (J=8Hz)	102.1	150.8	3.15
2″	3.15*m*	70.6	77.4	3.17/4.60/5.18
3″	3.17*m*	73.8	61.2	3.15/3.20/5.00
4″	3.20*m*	77.2	61.2/70.6	3.17/3.28/4.94
5″	3.28	77.4	73.8	3.65/3.45/3.20
6″	3.65/3.45	61.2	77.2	3.28/4.51
2″-OH	5.18*d*	-	-	3.15
3″-OH	5.00*d*	-	-	3.17
4″-OH	4.94*d*	-	-	3.20
6″-OH	4.51*t*	-	-	3.45/3.65

* The proton to carbon direct couplings were determined from the HSQC experiment (Figure 12).

The other quaternary, oxygen-bearing carbon (150.8ppm) of the aromatic ring had a ^{3}J coupling to the aromatic proton δ_H 6.65 and to the anomeric proton of the sugar δ_H 4.60 indicating a glycosidic linkage through oxygen to that carbon. These data indicated the structure (Figure 9).

The correlation (HMBC, Figure 13) between the proton at δ 4.60 (1``, the glucose anomeric H) and the ^{13}C at 150.8 ppm on the aromatic ring confirms the attachment of the glucose as a glucoside at position- 1 of the benzene ring. This compound was identified as 3-(3-methylbut-2-enyl)-arbutin.

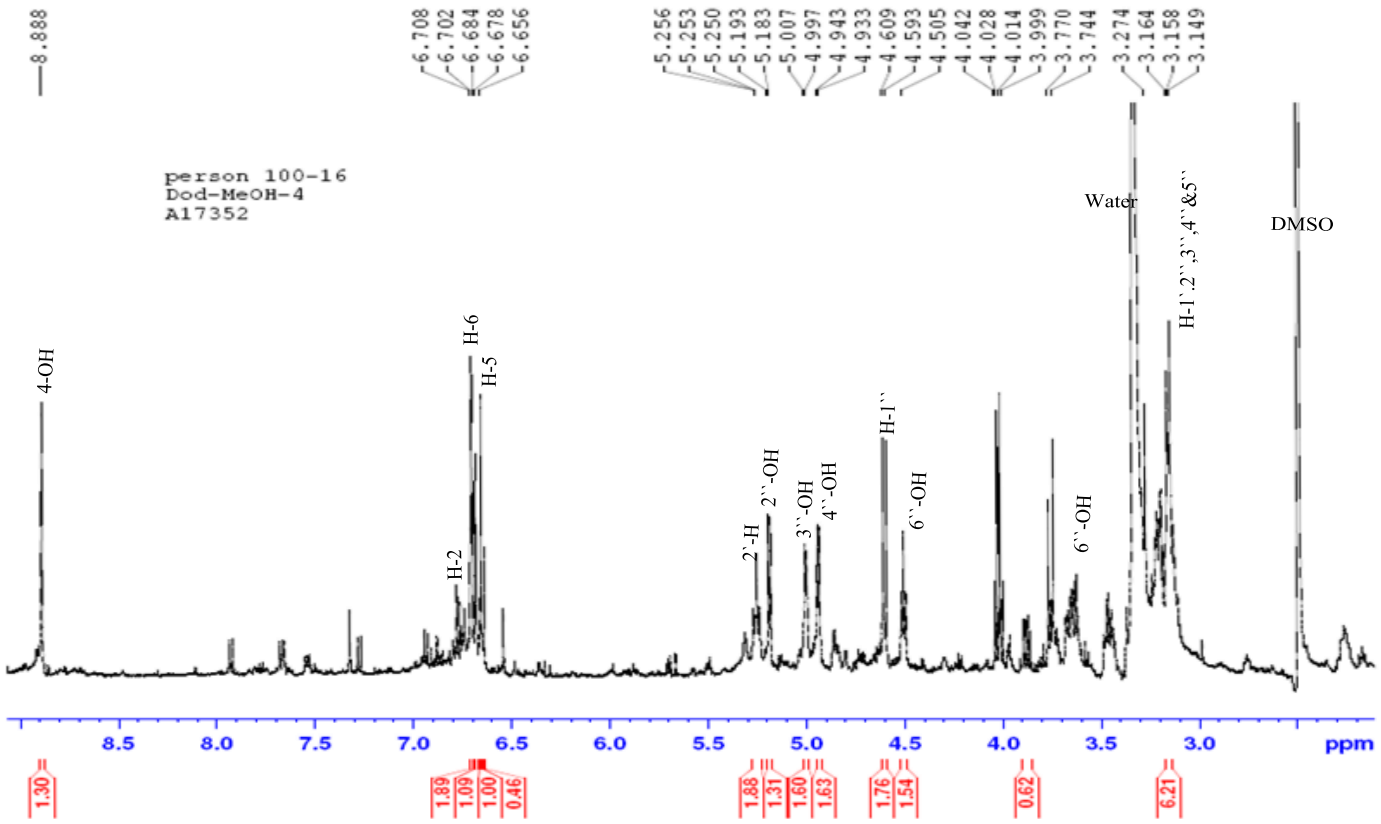


Figure 10: The ^{1}HNMR spectrum of SA-1 in DMSO-d6 at 400 MHz.

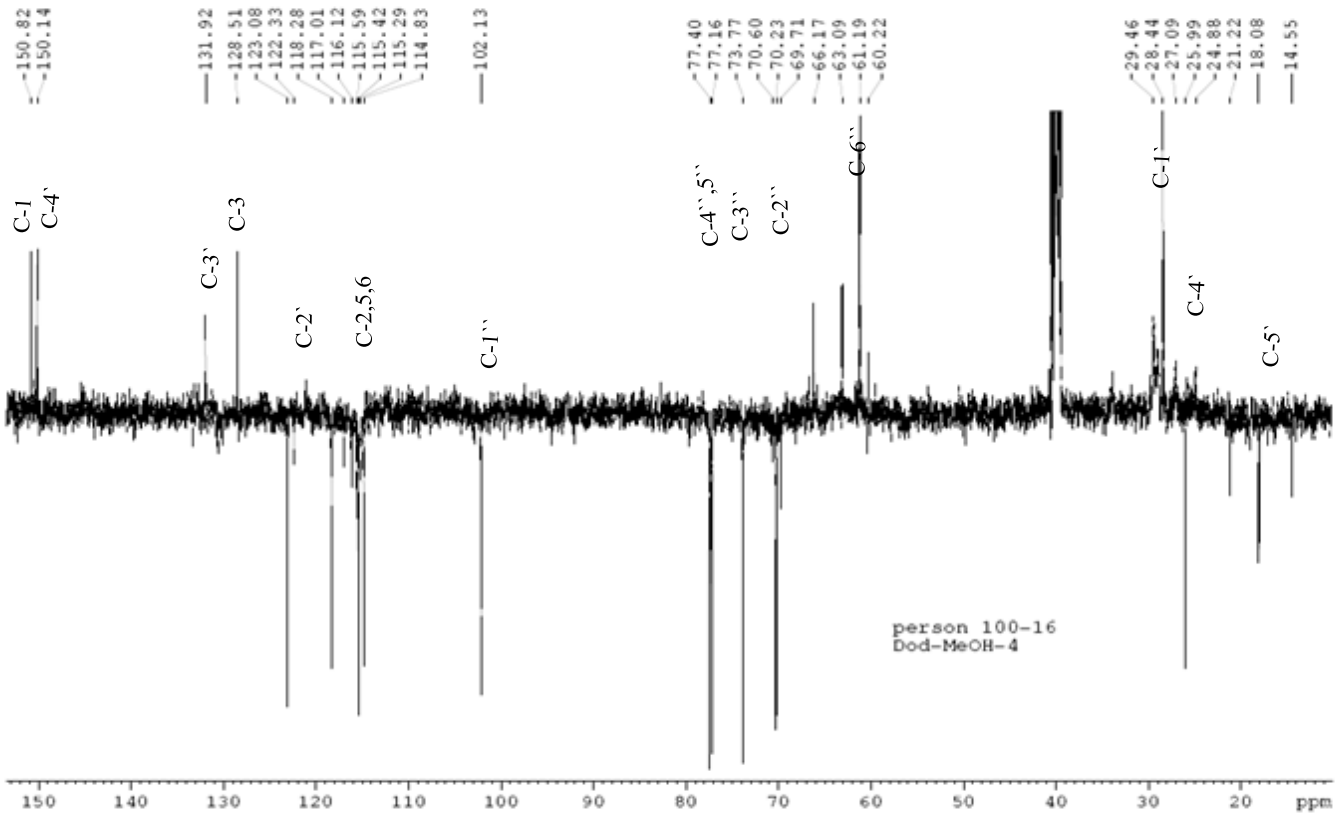


Figure 11: The 13C NMR spectrum of SA-1 in DMSO-d6 at 100 MHz.

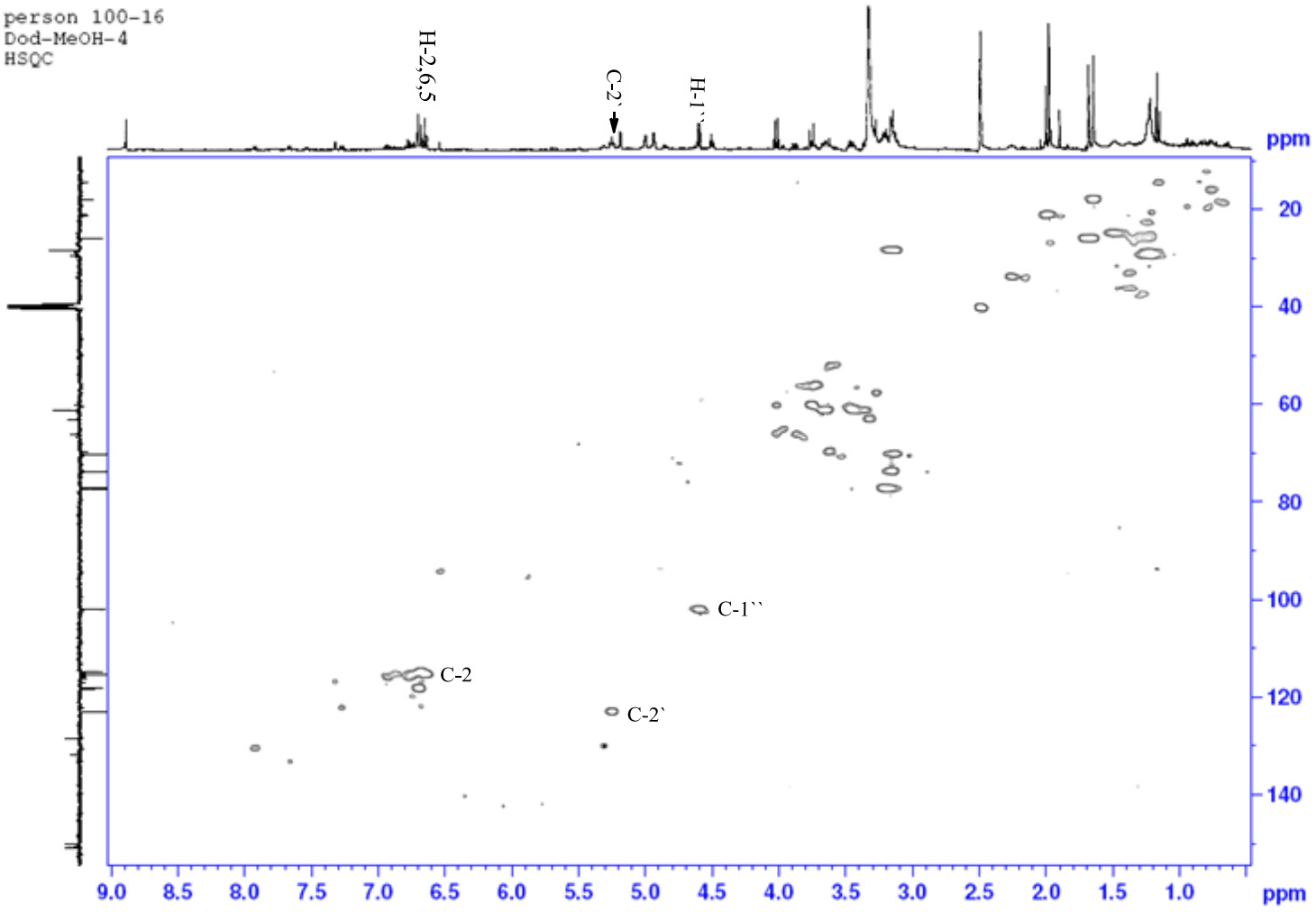


Figure 12: The HSQC spectrum of SA-1 in DMSO-d6 at 400 MHz.

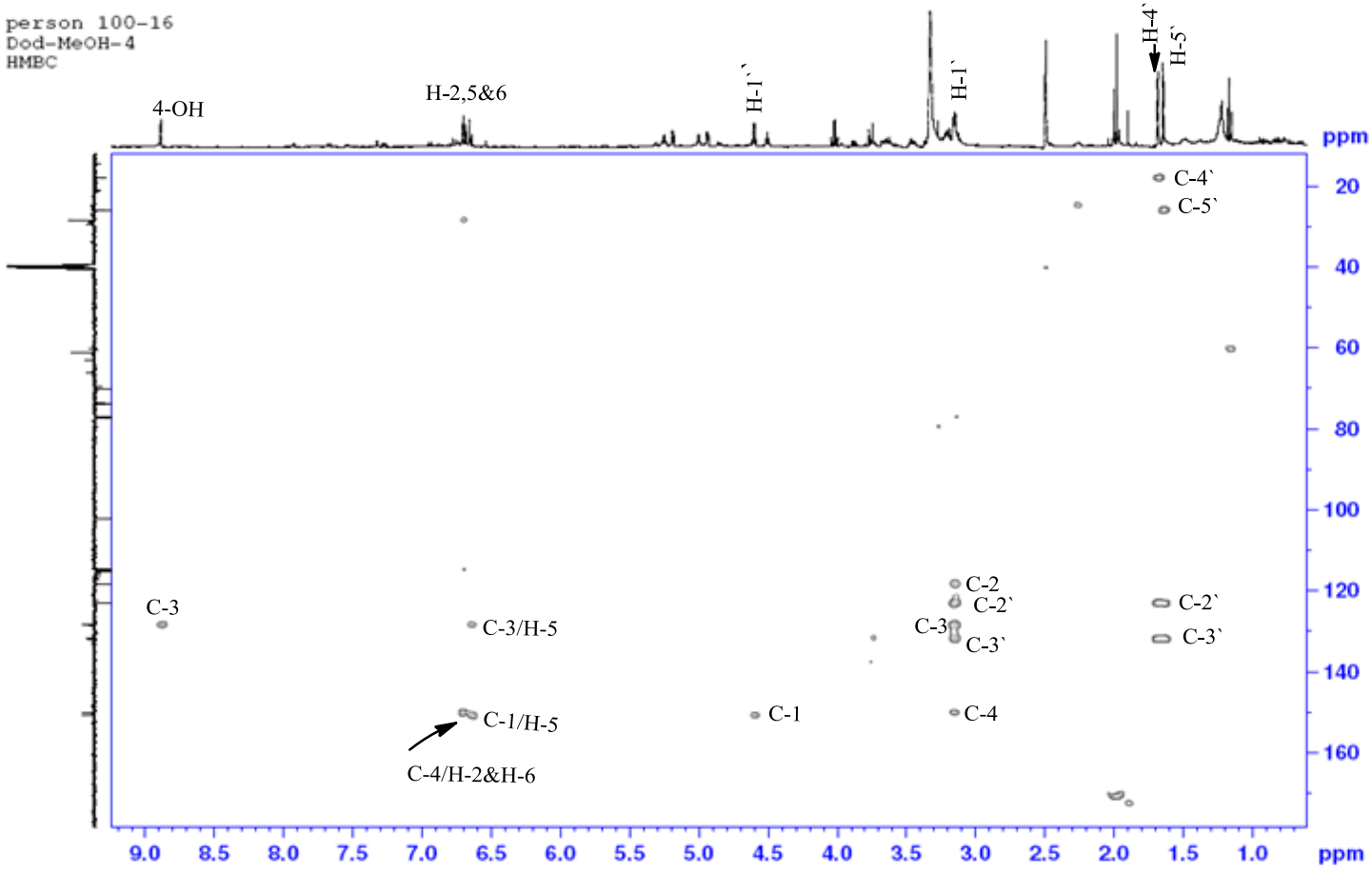


Figure 13: The HMBC spectrum of SA-1 in DMSO-d6 at 400 MHz.

3.6 Characterization of Compound SA-2 as 3, 6-dimethoxy-5, 7, 4`-trihydroxyflavone.

This compound was isolated from the leaves of *Dodonaea viscosa* ethyl acetate extract using a Sephadex column (SC). After spraying with *p*-anisaldehyde-sulphuric acid reagent and heating, a yellow to greenish spot appeared with R_f value of 0.22. The mass spectrum in negative ion mode gave $[M-H]^- = 281 \equiv C_{17}H_{13}O_7$ which showed that the molecular formula of this compound was $C_{17}H_{14}O_7$.

The ^1H NMR spectrum (400MHz, DMSO-d_6, Figure 15) showed two methoxy signals at δ_H 3.77 and 3.74 (OMe-3, OMe-6). Four aromatic protons representative of an AA`BB` coupling pattern were observed at δ_H 7.92 (2H, *d*, H-2`.6`), 6.93 (2H, *d*, H-3`, 5`) and another singlet at δ_H 6.54(1H, *s*, H-8,) was from a second aromatic ring. The presence of a highly de-shielded proton at δ_H 12.77 could be attributed to a H- bonded phenolic –OH group found at position-5 of flavonoids. The ^{13}C NMR spectrum (Figure 20) indicated the presence of 17 carbons including a carbonyl at δ_c 178.7 (C-4) and two methyls of methoxy groups in sterically hindered positions at δ_c 60.2 and 60.4 ppm, five methine carbons at 115.6 (2XC), 130.3 (2XC) and 94.5ppm (C-3`/C-5`,C-2`/C-6` and C-8, respectively). Seven phenolic carbons were observed at 156.2, 137.8, 152.9, 131.6, 152.0, 157.8, and 160.7 ppm (C-2, C-3, C-5, C-6, C-7, C-9 and C-4`) and two quaternary carbons at 105.0 and 121.1ppm (C-10 and C-1`).

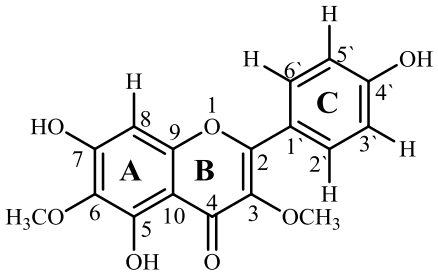


Figure 14: Structure of SA-2

Using 2D NMR (COSY, HSQC and HMBC) the compound was confirmed as follows: The long range correlation (HMBC) of -OH to C10, C5 and C6 indicates the carbons near its own carbon. The –OH bearing one and the two adjacent carbon atoms since C10 is at 105.0, it is not phenolic and cannot be bearing the -OH. Therefore, OH is either at position 5 or 6 at (152.9 or 131.6ppm). The methoxy group at 3.74 correlates with the carbon at 131.6 but the –OH at position 6 cannot be H-bonded with the -C=O at position 4. Therefore, the –OH must be at position 5 and its carbon is 152.9 and C6 must be 131.6ppm. Therefore, this is the carbon bearing this methoxy as the CH_3O can correlates with only one carbon i.e. the carbon bearing it. This confirming C6 to be a methoxy bearing carbon. Correlations for the proton at 6.54 to C6, C7, C9 and C10 at 131.6, 152.0, 157.8 and 105.0, respectively indicate that this proton is at position 8 as it has a 3*J* coupling to C10 and C6 and the other correlations must be 2*J* to C7 and C9 of the compound. The second methoxy correlates (HMBC) with the C3 at 137.8 and this must be the methoxy bearing and since none of the aromatic protons correlate to this carbon, it must be at C3 of the compound. Therefore, the phenolic carbon at 160.7 must be in

ring C of the compound and at position C4` to give the AA`BB` coupling of the proton in this ring.

The ^{1}H &^{13}C NMR spectral data are in agreement, eventhough the data were collected in different NMR solvents, with those reported earlier (Teffo *et al*., 2006, Table 5) and the structure identified as 3, 6-dimethoxy- 5, 7, 4`-trihydroxyflavone (Figure 14).

Table 4: ^{1}H (400MHz), ^{13}C (100MHz), HMBC and COSY data of 3, 6-dimethoxy- 5, 7, 4`-trihydroxyflavone in DMSO-d_6.

Position	^{1}H	^{13}C	HMBC	COSY
1	-	-	-	-
2	-	156.2	-	-
3	-	137.8	-	-
4	-	178.7	-	-
5	-	152.9	-	-
6	-	131.6	-	-
7	-	152.0	-	-
8	6. 54*s*	94.5	105.0,131.6,152.0,157.8	
9	-	157.8	-	-
10	-	105.0	-	-
3-OCH_3	3.77	60.2	137.8	-
6-OCH_3	3.74	60.4	131.6	-
5-OH	12.77	-	152.9,105.0,131.6	-
7-OH	10.25 *brs**	-	-	-
1`	-	121.1	-	-
2`, 6`	7.92*d*	130.3	160.7,130.3	6.93
3`, 5`	6.93*d*	115.6	122	7.92
4`	-	160.7	-	-
4`-OH	10.80 *brs**	-	-	-

* = interchangeable.

Table 5: Comparison of the ^{1}H & ^{13}C NMR data of SA-2 in DMSO-d_6 and the same compound (3, 6-dimethoxy-5, 7, 4`-Trihydroxy-flavone) found in the literature (Teffo, 2006; Heerdena, 2000).

Position	^{1}H of SA-2 (DMSO-d_6)	^{13}C of SA-2 (DMSO-d_6)	^{1}H of 5, 7, 4`-Trihydroxy-3,6 dimethoxyflavone(CD_3OD) (Heerdena *et al*., 2000)	^{13}C of 5, 7, 4`-Trihydroxy-3,6-dimethoxyflavone (Acetone –d_6) (Teffo, 2006)
1	-	-	-	-
2	-	156.2	-	157.6
3	-	137.8	-	138.8
4	-	178.7	-	179.8
5	-	152.9	-	153.6
6	-	131.6	-	131.8
7	-	152.0	-	153.1
8	6. 54*s*	94.5	6.45*s*,1H	94.4
9	-	157.8	-	156.9
10	-	105.0	-	106.3
3-OCH_3	3.77	60.2	3.86*s*,3H	60.2
6-OCH_3	3.74	60.4	3.75*s*,3H	60.7
1`	-	121.1	-	122.7
2`,6`	7.93	130.3	7.93*d*,2H	131.2
3`,5`	6.94	115.6	6.88*d*,2H	116.4
4`	-	160.7	-	160.9

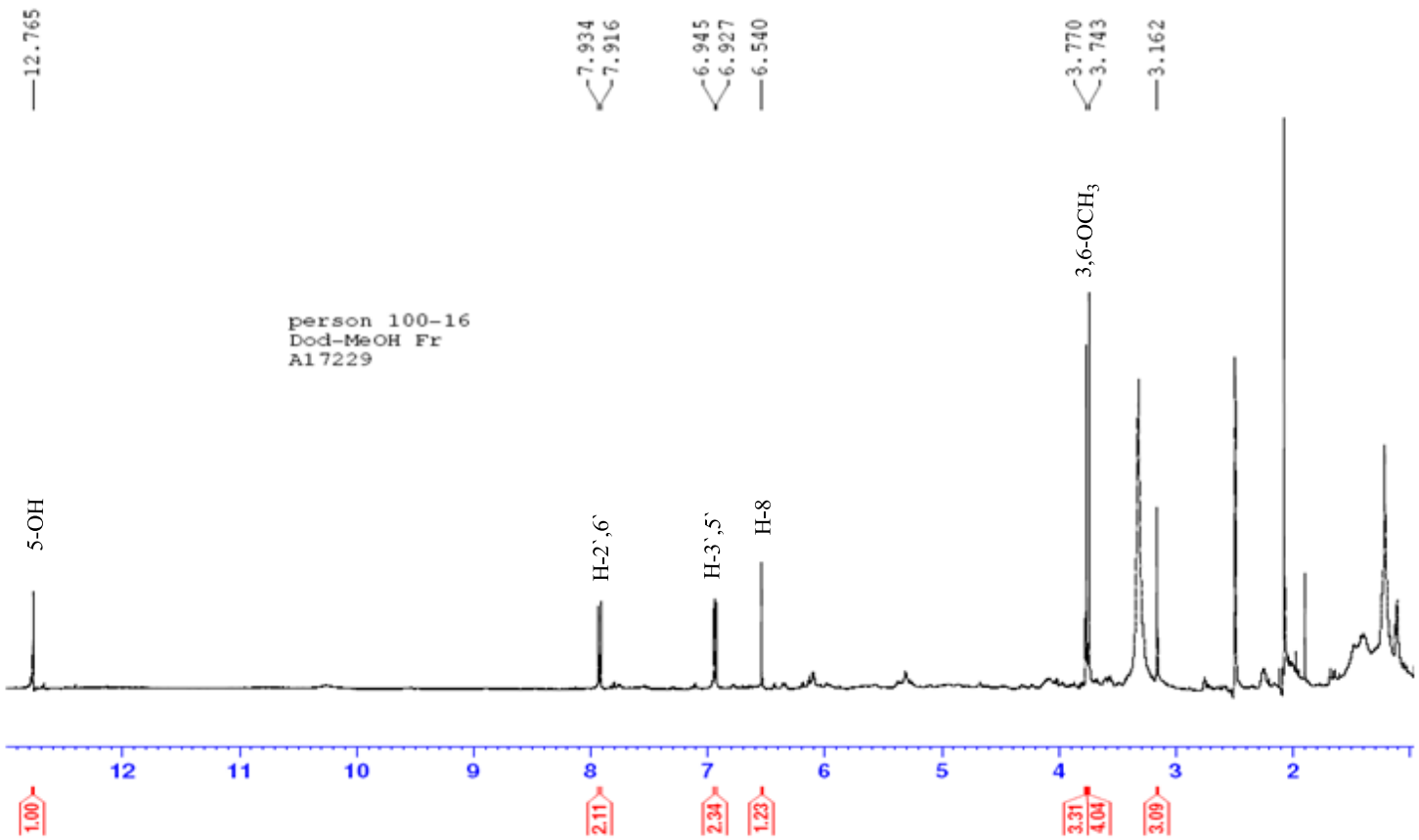


Figure 15: The ^{1}HNMR spectrum of SA-2 in DMSO-d6 at 400MHz.

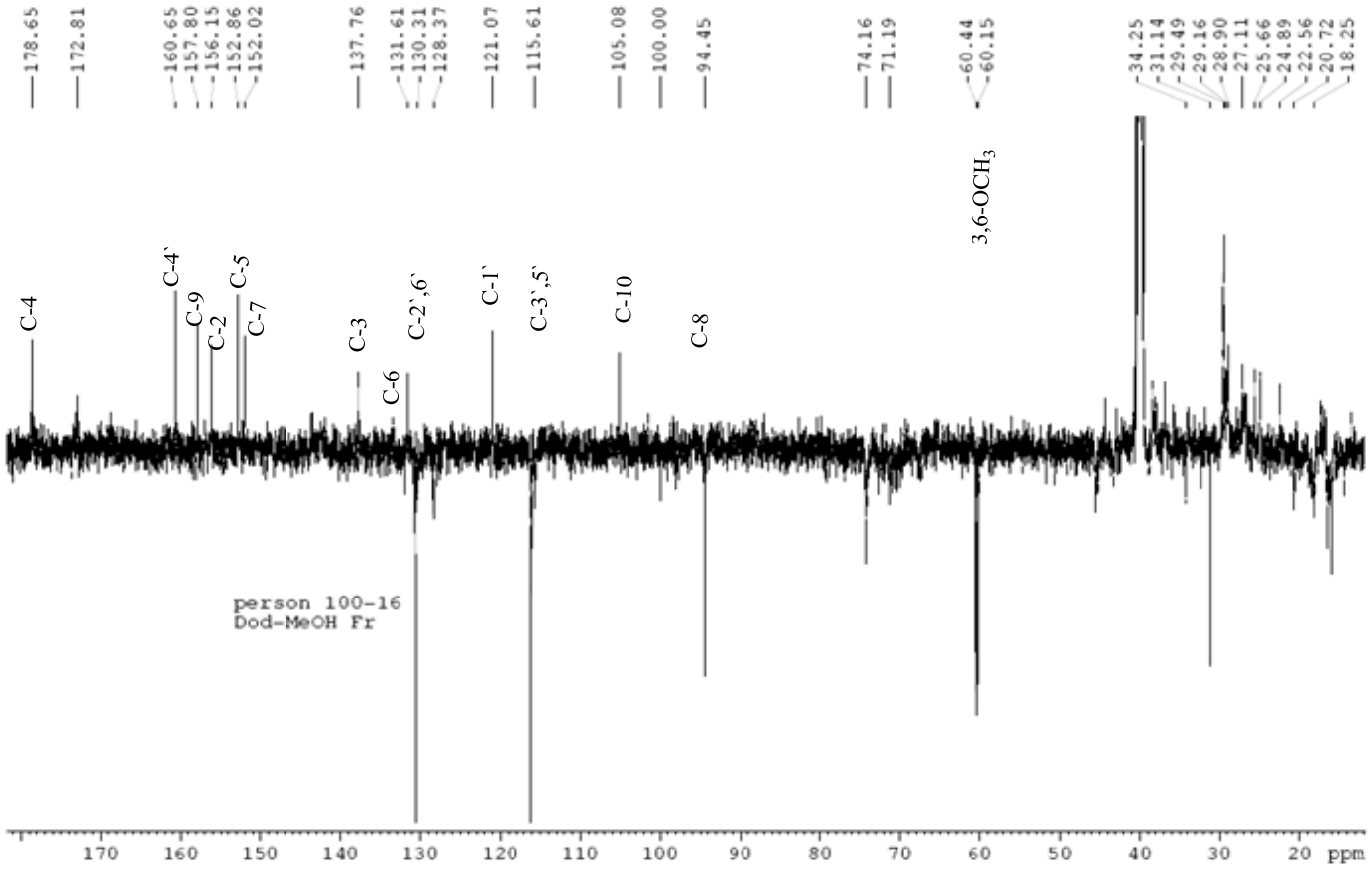


Figure 16: The 13C NMR spectrum of SA-2 in DMSO-d6 at 100 MHz.

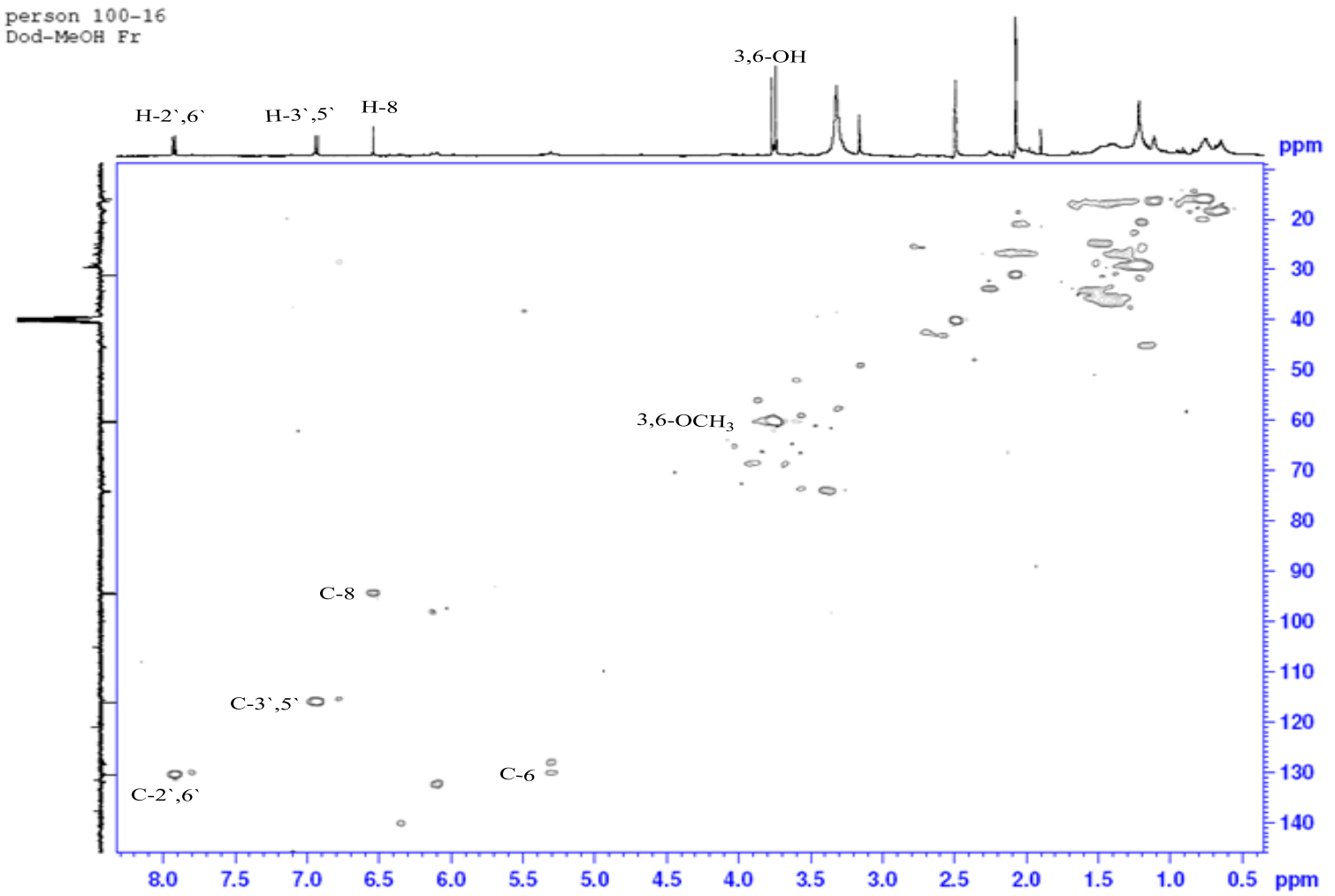


Figure 17: The HSQC spectrum of SA-2 in DMSO-d6 at 400 MHz.

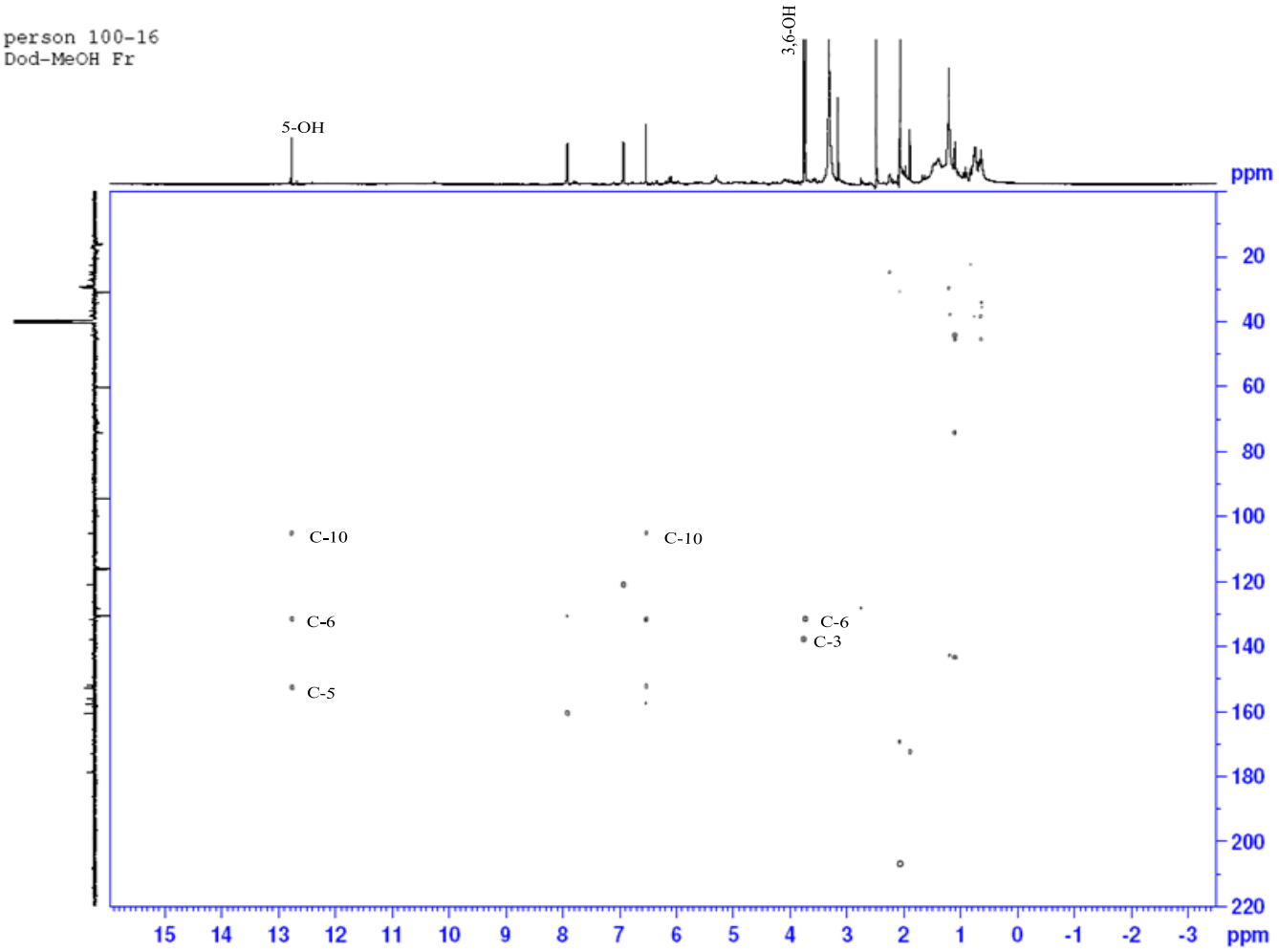
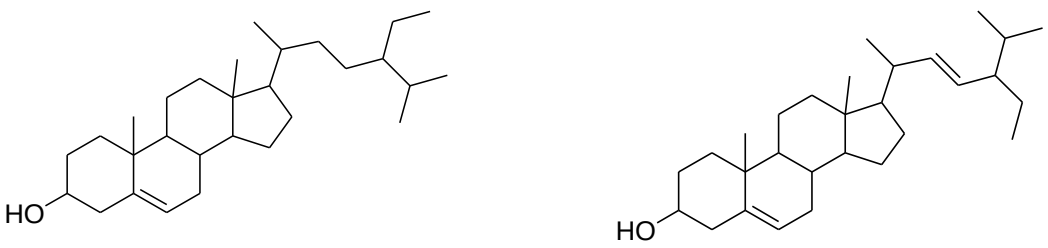


Figure 18: The HMBC spectrum of SA-2 in DMSO-d6 at 400 MHz.

3.7 Characterization of fraction SA-3 as a Mixture of Two Steroids (β-sitosterol and stigmasterol)

The fraction SA-3 was isolated from the hexane extract and early fractions of ethyl acetate extract of *A. setaceus* and identified as a mixture of β-sitosterol and stigmasterol **(**Figure 19). It appeared as white crystals that gave a pink colour on TLC, after spraying with anisaldehyde reagent. This was confirmed by comparison with a standard β-sitosterol (Sigma Aldrich, UK).



β-Sitosterol Stigmasterol

Figure 19: chemical structures of β-sitosterol and stigmasterol.

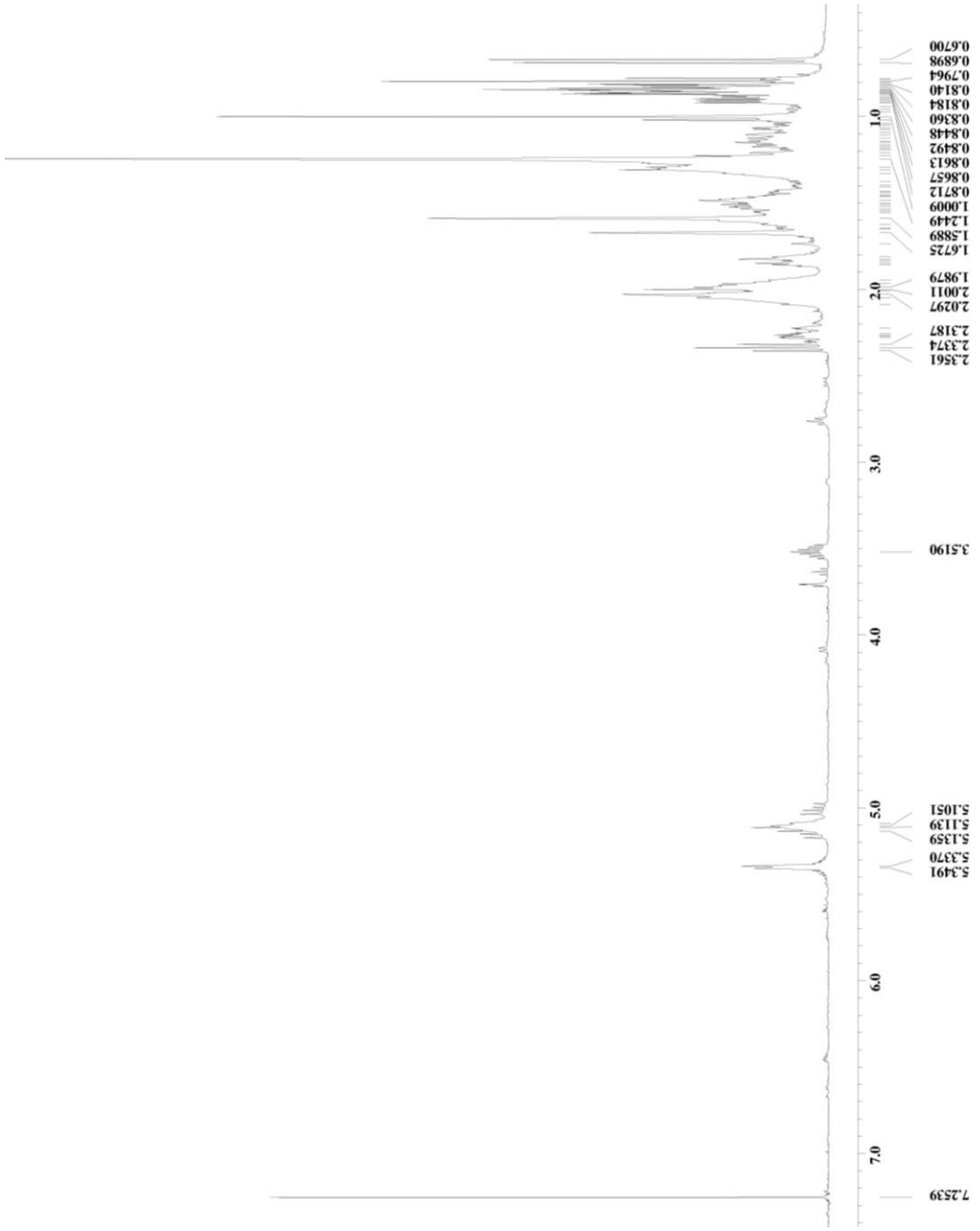


Figure 20: The ^{1}H NMR spectrum of SA-3 in Chloroform-d at 400MHz.

3.8 Cytotoxicity of the *D. viscosa* fractions using Trypan blue exclusion test

Fractions SA-1 and SA-2 obtained from VLC and SC were so low in quantity that it was not possible to investigate their cytotoxicity using the resazurin method used previously for the crude extracts. Following the first 24 hour incubation period, cell viability was assessed using a trypan blue dye exclusion test. Any fraction that caused cell viability to decrease to less than 50% was considered cytotoxic. Trypan blue was used on both tested fractions at a concentration of 0.5mg/ml. The results, which can be seen in Figure 21, show that none of the fractions were toxic, with the majority of cells still viable. Fraction SA-2 caused the lowest cell viability, at 70%, which is still significantly above the threshold.

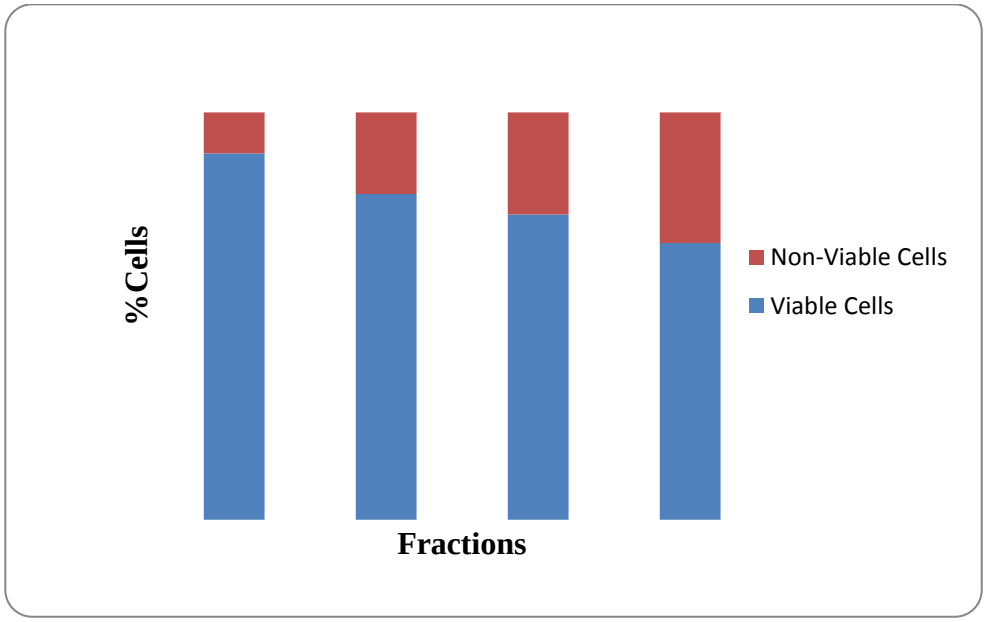


Figure 21: Percentage of viable and non-viable cells after a 24 hour incubation period with 0.5mg/ml of both fractions (SA-1 and SA-2). These results show that none of the plant fractions at that concentration were cytotoxic. Each bar represents the mean of three wells (duplicate reading from each).

3.9 Preliminary Anti-inflammatory Assessment

3.9.1 TNF-α and IL-1β Assessment for Compound SA-1 of the *Dodoneae* Methanol Extract

The isolated phenolic glycoside 3-(3-methylbut-2-enyl)-arbutin (SA-1) was tested to evaluate anti-inflammatory activity at a concentration of 0.5mg/ml. As seen in Figure 22, SA-1 significantly (p= 0.0001) reduced the levels of TNF-α produced by stimulated cells, from 296±4.8pg/ml (control) to 50±13.5pg/ml. Figure 23 shows SA-1 inhibited IL-1β significantly (p=0.003) from 778±100pg/ml (control) to 0.37±0.75pg/ml and this was indicative of anti-inflammatory activity. No pro-inflammatory response was observed.

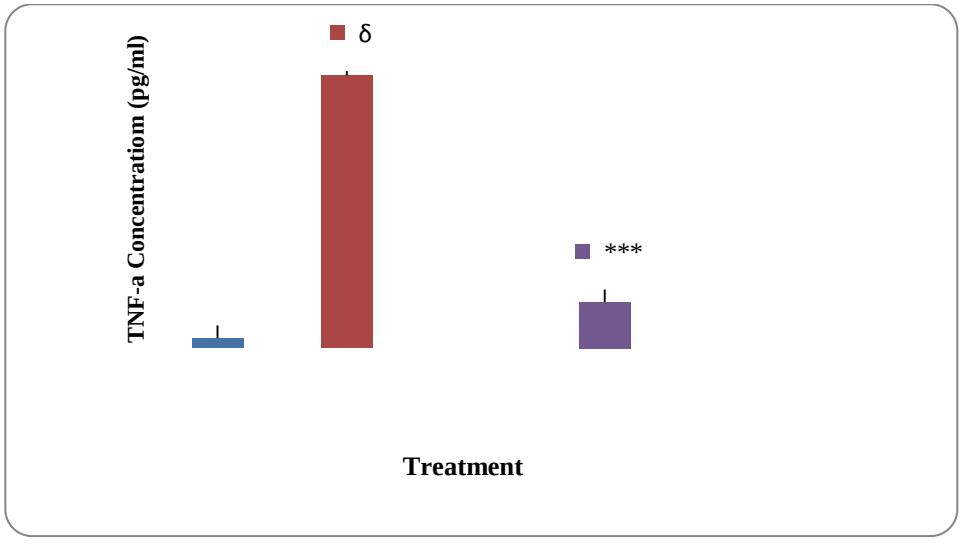


Figure: 22 Levels of TNF-α produced by the THP-1 cells stimulated with LPS (0.05mg/ml) and without LPS in the presence and absence of 0.5mg/ml SA-1. Each bar is representive of the mean of four readings ± SD; the experiment was carried out at least four times. $^{\delta}$ Significant (p≤0.0001) increase in TNF-α compared with cells only control. ***Significant (p≤0.0001) decrease in TNF-α compared with cells + LPS control.

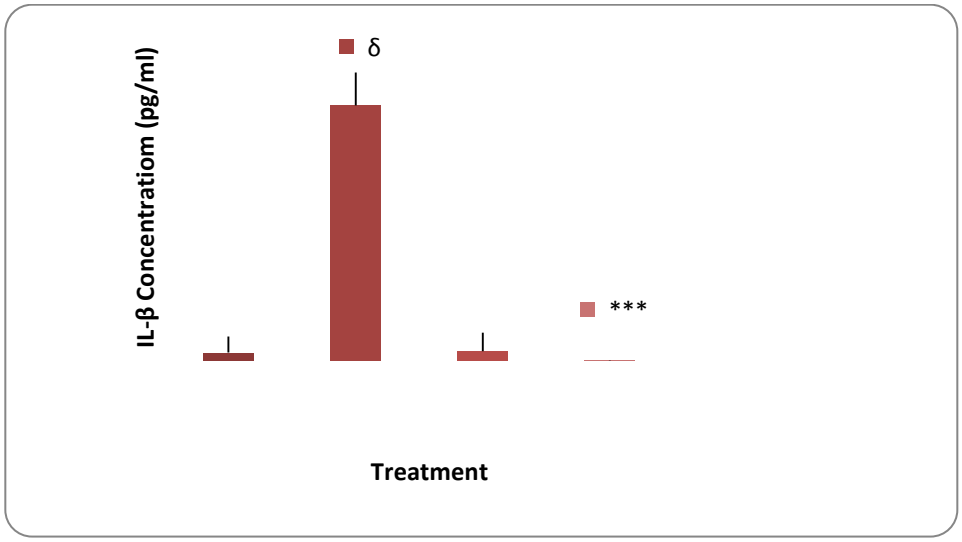


Figure 23: Levels of IL-1β produced by the THP-1 cells stimulated with LPS (0.05mg/ml) and without LPS in the presence and absence of 0.5mg/ml SA-1. Each bar is representive of the mean of four readings ±SD; the experiment was carried out at least three times. $^{\delta}$ Significant ($p \leq 0.0001$) increase in TNF-α compared with cells only control. ***Significant ($p \leq 0.003$) decrease in TNF-α compared with cells + LPS control.

3.9.2 TNF-α and IL-1β Assesement for Compound SA-2 of *Dodonaea* Ethyl Acetate Extract

Figures 24 and 25 show the effect of the flavone SA-2 on THP-1 cells stimulated with LPS. The compound tested at 0.5mg/ml significantly ($p \leq 0.0001$) inhibited TNF-α concentrations from 296±4.8 pg/ml (control) to 11.77±23.5 pg/ml. Similarily, it significantly ($p \leq 0.008$) reduced the levels of IL-1β produced by stimulated cells, from 778 ±100 pg/ml to 68.541 ±70 pg/ml. These results were indicative of an anti-inflammatory effect of this compound and no pro-inflammatory response was detected.

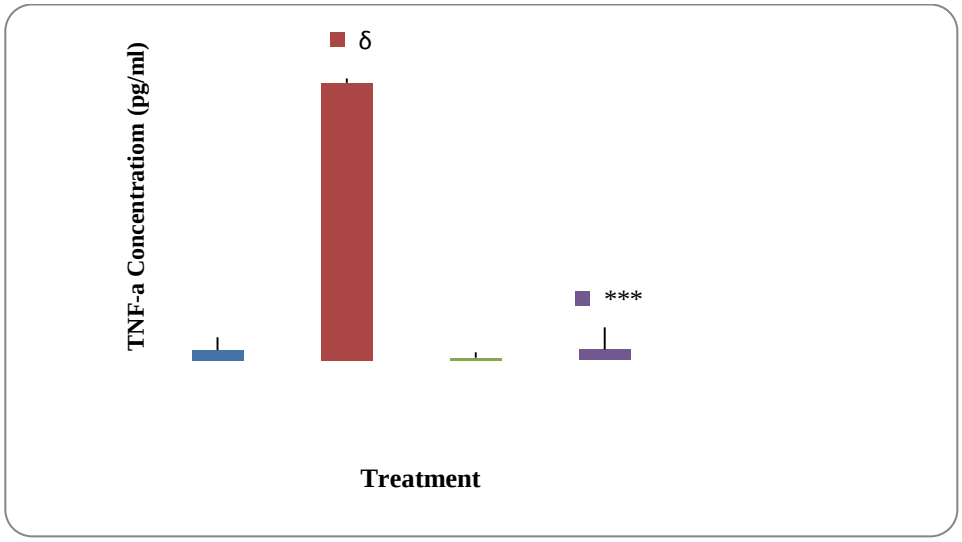


Figure 24: Levels of TNF-α produced by the THP-1 cells stimulated with LPS (0.05mg/ml) and without LPS in the presence and absence of 0.5mg/ml SA-2. Each bar is representive of the mean of four readings ± SD; the experiment was carried out at least four times. $^{\delta}$ Significant ($p \leq 0.0001$) increase in TNF-α compared with cells only control. ***Significant ($p \leq 0.0001$) decrease in TNF-α compared with cells + LPS control.

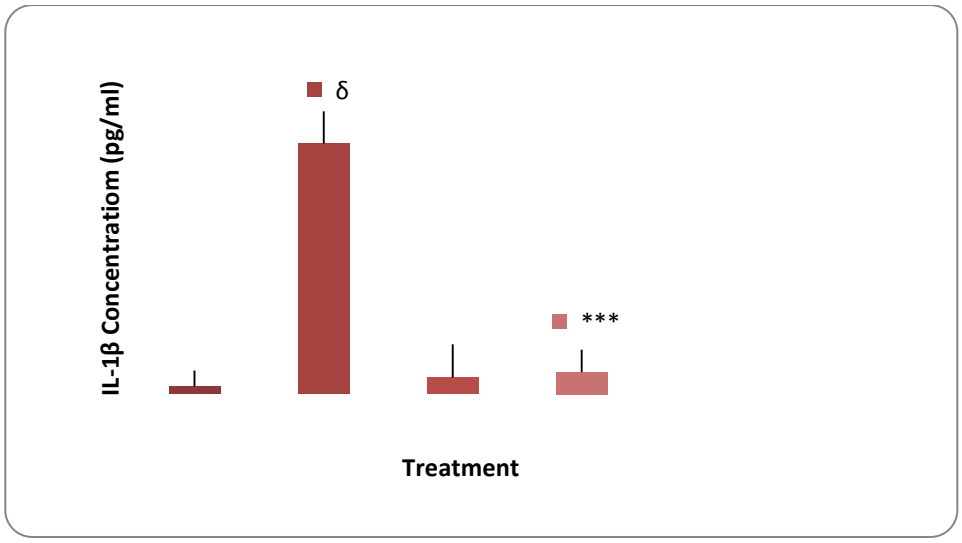


Figure 25: Levels of IL-1β produced by the THP-1 cells stimulated with LPS (0.05mg/ml) and without LPS in the presence and absence of 0.5mg/ml SA-2. Each bar is representive of the mean of four readings ± SD; the experiment was carried out at least three times. $^{\delta}$ Significant ($p \leq 0.0001$) increase in TNF-α compared with cells only control. ***Significant ($p \leq 0.008$) decrease in TNF-α compared with cells + LPS control.

4.0 Discussion

D. viscosa (Sapindaceae) and *A. setaceus* (Asparagaceae) were selected for discovering of compounds with potential anti-inflammatory activity because of the fact that both plant families have been reported to have therapeutic value in these areas (Simpson *et al.*, 2010; Khalil *et al.*, 2006). Carrying out research on *A. setaceus*, which has not been previously studied for anti-inflammatory activity, could provide novel compounds with anti-inflammatory properties.

Maceration with ethanol for both plants was carried out overnight. This step was performed to provide information about the total extractable constituents. Hot solvent extraction was then performed using hexane, ethyl acetate and methanol. The yields obtained from *A. setaceus* extraction were 2.7%, 2.0% and 8.4% (w/w) with the hexane, ethyl acetate and methanol, respectively while for *D. viscosa* extraction the yields were 4.4%, 7.4% and 6.8% (w/w), respectively. The lowest yields were observed in both hexane and ethyl acetate *A. setaceus* extraction and hexane *D. Viscosa* extraction. Overall, low yields of extract from both plants may be associated with several factors such as the plant was not dry enough, the extraction process was not carried out for enough time or the solvent used was not suitable for extracting sufficient quantities (Handa, 2008).

THP-1 cells provide a good model for this study as they produce TNF-α and IL-1 pro-inflammatory cytokines when stimulated with LPS (Lee *et al*., 2008). Determination of the cytotoxicities of the crude ethanolic extract of both plants (*Dodonaea viscosa* and

Asparagus setaceus) revealed that they did not exhibit cytotoxicity on THP-1 cells at the maximum concentration of 2mg/ml over the two days. This is an important assay and was performed to calculate the highest concentration that could be used in the cell stimulation assay without killing the cells. On the other hand, trypan blue dye exclusion is a quicker but less reliable test to evaluate cell viability.

A TNF-α ELISA was carried out for the crude extracts of the two plants and the pro-inflammatory cytokine (TNF-α) was analysed. The ethanolic extract of *A. setaceus* (2mg/ml) exhibited no effect on THP-1 cells and no pro- or anti-inflammatory effects were observed, while the crude *D. viscosa* ethanol extract showed significant ($p \leq 0.0001$) anti-inflammatory and no pro-inflammatory effects on THP-1 cells at a concentration of 2mg/ml. TNF-α ELISAs were also carried out with the crude hexane, ethyl acetate and methanol extracts (results not shown), the level of this cytokine produced was too high to give accurate results. This may have been due to a problem with the ELISA kit used. No previous reports have described these activities *in vitro* before. On the other hand, there are studies that have examined the anti-inflammatory effects of crude *D. viscosa in vivo*. For example, an oral dose of 300 mg/kg inhibited the paw oedema induced in rats in a carrageenin model (Alagarsamy *et al.,* 2007; Vedavathy *et al.*, 1995). Therefore, the present study and other studies (Venkatesh *et al.,* 2008) support the ethnomedicinal use of this plant as a potential anti-inflammatory treatment. From the TNF-α ELISA results of the crude extracts, all three demonstrated anti-inflammatory properties. Therefore, all the extracts were chosen for further fractionation due to the low cytotoxicity of their compounds.

Fractionation of the methanol extract of *D. viscosa* was performed using VLC. The individual compounds were seen as coloured bands moving up the column and collected in vials. However, better separation may have been achieved if a traditional column was used, which uses gravity to pull through the solvent and compounds, although it takes a longer time. An arbutin derivative compound (SA-1) was obtained from this fractionation. SA-1 is a phenolic glycoside and isolated for the first time from *D. viscosa* leaves. This compound has only been isolated by Cottiglia *et al*. (2005) from the aerial parts of *Ephedra nebrodensis* as a new phenolic glycoside (nebrodenside A). Although they assigned 4`-$CH_3$ as trans to the alkene-H at 5.25ppm, whereas the 4`-CH_3 is always shown to be cis to the alkene-H and this cannot be correct based on this study and other reports (Gray,1993). No studies have described any bioactivity of this compound. However, many studies have reported different therapeutic uses of arbutin and arbutin derivatives. For example, Petkou (2002) and Myagmar (2004) demonstrated that arbutin has hypopigmentation activity where it has an effect on tyrosinase activity. For this reason, it has been applied in many cosmetic products. It is a diuretic, urinary tract disinfectant and these activities are related to the presence of hydroquinone (HQ). Arbutin is rapidly metabolized and excreted in humans as HQ, HQ glucuronide, and HQ sulfate and HQ possesses disinfectant, antimicrobial and astringent activities. It is also an inhibitor of melanin formation and is used in some skin-lightening products and can effectively control the activity of tyrosinase in the skin and block the formation of melanin (Petkou, 2002; Myagmar, 2004). All these bioactivities may also be proposed for the isolated compound due to structure similarity between arbutin and this compound.

Further work would be carried out on this compound, to test for different bioactivities. SA-1 is more fat soluble and this property may make the compound more potent than arbutin. For example, arbutin has antimicrobial activity against *Mycobacteria* species which are characterized by a hydrophobic and waxy cell wall and SA-1 may penetrate the cell wall of those microbes more easily and therefore produce stronger antimicrobial effects than arbutin. With further investigation into this compound, its presence could prove to be a very useful find.

Fractionation of *D. viscosa* hexane extract was performed using column chromatography and nothing was obtained. Sephadex column chromatography was used to separate the ethyl acetate extract of *D. viscosa* leaves and further analysis of the fraction was carried out by 1D and 2D NMR to try identifying the compound. The ^{1}H and ^{13}C spectral data described by Heerdena *et al.* (2000) and the ^{13}C NMR spectral data described by Teffo (2006) confirm that the SA-2 is a 3, 6-dimethoxy-5, 7, 4`-trihydroxy-flavone. Compound SA-2 was isolated from the ethyl acetate extract and was also obtained from early fractions of the methanol extract. Venkatesh *et al*. (2008) supported the presence of flavonoids from the ethyl acetate extract of *D. viscosa* leaves. This flavanoid was first identified from the leaves and steams of *Gutierrezia wrightii* (Fang *et al.,* 1986) and reported as a constituent from *D. attenuata, D. angustifolia* and *D. viscosa* (Heerdena *et al.*, 2000). Flavonoids are polyphenolic compounds present in plants and are categorized into flavonols, flavones, catechins, flavanones, anthocyanidins and isoflavonoids. They have been reported to have a variety of biological activities such as anti-inflammatory and antioxidant activities (Guardiã *et al*., 2001 ; Hämäläinen *et al*., 2007). Teffo (2006) demonstrated antibacterial activity of this compound against some microorganisms such

as *E. coli*, *E. faecalis* and *P. aeruginosa* at concentrations of 5 and 10µg/ml. Also, in the same study, the flavone has been shown to have high antioxidant activity. Moreover, Queiroza *et al*., (2010) isolated this compound from aerial parts of *Piptadenia stipulacea* and reported significant antinociceptive and anti-inflammatory effects of the isolated flavonoid in laboratory animals. Fractionation of *A. setaceus* extracts was carried out using normal column chromatography for three extracts. Only a mixture of β-sitosterol and stigmasterol (SA-3) was obtained from the hexane extract and early fractions of the ethyl acetate extract. It was confirmed by comparison with a standard sample of β-sitosterol using TLC. To determine whether SA-1 and SA-2 had anti-inflammatory properties, both compounds were investigated in a THP-1 cell assay stimulated with LPS. TNF-α and IL-1β production was measured by ELISA. SA-1 showed significant ($p= 0.0001$) anti-inflammatory activity on THP-1 cells since both TNF-α and IL-1β levels were reduced by 6 and 2102 fold, respectively. It is not known to be anti-inflammatory and no previous data was available on this compound, as this was the first time this research has been carried out.

SA-2 has been examined for potential anti-inflammatory activities and about 24 and11-fold significant ($p\leq 0.0001$, $p\leq 0.008$) decrease in TNF-α and IL-1β production, respectively, was demonstrated. For the *in vitro* studies, no reports have been made on the anti-inflammatory properties of this compound. On the other hand, *in vivo* investigations have described the anti-inflammatory effect of this flavanoid. For example, de Queiroz *et al*. (2010) demonstrated significant anti-inflammatory effects of

this flavonoid when an experiment was conducted on Swiss mice using the zymosan A-induced peritonitis test.

5.0 Future Work

The most significant problem in this study was the low yields achieved from the hot solvent extraction of both plants. Therefore, if further studies are to be performed on these plants, alternative extraction methods should be applied such as cold maceration to extract more plant material (Handa *et al.,* 1995). Separation of some extracts using traditional methods such as column chromatography was not successful because nothing was isolated. Also, SA-1 and SA-2 were yielded from methanol and ethyl acetate extracts of *D. viscosa* in small amounts with some impurities. Therefore, if further work is to be carried out on these plants, alternative separation techniques such as gel filtration chromatography, which separates compounds based on size exclusion could be used. The next step for these compounds is to examine their anti-inflammatory effects in *in vivo* models of inflammation such as carrageenan-induced oedema in the rat hind paw, and in the zymosan A-induced peritonitis test (Khalil *et al.,* 2006; Queiroz *et al*., 2010). Since other properties such as antimicrobial and antioxidant activities are common in compounds from the Sapindaceae family, tests could be carried out to evaluate these properties as well. This would involve techniques such as a both dilution and disc diffusion assay (Anilreddy, 2009) and the spectrophotometer and micro plate methods (Ramzi *et al.,* 2008; Brand *et al.,* 1995).

6.0 Conclusion

To sum up, There is very little literature on the anti-inflammatory properties of *D. viscosa* (Getie *et al.*, 2003; Khalil *et al.*, 2006). Therefore, any publications that arise from this work will be important. From this study, the experiments revealed that the ethyl acetate and the methanol crude extracts are anti-inflammatory. Three fractions were isolated and identified; the first revealed compound SA-1 which is an arbutin derivative and was isolated from the ethyl acetate extract of *D. viscosa.* It exhibited significant ($p \leq 0.0001$, $p \leq 0.003$) anti-inflammatory (TNF-α and IL-1β) activity at a concentration of 0.5mg/ml. The anti-inflammatory properties of SA-1 and its presence in *D. viscosa* have not been previously established. However, arbutin derivatives are documented as having a wide range of therapeutic activities, including anti-inflammatory activity (Cheenpracha *et al.,* 2010). The second fraction provided SA-2 which is a flavonoid and was isolated from the methanol extract of *D. viscosa*. It also demonstrated significant ($p \leq 0.0001$, $p \leq 0.008$) anti-inflammatory activity at the same concentration and no pro-inflammatory activity. These activities with the presence of the already established therapeutic compounds, makes *D. viscosa* an important source of useful compounds for future drug development.

7.0 References

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