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A Comparison of the Chemical and Potential Anti-Inflammatory Effects of Freeze Dried and Spray Dried Aloe Vera

A thesis presented by

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Signed:…….. **Date:……**

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ABSTRACT

Many of the therapeutic activities associated with Aloe vera have been attributed to polysaccharides contained in the inner gel of the leaves. The main reason for this study was to investigate the effect of processing on the potential anti-inflammatory properties of Aloe vera inner gel. This was evaluated using human monocytes (THP-1 cells) stimulated with lipopolysaccarides (LPS). The two different processes evaluated were freeze-dried (FD) and spray-dried (SD) preparations of Aloe vera inner gel. Initially TLC and NMR analysis was carried out. TLC showed no difference in profile whereas ^{1}HNMR showed differences between the two products. In order to see if this affected biological activity comparison of potential anti-inflammatory activities (TNF-α and IL-1β) and further NMR analysis was carried out. The FD preparation was solvent extracted (methanol/water), and then fractionated by Flash chromatography. Nine different fractions (A1-A9), were obtained and examined by nuclear magnetic resonance (NMR) and were tested in bioassays to identify which showed anti-inflammatory responses.

The bioassays consisted of an initial cytotoxicity screen. The crude Aloe vera inner gel was found to be non-toxic. There were no differences in reduction of TNF-α released from THP-1 cells stimulated with LPS (from 76±1.78pg/ml for cells treated with LPS to 58±3.24 and 54±2.9pg/mg for FD and SD, respectively; $p \leq 0.001$). On the other hand, some fractions (A7 and A9), showed potent anti-inflammatory activity in comparison with crude FD from 1369±57pg/ml in treated cells to 1008±12 (A7) and 1090±103 pg/mg (A9), respectively; $p \leq 0.01$, while A4, A5 and A6 showed 415±241, 1275±3.75 and 1893±1.56 pg/mg TNF-α, respectively; compared with cells only

(56±3.44pg/mg) which were exhibit an pro-inflammatory property. Additionally fractions A2, A4, A6, A7, A8 and A9 showed significant ($p \leq 0.001$) decrease in production of IL-1β than all the fractions (322±8.14, 420±9.37, 407±7.29, 317±46.94, 332±79.73 and 278±0.95pg/ml respectively.

The differences in ^{1}HNMR of FD and SD indicated chemical variations which might be caused by the application of heat during processing. However, initial examination did not show any difference on biological activity of the crude preparations. Fractionation of the crude preparations enabled identification of a few compounds such as acemannan, glucose and malic acid.

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ABBREVIATIONS

1D ^{1}H NMR	One Dimensional Proton NMR Spectra
AIDS	Acquired Immunodeficiency Syndrome
°C	Degree Celsius
Ab	Antibody
Ag	Antigen
B.C	Before Christ
C.E	Common Era
CAM	Complementary and Alternative Medicines
CC	Column Chromatography
cm	Centimeter
CM	Complete Medium
CO_2	Carbon dioxide
COSY	Correlation Spectroscopy
CRP	C reactive protein
DMSO	Dimethylsulphoxide
ELISA	Enzyme linked immunosorbant assay
FBS	Foetal Bovine Serum
FC	Flash Chromatography
FD	Freeze Dried
FDA	Food Drug Administration
FCC	Flash Column Chromatography
gm	Gram
IL-1β	Interlukin 1 beta
IL-2	Interleukin 2

ABBREVIATIONS (*Continued*)

IL-6	Interleukin 6
INF-γ	Interferon-gamma
kDa	kilo Dalton
LBP	LPS binding protein
LPS	Lipopolysaccharides
M	Molar concentration
MEM	Minimal Essential Medium
MeOH	Methanol
mg	Milligram
ml	Milliliter
mM	Millimolar
NaCl	Sodium Chloride
$NaHCO_3$	Sodium Bicarbonate
NaOH	Sodium Hydroxide
NF-κB	Nuclear Factor kappa
NMR	Nuclear Magnetic Resonance
NP	Normal Phase Chromatography
OTC	Over The Counter
PBS	Phosphate-Buffered Saline
pg	picogram
RP	Reverse Phase
SD	Spray Dry
TLC	Thin Layer Chromatography
TNF-α	Tumor Necrosis Factor-alpha
UV	Ultra Violet

v/v	Volume per volume
w/v	Weight per volume
WHO	World Health Organisation
µg	Microgram
µl	Microliter

1. Introduction

1.1. Herbal medicines

Herbal medicine is one branch of traditional medicine or Complementary and Alternative Medicines (CAM), which include herbs, herbal preparations, and finished herbal products. Various infectious and chronic conditions can be treated by traditional medicine for example; new anti-malarial drugs were a result of the discovery and isolation of artemisinin from *Artemisia annua* L., a plant used in China for almost 2000 years. It is well known that many modern drugs are derived from plant sources (Capriotti, 1999). One of the well known drugs in the treatment of fever, pain and inflammatory conditions (Aspirin™) which originates from salicylate-containing plants, was extracted from the bark of willow trees (*Salix alba* and other members of the *Salix* species) (Rainsford, 2004).

Internationally, the use of botanical medicines is generally higher, drugs derived from plants contribute thirty percent towards all modern drugs (Winslow and Kroll, 1998). In some countries, use of herbal medicine is popular compared with modern medicine for example, in some Asian and African countries, 80% of the population depend on traditional medicine for primary health care (WHO, 2008). Studies concluded that between 3% and 93% of the US population uses herbs, and the variability of these estimates is due to discrepancies in definitions of herbs (Winslow and Kroll, 1998). Additionally, it was reported that 80% of the world's population relies primarily on traditional medicines for their health care needs (Gesler, 1992; Winslow and Kroll, 1998). There are a considerable number of people in the UK (around 46%) that are expected to use one or more CAM therapies in their lifetime, or are turning to use of CAM (Bishop and Lewith, 2008).

From an economical perspective, herbal medicines are probably the most commonly used alternative medical treatment (Capriotti, 1999), and are the most popular of all traditional medicine therapy, which also produces large profits in the international market. According to the World Health Organization (WHO), the annual revenue in Western Europe amounted to 5 billion U.S dollars in the period 2003-2004, while China's sales of natural products yielded 14 billion U.S. dollars in 2005. In Brazil, herbal medicine income in 2007 was 160 million U.S dollars (WHO, 2008). The amount of money spent on herbal remedies is significant. Americans spent $553 million in 8000 health food stores in 1994 (Marwick, 1995). While in 2007 it was reported that consumers spent an estimated $34 billion. In fact, alternative medicine accounts for over 11 percent of out-of-pocket spending on health care in the United States(Dyess, 2009). Sales of herbal medicines are increasing by 20% a year and herbs are the largest growth area in retail pharmacy, far exceeding growth in the conventional drug category.

There is a lack of policy and regulation of herbal remedies, and the difficulty in achieving this is due to differences in definitions and classifications of traditional medicine. The source of herbal products can be defined as either food, dietary supplements or herbal medicines, depending on the country concerned. More than 100 countries have different regulations for herbal medicines (Calixto, 2000).

At the present time, it is easier to determine efficacy and safety of herbal remedies, because it is known which chemical compounds are present in these plants (Rodriguez-Fragoso *et al.*, 2008). The Food and Drug Administration (FDA) proposed regulations for "good manufacturing practices" in the dietary

supplement industry (Capriotti, 1999); there are still herbal products recognised as dietary supplements, rather than drugs within the United States.

1.2. Aloe vera background

Aloe vera, also known as Aloe barbadensis Miller, belongs to the Asphodelaceae family (Esua and Rauwald, 2006; Joseph and Raj, 2010) and it has been used medicinally for thousands of years. There are several hundred species of the genus Aloe world-wide, and only three or four species are used commercially. Aloe vera Linne is the most famous and has been widely applied for commercial purposes as it has considerable healing or therapeutic properties, is rich in vitamins, minerals, amino acids, enzymes and active polysaccharides (Grindlay and Reynolds, 1986; Reynolds and Dweck, 1999).

The word "aloe" is derived from the Arabic alloeh, meaning “bitter, shiny substance", while "vera" is the Latin word for "true". "True aloe" was probably given to this particular plant, because it is reputed to be the most medicinally beneficial and therapeutic since ancient times.

Aloe vera is widespread in sub-Saharan Africa, the Arabian Peninsula and a number of Indian Ocean islands and in tropical and subtropical latitudes. It is cultivated today in huge amounts in order to meet consumer demand (Vogler and Ernst, 1999). Currently, production is distributed among plantations in Mexico, the Dominican Republic, Venezuela, Guatemala and North Africa (Reynolds, 2004). In addition the plant is cultivated in India, China, South and Central America, the Caribbean, Spain, Mexico, North America primarily Texas and Florida and other tropical and semitropical regions in the world (Grindlay and Reynolds, 1986).

The Aloe vera plant has thick, thorn-edged leaves, ranging in colour from grey to bright green, giving an appearance of a cactus. It is an almost sessile perennial herb, leaf-succulent xerophyte with structural and physiological adaptations for survival in dry regions. The leaves are 30-50cm long and 10cm broad at the base. It has bright yellow tubular flowers 25-35cm in length arranged in a slender loose spike; stamens frequently project beyond the perianth tube (Figure 1-1). It has large thin-walled cells in which water is held in mucilage (water storage tissue).

Figure 1-1 *Aloe vera* Barbadensis Miller in the field.
http://www.naturephoto-cz.eu/pic/andera/aloe-vera-8935b.jpg

Aloe vera leaf products consist of materials such as bitter exudates leaf yellow sap, leaf gel (inner pulp, parenchyma tissue), whole leaf extracts in liquid or powder form. The longitudinal transitional section of the leaf layer is clearly visible in Figure 1-2. Green outer leaf layers are called the rind, while the soft

colourless parenchyma layer is the inner gel. The inner gel is clear, thick, and has a slimy consistency, and is obtained by cutting away the outer rind.

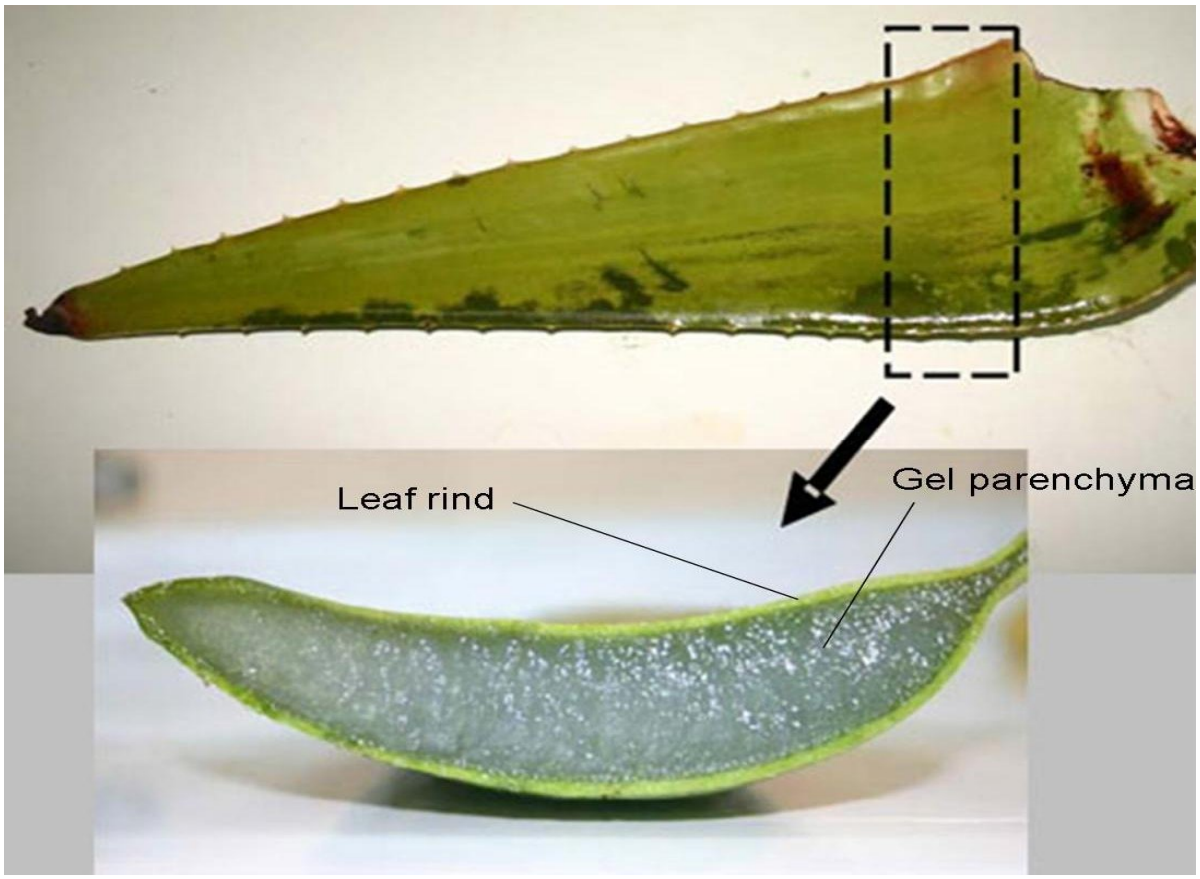


Figure 1-2 Transitional sections of an Aloe vera leaf.
http://lbriskincare.info/Aloe.aspx

1.3. History and traditional use

Aloe vera has been documented as being used by Egyptian queens, Nefertite and Cleopatra, as beauty aids. Aloe was used by Dioscorides to heal skin disorders and haemorrhoids. It was also reported that many famous physicians such as Celsus and Glen used to treat wounds and gastrointestinal disturbances. Aloe's medical uses were first reported on a Mesopotamian clay tablet dating from 2100 B.C. Later, in 1862 a papyrus found in a sarcophagus near Thebes mentioned at least twelve preparations for preparing Aloe vera to treat internal

and external ailments (Atherton, 1997; Reynolds, 2004). Furthermore, Greek literature before the first century reported Aloe vera was used as a laxative. In the first century it was reported that Dioscorides wrote of the use of Aloe vera in wound treatment, hair loss, genital ulcers, haemorrhoids, boils, mouth irritation and inflammation (Shelton, 1991). In the seventh century, Aloe vera was used to treat eczema, sinusitis, constipation and skin ailments and late in the 1930s it was used to treat radiation burns. Aloe vera was introduced as a laxative in the U.S. pharmacopoeia in 1820. Table 1-1 summarises some of the therapeutic history of Aloe vera.

Table 1-1 History of Aloe vera therapy.

1750 B.C.E	Aloe pictured as medicinal treatment on Mesopotamian clay tablets
550 B.C.E	Egyptian books refer to treatment of skin infections
74 C.E	Greek physician writes about his success in treating wounds, hair loss, and haemorrhoids
700 C.E	Aloe used for sinusitis and eczema
1200 C.E	Predominantly used as a cathartic medicine
1935 C.E	Modern experimentations begin with radiation-induced ulceration

Adapted from Shelton (1991).

1.4. Modern medicinal use

The topical and internal effect of Aloe vera has been known since ancient times. Aloe vera as a medical plant has maintained its activity, high reputation and popularity over a long period of time. In western and under-developed countries, Aloe vera is still being used for self-medication and is highly popular (Reynolds, 2004).

Aloe vera medical preparations are divided into three different main categories yellow sap; inner gel and whole leaf extract. Constituents of aloe have been reported to have anti-inflammatory, antibacterial, antitumor, anti-allergy, and immunostimulatory properties. Aloe yellow sap is used to relieve stomach constipation as a laxative. The major active ingredient present in the yellow sap, is anthraquinone that has been used as a natural drug for its cathartic effect and is widely employed as a bitter agent in alcoholic beverages (Pugh *et al.*, 2001), Today, this component is still recognised as a drug by the United States, in the U.S. pharmacopoeia (Gage, 1996).

While the inner gel, has different pharmacological activities due to a variety of chemical compounds it is also the most common part used for treatments; topically, in treatment of wound healing, genital herpes, burns, abrasion, bruises, cuts, and psoriasis and internally via oral administration. It is beneficial for healing gastric and mouth ulcers and for lowering blood glucose in diabetic patients and reducing blood lipid levels in patient with hyperlipidemia. The extract is reported to be beneficial in treatment of asthma, and potentially useful in treatment of cancer and AIDS. Additionally, Aloe is shown to increase collagen and elastin formation, which may reduce wrinkling, aid in wound

healing, and protect against damage from ultraviolet rays (Reynolds and Dweck, 1999; Vogler and Ernst, 1999).

From a literature review carried out by Reynolds and Dweck (1999), they reported that Aloe vera has been extensively used in treating many external and internal inflammatory diseases, such as burns, sun burn and certain skin conditions as psoriasis and ultraviolet-induced erythema and gastrointestinal ulcer like peptic ulcers.

Table 1-2, shows some pharmacological and therapeutic value of Aloe vera, the parts used and the mechanism of action suggested by some authors.

Table 1-2 Pharmacological and therapeutic activity of Aloe vera.

Pharmacological Properties	Leaf part used	Active ingredient	Mechanisms of action	References
Laxative	Yellow sap	Anthraquinones, aloe-emodin, isobarbaloin and chrysphanic acid	Increases mucosal permeability	Canigueralii and Vilar (1993); Capasso *et al.* (1998); Capasso and Gaginella (1997); Tavares *et al.* (1996)
Wound healing	Aloe gel	Mucilage substance polysaccharides (acetylated glucomannans) Acemannan and antitumor mucopolysaccharide	Skin protective barriers Stimulates the production of TNF-α and cytokines by macrophages	Blitz *et al.* (1963); Harris *et al.* (1991; Manna and McAnalley (1993); Peng *et al.* (1991)
Anti-cancer	Aloe gel	Vitamin A and E aloctins A aloe-emodin salicylic acid, barbaloin, emodin and amolin	Chemopreventive curative properties Reduces the severity of chemical hepatocarciogenicity Active against P-388 leukemia in mice induces apoptosis Potent antileukemic effect in human cells decreasing thromboxane A2 THA2 and THB2	Gribel and Pashinskii (1986); Ralamboranto *et al.* (1982) Shamaan *et al.* (1998) Lee *et al.* (2000a) Hiroko *et al.* (1989)
Anti-inflammatory	Aloe gel	Magnesium lactate	Inhibition of prostanoids Inhibits polymorphonuclear leukocytes infiltration. Inhibits histamine formation Inhibits bradykinin activity Blocks generation of histamine and bradykinin.	Hiroko et al. (1989; Penneys (1982) Davis and Maro (1989) Klein and Penneys (1988) Fujita *et al.* (1976) Davis *et al.* (1991)
Antibacterial	Aloe gel	Acemannan	Inhibits bacterial growth of Gram negative and Gram positive organisms	Heggers *et al.* (1979) Lorenzetti *et al.* (1964) Ferro *et al.* (2003); Fly and Kiem (1963)
Antiviral	Yellow sap	anthraquinone derivitives dianthrones, rhein and emodin acemannan modified polysaccarieds (MPS)	Increases the production and function of cytotoxic T cells Inhibits glycosylation of viral glycoproteins Inhibits the replication of HIV and HSV-1	Sydiskis *et al.* (1991) Kahlon *et al.* (1991) Kahlon *et al.* (1991) Syed *et al.* (1996b)
Antihperlipidaemia	Aloe gel	aloe ulcin	Suppression of L-histidine decarboxylase Decreases the total serum of cholestrol	Yamamoto (1973) Nassif *et al.* (1993)

Table 1-2 Pharmacological and therapeutic activity of Aloe vera (*Continued).*

Immunomodulant	Aloe gel	Acemannan, modified polysaccharides (MPS)	Stimulates the immune system Stimulates macrophages to produce nitric oxide and cytokines Enhances phagocytosis Increases the number of circulating monocytes and macrophages	Womble and Helderman (1988) Karaca *et al.* (1995) Shida *et al*. (1985) Qiu *et al*. (2000)
Antidiabetic	Aloe gel	anthrones	Stimulates release or synthesis of insulin Lowers blood glucose in patients with diabetes	Ajabnoor (1990); Bunyapraphatsara *et al.* (1996); Yongchaiyudha *et al*. (1996)
Antipsoriatic	Aloe gel	anthrones	Inhibits oxygen consumption of cells Reduces the size of the intracellular spaces Induces mitochondrial damage Retards increases of cell division in psoriatic epidermis	Anton and Haag-Berrurier, (1980); Friedman (1980); Muller (1996); Verhaeren (1980)
Gastric ulcer	Aloe gel	glycoprotein (Lectin)	Prophylactic effect Suppression of ulcer growth Treatment of peptic ulceration	Galal *et al.* (1975; Parmar *et al*. (1986) Yamamoto (1973) Blitz *et al*. (1963)

1.5. Inflammation

Inflammation is normally a highly complex characterised by swelling, pain, redness, heat and loss of function, and inflammatory condition recognised as interdependent biochemical and physiological protective responses of the immune system to tissue injury or infection. Invading pathogens as well as harmful internal environments such as ischemia or autoimmune diseases result in leukocyte secretion of inflammatory mediators such as cytokines and chemokines (Medzhitov, 2008). Outer membrane components of foreign pathogens bind to receptors on leukocytes and activate the inflammatory response (Ryan and Majno, 1977). Controlled inflammation is an advantageous and necessary process which functions to protect the body against infection and injury. Basically, inflammation resolves itself with inconsequential damage to the host (Baumann and Gauldie, 1994; Serhan, 2007). The loss of control of inflammation can cause chronic disorders such as inflammatory bowel disease, rheumatoid arthritis, and septic shock and can ultimately lead to irreversible damage to tissues and organs and, even death.

Inflammation is commonly divided into two categories: acute and chronic. Acute inflammation is temporary, lasting in the order of minutes to days, whereas chronic inflammation persists over longer periods of time. Although both are characterised by secretion of inflammatory mediators. A characteristic of acute inflammation is the significant increase in acute phase serum proteins such as LPS binding protein (LBP), C reactive protein (CRP), and serum amyloid A (Gabay and Kushner, 1999). Furthermore, acute inflammation can be local, limited to a specific area or it can be systemic; in both cases, it commonly persists only for as long as the stimulus is present. Upon inflammation cytokines are secreted by resident cells at the site of injury or infection, leading

to the rapid and short-lived constriction of blood vessels. This is immediately followed by vasodilatation, which results to an increased blood flow and pooling of leaked fluids in the area of injury. These processes cause the redness and warmth (*rubor* and *calor*) observed in acute inflammation. Oedema is visible as a swelling (*tumor*) when the increased permeability of the microvasculature allows for the leakage of plasma proteins and other fluids into the injured tissue.

Additionally, resident cells secrete chemokines to prompt coordination and recruitment of neutrophils to the site of injury to remove the invading pathogen by phagocytosis as well as causes release of bacteriocidal granules. While chronic inflammation develops when acute inflammation fails to be resolved, which can be caused by persistent pathogen presence, the inability of phagocytic cells to degrade foreign substances, or autoimmune responses (Serhan, 2007). It can also be induced by either exogenous or endogenous stimuli. Chronic inflammation mainly results from persistent endogenous stimuli (Kitchens *et al.*, 2003) and causes destructive and often irreversible tissue damage (Drayton *et al.*, 2006).

There are many stimulators of inflammatory response. LPS is a potent activator of the immune system, provoking a cascade of events in the cell, resulting in inflammation mediators released, including TNF-α and IL-1β, which are the two cytokines monitored in this study as a marker of inflammation. TNF-α is produced in response to direct stimulation, and can also induce various other cytokines including IL-1β and IL-8 (Cassatella et al., 1993; DeForge et al., 1992) Although IL-1α and IL-1β are both induced upon stimulation of the inflammatory response, IL-1α remains in the cytosol, whereas IL-1β is processed and cleaved into its active form by IL-1β converting enzyme (Hanley *et al.*, 1982)

Inflammation responses resulting in the release of TNF-α and IL-1β are complex (see Figure 1-3), involving several inflammation mediators and regulation factors, some of which are still under investigation.

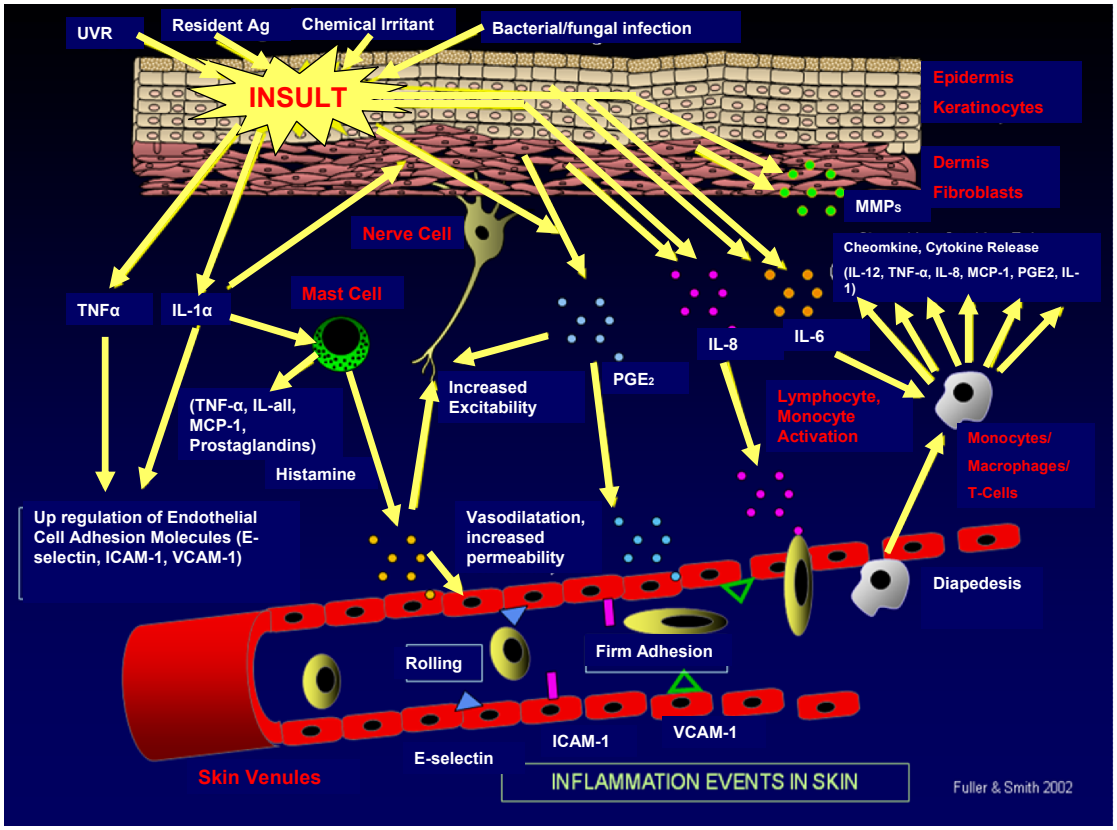


Figure 1-3 Inflammation cascade and cytokines release.
http://www.dermamedicsprofessional.com/inflammation_id52.html

1.6. Anti-inflammatory activity

Traditionally, Aloe vera is used to treat some anti-inflammatory conditions (Reynolds and Dweck, 1999) as mentioned in section 1.4. In order to examine the anti-inflammatory activity of aloe gel a considerable amount of literature has been published on *in vitro* and *in vivo* studies that have attempted to find the responsible components and their mechanism of action. For this reason several models of inflammation have been used to assess the acute and chronic anti-inflammatory activity (adjuvant arthritis and hind-paw oedema in rats, ear swelling in mice and rabbits, synovial pouch and burn in mice, rats and guinea-pigs) and phlogogen agents (carrageenan, kaolin, albumin, dextran, gelatin, mustard, croton oil, streptozocin) (Davis *et al.*, 1986; Davis *et al.*, 1989; Vazquez *et al.*, 1996).

1.6.1. Anti-inflammatory activity assessed *in vitro*

There are various studies carried out on pathological tissues from patients suffering from inflammatory conditions (active ulcerative colitis). This has been carried out with human mucosal biopsies. Anti-inflammatory activity of the Aloe vera gel leaf pulp, was assessed using a chemiluminescence assay to determine oxygen reactive metabolites and an ELISA to detect interleukin-8, prostaglandin E2 and thromboxane B2. Aloe vera was found to have a dose dependent inhibitory effect on reactive oxygen metabolites and interleukin-8 production and slightly decreased production of prostaglandin E2 and had no effect on thromboxane B2 (Langmead *et al.*, 2004b)

Other studies have looked at potential systemic anti-inflammatory activity of aloe vera. A pure glycoprotein known as Alprogen, was purified and isolated from fresh Aloe vera leaf. Guinea pig lung mast cells were activated with

specific antigen-antibody and Alprogen was found to strongly inhibit histamine and leukotriene release (Jai *et al.*, 2002).

A recent study in freeze-dried Aloe vera and a commercial health drink from the same source were tested, reported a significant suppression of production of TNF-α and IL-1β released from both human peripheral blood leukocytes cell and human monocytic cell-line (THP-1 cells) stimulated with bacterial lipopolysaccharide (Habeeb *et al.*, 2007).

1.6.2. Anti-inflammatory activity assessed *in vivo*

A considerable amount of literature has confirmed the anti-inflammatory activity of Aloe vera inner gel and its ability to reduce the severity of acute inflammation this has been evaluated in many different animal models (Davis et al., 1986; Davis et al., 1989; Vázquez et al., 1996).

For instance in a comparison of Aloe vera extract with vitamin C, thymus extract and deoxyribonucleic acid an adjuvant arthritis, animal model was used consisting of rats injected with heat killed *Mycobacterium butyricum* in mineral oil to induce immunological responses leading to adjuvant arthritis. The study showed varying degrees of effectiveness in reducing the inflammation. Aloe extract was the most potent in reducing inflammation (Hanley *et al.*, 1982).

A more detailed study, was carried out to evaluate the anti-inflammatory activity of decolorised Aloe vera (without anthraquinones), and Aloe vera as a vehicle for delivering hydrocortisone-21-acetate, using a mustard-induced paw-swelling assay for systemic assessment while an ear-swelling assay on rats was used for topical assessment. Different concentrations of Aloe vera and hydrocortisone were used and tested individually and mixed together. Anti-

inflammatory activity was significant for Aloe vera both systemically and externally, with or without hydrocortisone, but the result was more significant when Aloe vera gel was mixed with hydrocortisone. Reduction of oedema was 88.1% in the systemic assay and 97% reduction of oedema. From these results the authors suggested that Aloe vera has valuable potential as a delivery vehicle for steroid preparations, and may have synergistic anti-inflammatory activity to hydrocortisone to increase effectiveness and bioavailability and thus decrease use of hydrocortisone (Davis *et al.*, 1991).

In addition a study has reported that decrease of inflammation caused by mustard induced oedema was achieved in rats and mice fed with 10mg/kg compared with untreated controls. This suggested that the inhibited inflammation is due to blocking inflammatory mediators such as prostaglandins, thromboxane, kinin and enzymes and immune mechanisms (Davis *et al.*, 1993).

Additionally, three different extracts of Aloe vera; aqueous, chloroform and ethanol extracts were investigated on oedema in the rat paw as well as neutrophil migration into the peritoneal cavity induced by carrageenan. Both the aqueous and chloroform extracts were found to inhibit oedema formation close to that of well established anti-inflammatory agents (i.e. indomethacin and dexamethasone). Furthermore, the antioedema effects of these two extracts correlated well with their abilities to decrease the number of neutrophils migrating into the peritoneal cavity. The ethanol extract did not show an effect on the oedema, but reduced the number of migrating neutrophils. Further experimentation on the mechanism of action suggested that the anti-inflammatory activity of the extracts of Aloe vera gel probably occurs via an

inhibitory action on the arachidonic acid pathway (Figure 1-4) through cyclooxygenase (Vázquez *et al.*, 1996).

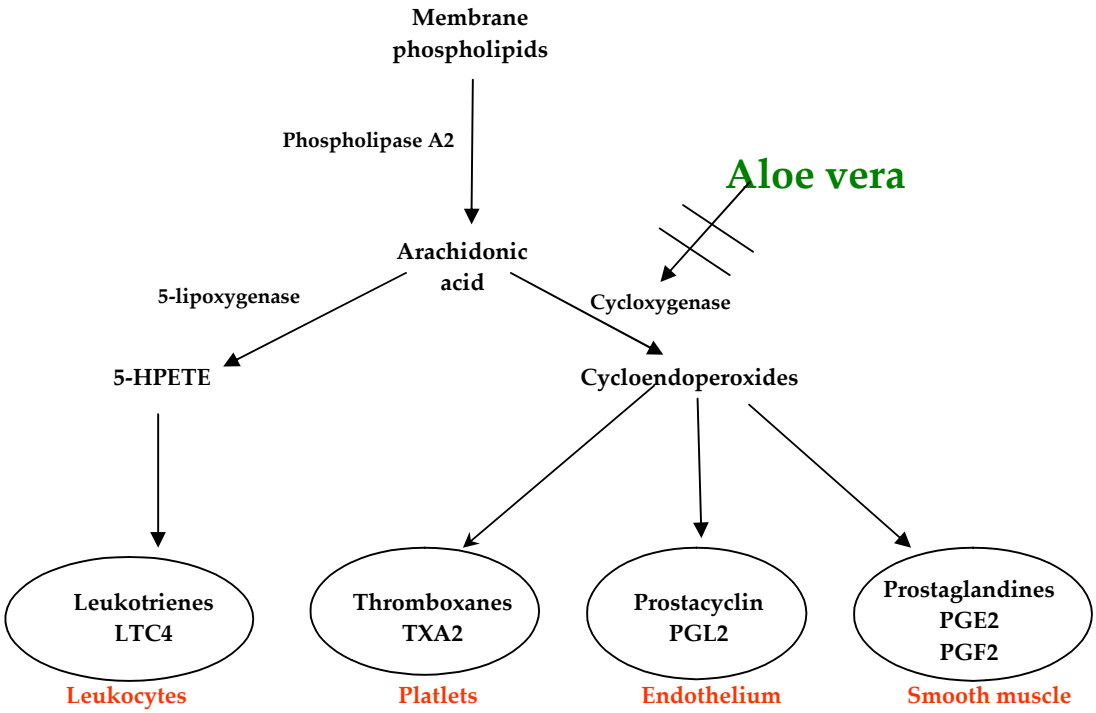


Figure 1-4 Arachidonic acid pathway responsible for the inflammatory mediators associated with pain.

In anther study using a croton oil induced ear-swelling assay on mice, cinnamoyl-*C*-glucosyl chromone isolated from Aloe vera leaves was shown to have a topical anti-inflammatory activity. In this study, anti-inflammatory activity was exhibited by reduction of ear swelling, at a dose of 200µg/ear and afforded anti-inflammatory activity that was comparable to 200µg/ear of hydrocortisone at 6 h after treatment (Hutter *et al.*, 1996).

Furthermore, the acute anti-inflammatory activity of Aloe vera was assessed using carrageenan and dextran while its chronic anti-inflammatory effect was investigated in a complete Freund's adjuvant-induced model of arthritis. Aloe

vera reduced carrageenan and dextran inflammation in rats by 61.9% and 61.7%, respectively and in the Freund's adjuvant-induced model, anti-inflammatory activity was demonstrated, but failed to decrease the arthritic index, indicating the absence of anti-arthritic activity. The parameter used to indicate the inflammation was a measurement of nitric oxide (NO) production in macrophages (Sarkar *et al.*, 2005).

Eamlamnam *et al.* (2006) confirmed the gastric ulcer healing properties of Aloe vera compared with sucralfate (used for the treatment of peptic ulcers) in a study on rats given an oral administration of 20% acetic acid to induce gastric ulcer inflammation (increase leukocyte adherence in postcapillary venules and TNF-α levels and reduce IL-10 levels). The Aloe vera oral dose was 200mg/kg, with twice daily administration and sucralfate was orally administration, 200 mg/kg/dose, twice daily. Aloe vera and sucralfate showed a significant reduction in leukocyte adherence and TNF-α levels, with elevated IL-10 levels; therefore promoting gastric ulcer healing (Eamlamnam *et al.*, 2006). Additionally, in a study on *Helicobacter pylori*-infected rats showed that treatment with Aloe vera significantly reduced leukocyte adhesion and TNF-α levels. The results suggested that Aloe vera showed potential in the treatment of the inflammatory responses of the gastric mucosa due to *H. pylori* infection (Prabjone *et al.*, 2006).

Lupeol isolated from the Aloe vera has been reported to be associated with anti-oxidative and anti-inflammatory activities (Grindlay and Reynolds, 1986; Waller et al., 1978), and has significantly inhibited oedema induced by carrageenan in rat paw (Nguemfo *et al.*, 2009).

In terms of the possible mechanism of action by which Aloe vera exert its anti-inflammatory effect there are several distinct pathways that have been described in the literature. Firstly inhibition of inflammation, in a dose-response manner, in streptozotocin-induced diabetic mice by inhibition of polymorphonuclear leukocyte infiltration into a site of gelatine-induced inflammation was attributed to gibberellin or a gibberellin-like substance as an active anti-inflammatory component (Davis and Maro, 1989).

Secondly, another suggested anti-inflammatory mechanism is a result of classical and alternative pathway complement activity ('t Hart *et al.*, 1988), the inhibition appears to be due to alternative pathway activation attributed to mannose-rich polysaccharides ('t Hart *et al.*, 1988). Another possible mechanism suggested involves the inhibition of the activity of some β2 integrins to block neutrophil emigration into inflamed tissues by mannose-rich carbohydrate solutions (Bowden, 1995)

1.6.3. Clinical studies

There are considerable attempts, at clinical evaluations and assessments of Aloe vera gel in order to investigate the therapeutic approach and management of some medical conditions. However, clinical trials are now in progress to provide conclusive evidence in many diseases such as; arthritis, gastric ulcer, cancer, AIDS and colitis (Reynolds, 2004).

Aloe extract has been applied for cathartic and topical uses as an OTC-drug in the Japanese market, and one of the active components is barbaloin (Reynolds, 2004). Other clinical studies were reported as follows;

A recent systematic review by Maenthaisong *et al.* (2007) suggests that there is clinical evidence showing the efficacy of aloe extract in burn wound healing. The effect of aloe extract seems to be due to increasing the rate of success of healing, and the rate of epithelialisation in first and second degree burns when compared with conventional treatments (Maenthaisong *et al.*, 2007). Additionally, a randomized double-blind, placebo-controlled study was carried out on sixty patients with slight to moderate chronic plaque-type psoriasis; topical Aloe vera extract 0.5% in a hydrophilic cream or placebo were tested. Aloe vera extract cream alleviated 25/30 patients (83.3%) compared to the placebo rate of 2/30 (6.6%) and significant clearing of the psoriatic plaques (328/396 (82.8%) vs placebo 28/366 (7.7%) was observed. Externally applied Aloe vera extract was considered more effective than the placebo, and did not show any toxic or other side-effects (Syed et al., 1996a).

In a pre-clinical trial of four hospitalised elderly patients identified with I, II and III degree skin ulcers due to bedsores, external application of Aloe vera gel to investigate its healing wound properties was carried out. Gentamicin ointment was applied as a positive control. Clinical assessment parameters were used in an ulcer square and humidity test. The results showed that Aloe vera has a high potential to treat bedsores. The authors concluded that the treatment of bedsores might be attributed to wound healing and anti-inflammatory properties of the gel, and it may be related to polysaccharides with an acetyl group and glycoprotein fraction, indentified as verectin (Matsuo *et al.*, 2009).

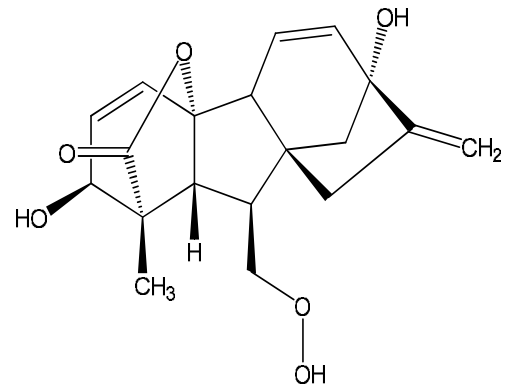
Recently, thirty patients with second degree burns on different parts of the body, were used to evaluate the efficacy of Aloe vera inner gel cream compared with silver sulfadiazine as the control drug for partial thickness burn wounds. Each patient had one burn treated with topical silver sulfadiazine and one

treated with Aloe vera. Aloe vera showed significantly faster healing than silver sulfadiazine (15.9 ± 2 vs 18.73 ± 2.65 days, respectively), and the results clearly demonstrated the greater efficacy of loe vera gel cream over silver sulfadiazine cream in the rate of re-epithelialization and healing of partial thickness burns and consequently in treating second-degree burns (Khorasani *et al.*, 2009).

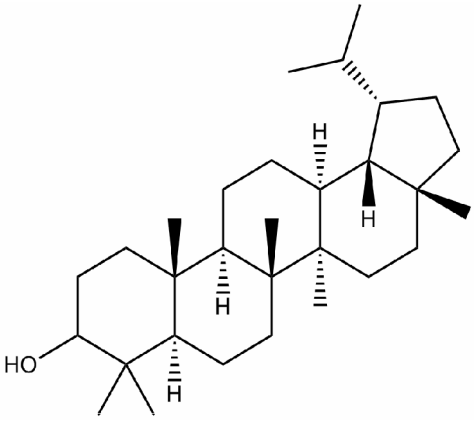
Another study was carried out on 44 patients suffering from ulcerative colitis, who were given oral Aloe vera gel or a placebo. The result was that oral gel administered for 4 weeks produced an improvement and clinical response more often than the placebo. In addition, it reduced histological disease activity (Langmead *et al.*, 2004a).

1.7. Phytochemical review on Aloe vera active components related to anti-inflammatory activity

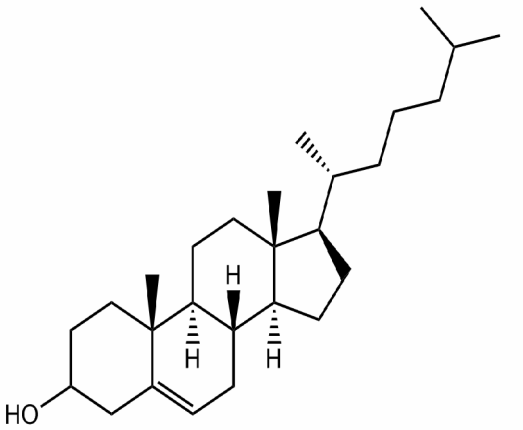
According to a phytochemical review of Aloe vera mentioned in the literature, there are many active components of Aloe vera isolated by different chromatographical techniques and attributed to anti-inflammatory activity. Gibberellin (1) has shown an ability to inhibit inflammation and stimulate antibody production and wound healing in a dose-response manner (Davis and Maro, 1989). Sterols were isolated from aloe such as; Lupeol (2) (Davis *et al.*, 1994), cholesterol (3) , β-sitosterol (4) , and campesterol (5) (Davis et al., 1994; Yamamoto et al., 1991). Three maloyl glucans were isolated by Esua *et al*. (2006): Veracylglucan A, Veracylglucan B **(6)** and Veracylglucan C **(7).** Veracylglucan B demonstrated potent anti-inflammatory activity.



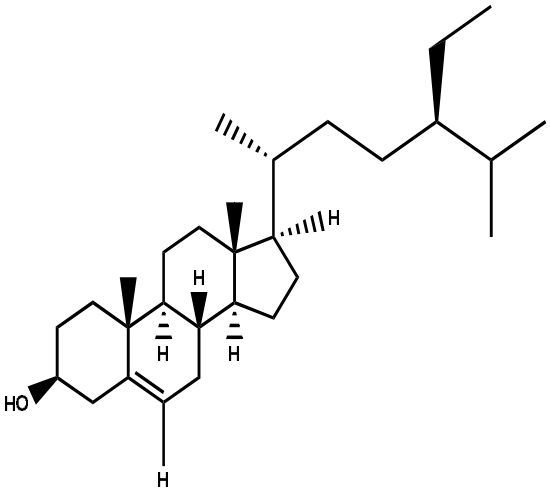
(1) Gibberellin



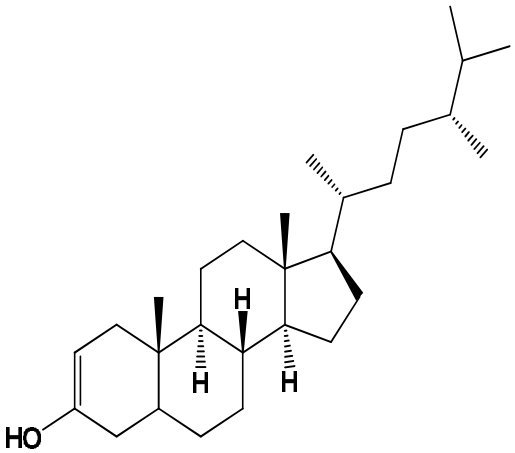
(2) Lupeol



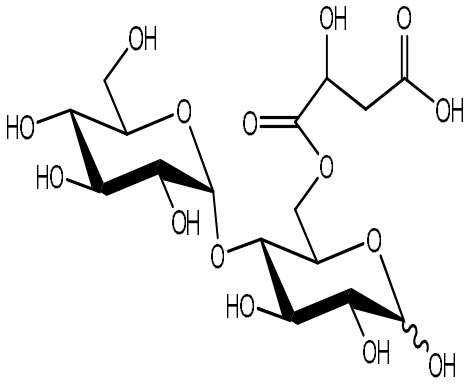
(3) Cholesterol



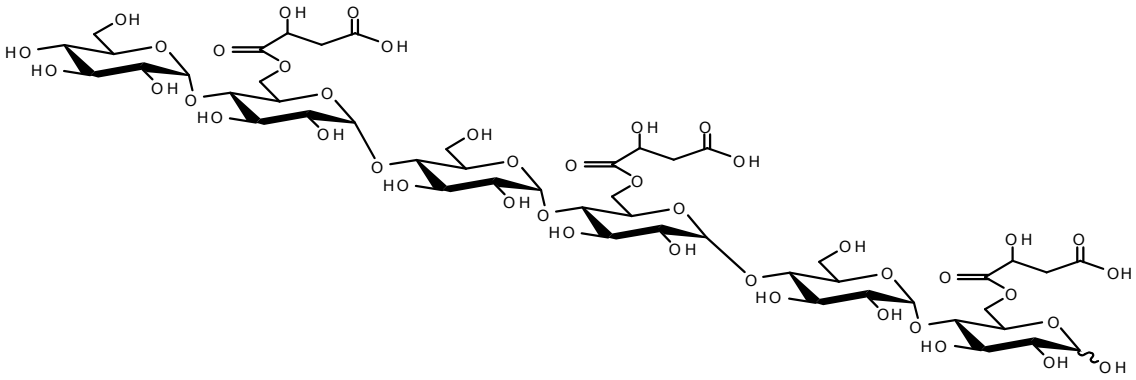
(4) β-sitosterol



(5) Campesterol



(6) Veracylglucan B



(7) Veracylglucan C

1.8. Chemical compositions of Aloe vera inner gel.

There are a number of folk medicine uses of Aloe vera gel since ancient times in the treatment of different diseases. Recently, various biological properties have been demonstrated *in vivo, in vitro* and in clinical trials to investigate the chemical compositions attributed to biological activities. Over 75 active ingredients from the inner gel have been identified, but therapeutic effects have not been correlated well with each individual component. Polysaccharides are the main components which contribute to the glutinous consistency of the inner gel (Reynolds, 2004; Vogler and Ernst, 1999). The gel primarily consists of more than 98% of water and polysaccharides (pectins, cellulose, hemicellulose, glucomannan, acemannan and mannose derivatives). Acemannan is considered the main functional component and is composed of a long chain of acetylated mannose (polydispersed β-1, 4-linked mannan substituted with *O*-acetyl groups) (Bozzi et al., 2007; Djeraba and Quere, 2000; Femenia et al., 1999; Lee et al., 2001) (Tables 1-3 and 1-6).

The therapeutic properties of Aloe leaf extracts have been attributed mainly to the polysaccharides, such as immunostimulation, anti-inflammatory effects, wound healing, promotion of radiation damage repair, anti-bacterial, anti-viral, anti-fungal, anti-diabetic and anti-neoplastic activities, stimulation of haematopoiesis and anti-oxidant effects (Reynolds and Dweck, 1999; Talmadge et al., 2004), but it is believed that these biological activities could also be assigned to a synergistic action of the various compounds contained therein rather than a single chemical substance (Dagne *et al.*, 2000).

Aloe vera leaf parts have different active constituents, leaf rind (exudate most compounds are identified as chromone, anthraquinone or anthrone derivatives anthrone- *C*-glucoside, barbaloin (Birch and Donovan, 1955; Hay and Haynes, 1956), while phenolic components do not occur in the parenchyma cells within the leaf gel, where polysaccharides and glycoproteins are characteristic. Thus, during processing, the discarding of the rind may lead to throwing away the majority of active constituents of inner gel. Mannan is the one of the most widely studied polysaccharides from aloes and was mostly isolated from the pulp of all the *Aloe* species analysed. Femenia (1999) interestingly reported that the presence of mannan in the rind may be significantly different from that found in the pulp.

Table 1-3 The amounts of some major components in the Aloe vera pulp.

	Amount (% dry weight)		
	Intact pulp	**Liquid gel**	**References**
Total polysaccharide	-	10–20	Yaron (1993)
	30	-	Roboz and Haagen-Smit (1948)
Total soluble sugar	16.48 ± 0.18	26.81 ± 0.56	Femenia *et al*. (1999)
	–	20–30	Yaron (1993)
	6.5	-	Rowe and Parks (1941)
	25.5	-	Roboz and Haagen-Smit (1948)
Total protein	7.26 ± 0.33	8.92 ± 0.62	Femenia *et al*. (1999)
	2.78	-	Roboz and Haagen-Smit (1948)
Total lipid	4.21 ± 0.12	5.13 ± 0.23	Femenia *et al*. (1999)
	4.76	-	
Malic acid	5.4 ± 0.85 – 8.7 ± 3.0	-	Paez *et al.* (2000)
Calcium	5.34 ± 0.14	3.58 ± 0.42	Femenia *et al*. (1999)
Sodium	1.98 ± 0.15	3.66 ± 0.07	Femenia *et al*. (1999)
Potassium	3.06 ± 0.18	4.06 ± 0.21	Femenia *et al*. (1999)
Ashes	15.37 ± 0.32	23.61 ± 0.71	Femenia *et al*. (1999)
	13.1	-	Rowe and Parks (1991)
	8.63	-	Roboz and Haagen-Smit (1948)

Adapted from Reynolds (2004).

Ni *et al.*(2004) carried out a study on Aloe vera leaf pulp using electron microscopy and carbohydrate analysis to determine chemical composition, in particular polysaccharides in three different structural components (cell wall pellet, microparticles pellet and liquid gel). Carbohydrate analysis of the three pulp components are different to each other in sugar composition, with the liquid gel containing mannan, the microparticles pellet containing galactose-rich polysaccharides and the cell wall pellet containing a high yield of galacturonic acid (Table 1-4).

The differences in carbohydrate analysis is associated with polysaccharide content (Table 1-6), and thus may potentially have different functionality, as

shown in Table 1-3. The mannan, is predominantly located in the liquid gel and is the most widely studied polysaccharide (Table 1-5) (Ni *et al.*, 2004).

Table 1-4 Proportions and carbohydrate of three primary components of Aloe vera L. pulp.

		% of dry pulp (w/w)	Carbohydrate content	Uronic acid (Gal A) content
Cell wall Fibers (pellet I)		16.2% (±3.8)	93% (w/w)	34% (w/w)
Microparticles (pellet II)		0.70% (±0)	50% (w/w)	8% (w/w)
Liquid Gel	Alcohol insoluble (precipitates)	7.5%	22% (w/v)	<0.015% (w/v)
	Alcohol-soluble	75.6%	-	-

Adapted from Ni *et al.*, (2004).

Table 1-5 Comparison of carbohydrate analysis of Aloe vera L. pulp.

	Liquid gel (ethanol precipitate) (%)	Microparticle (%)	Cell wall (%)
Mannose	62.9	32.2	20.4
Fucose	0	0	0
Glucose	13.1	20.6	12.3
Galactose	1.5	40.2	6.4
Gal. A	6	2.8	38.4
Others sugars	21.9	4.2	22.50
Total	100	100	100

Adapted from Ni *et al.*, (2004).

There are several factors affecting the results of experimental studies. The quality of Aloe vera is important. For example, the water content of plants in arid areas affects the quality of active components.

In terms of the cultivation method, Leung (1978) reported that no previous studies had ever considered seasonal, climatic and soil variations which may strongly affect composition of the gel (Leung, 1978). Natural environment cultivation is different from artificial irrigation. Mandal (1980), suggested that there are differences in the chemical compositions within the same species due to seasonal variation (i.e. leaves harvested in April yield 85% galacturonic acid while in October of the same year only gave 70% of galacturonic acid) (Mandal, 1980).

Table 1-6 Comparison of inner gel and whole leaf.

Property	Inner gel	Whole leaf
Soluble solids	0.62%	1.3 - 3.5%
pH	4.5	4.2
Nitrogen (as ammonia)	12	6
Ca^{++} (mg/l)	340	600
Mg^{++} (mg/l)	60	100
K^{+} (mg/l)	390	750
Conductivity	1200 - 2300 μ	1900 - 2500 μ
Taste	bland	salty
Methanol precipitable solids (MPS)	0.12 - 0.16%	0.45 - 1.3%
Monosaccharide	galactan and glucomannan	glucomannan

Adapted from (Agarwala, 1997).

1.9. Project aims

The high consumer demand for Aloe vera inner gel has resulted in improvements in the processing technology of the raw material involving in freeze dried and spray dried methods to stabilize the end product (Park and Lee, 2006). The production process follows a sequence of steps to ensure stabilization of the inner gel and to avoid mixing it with the bitter components (anthraquinones) from the rind (Waller *et al.*, 2004).

The inner gel can be dried by dehydration under low heat, and it is mostly dried by spray drying or freeze drying. The freeze drying or lyophilization process is carried out by drying frozen gel or whole extract under high vacuum pressure in a freeze drier. The water is removed by sublimation (evaporation of frozen solid water particles which is transient to the vapour phase without first passing to an intermediate liquid phase) (Waller *et al.*, 2004).

Spray drying as described by Waller *et al.* (2004) starts with matrix development. The matrix is pumped through a spray dryer chamber. The fluid is sprayed as a fine mist through a series of nozzles through this chamber. The chamber is heated between 50 to 90°C causing the water evaporate and the aloe matrix to dry. The high heating may affect the quality of the gel in term of biological activity; therefore the freeze dried process is the favored one.

Studies published on influence of heat on inner gel stability include one by Femenia (2003), who examined the effect of heat treatment on the stability of polysaccharides in inner gel; stability was observed at 70°C, and decreased either at high (90°C) or low (50°C) temperature.

Furthermore, the stabilization procedure of Aloe vera, collection and handling of the plant must be immediate after harvesting. Any delay in stabilisation will affect the quality and efficacy of active constituents (Grindlay and Reynolds, 1986). The kind of chemicals used in processing steps extensively affects the beneficial activity of some ingredients. In addition, manual processing is better than mechanical processing, but is more expensive and tedious. Pasteurization must be done carefully to prevent deactivation of aloe gel, and the most efficient way is HTST processing as it involves heating to 75-80 °C for less than 3min (Grindlay and Reynolds, 1986).

However, all the previously mentioned studies suffer from some limitations including if there is any relationship between the effect of heat treatment in the processing steps and the subsequent biological activity of Aloe vera inner gel.

The aim of this project is to study the effect of processing on the chemical and biological activity of Aloe vera inner gel. Aloe vera was chosen because of the well known anti-inflammatory activities amongst the different species of the Asphodelaceae family. This was achieved as follows;

1. Determine the chemical profiles of freeze-dried (FD) and spray-dried (SD) using TLC, NMR and compare the biological activity.
2. As time allowed fractionation of FD/SD Aloe vera to separate out the active compounds that possess anti-inflammatory properties using Flash chromatography.
3. To examine the anti-inflammatory properties of the separated compounds *in vitro* in an assess using THP1 cell stimulated with LPS to induce TNF-α.
4. Ascertain fractionated structures using nuclear magnetic resonance (NMR).

2. Materials and Methods

Materials and Equipment

0.22µm filter (Millipore, UK)

15ml sterile centrifuge tubes (Greiner Bio-one, UK)

20ml universal centrifuge tubes (Greiner Bio-one, UK)

24-well plates (TTP, Switzerland)

25cm^2 cell culture flasks (Corning Incorporation, USA)

50ml sterile centrifuge tubes (Greiner Bio-one, UK)

75cm^2 cell culture flasks (Corning Incorporated, USA)

7ml Bijoux (Greiner Bio-one, UK)

96 well tissue culture plates (TPP, Switzerland)

Alamarblue™ (Serotec, UK)

Aloe vera inner gel powder, (Rainbow Naturprodukte GmBH,Germany)

Aloin, glucose, fructose and mannose standards (Sigma, UK)

Anisaldhyde (FSA laboratory, UK)

Anti-bumping granules (BDH, UK)

Bulk Isolute sorbent Isolute® HM-N (Intl. Sorbent Tech. Ltd, UK)

Certoclav autoclave (Kelomat, Austria)

Chloroform, HPLC grade (Fisher Chemicals, UK)

Cryovials (Nunc, UK)

Deuterium oxide (D_2O) 99.9+atom % D (Sigma-Aldrich, USA)

Dimethylsulphoxide (DMSO), (Sigma Aldrich, UK)

Distilled water, HPLC grade (Sigma,UK)

Ethanol (Sigma Aldrich, UK)

F16 Maxisorp Loose Nunc-immuno modules (Nunc, Denmark)

Flash chromatography cartridges, C18-E 50g/150ml Giga Tubes (Strata, UK)

Flash Master Personal Apparatus (Jones Chromatography, UK)

Foetal calf serum (Sigma, UK)

IL-1β ELISA Ready-Set-GO! Kit (eBioscience, UK)

JEOL Delta GX 400MHz FT Neuclear Magnetic Resonance (NMR) Spectrometer (USA)

L-glutamine (Sigma, UK)

Lipopolysaccharide - *Salmonella abortus* equi (Sigma-Aldrich, UK

Methanol, HPLC grade (Sigma, UK)

Microcentrifuge (Centaur, SANYO, Japan)

Microscope (Olympus, Japan)

Neubauer-Improved Haemocytometer (Marienfeld, Germany)

Rotary evaporator (Büchi, Switzerland)

RPMI 1640 medium (Sigma, UK)

Sodium chloride (BDH, UK)

SpectraMax M5 microplate reader (Molecular Devices Corporation, California, USA)

Strata® SI-1 Silica (55µm, 70A) RP Flash column (Phenomenex®, USA)

Streptomycin/ Penicillin (Cambrex, UK)

Sulphuric Acid (Sigma, UK)

Syringes (Becton Dickinson UK Ltd, UK)

Thin walled NMR sample tubes (VWR® International, USA)

THP-1 cells (European Collection Animal Cell Culture, UK)

TLC aluminium sheets (20x20cm) silica gel 60 F_{254} N (Merk KgaA, Germany)

TLC aluminium sheets, RR-18 F_{254} (Merk KgaA, Germany)

TNF-α ELISA Ready-Set-GO! Kit (eBioscience, UK).

Tween 20 - Polyoxyethylenesorbitanmonolaurate (Sigma, UK)

UV-detector 254 and 364nm UVGL-58 (UVP, USA)

Water HPLC grade (Sigma, UK)

Wilmad® NMR tubes, 5mm, 300MHz, 7inL, 507-PP (Sigma-Aldrich, USA)

2.1. Analytical Methods

2.1.1. Reverse Phase Thin Layer Chromatography (TLC)

Reverse phase TLC was used prior to Flash chromatography to establish the solvent systems to be used in the separation process of crude Aloe vera inner gel. A pencil line was drawn 1 cm from the base of the plate, and 1 cm partitions were marked along the base line. The samples were dissolved in volatile solvents (b.p < 80°C), and spotted onto the plate. Methanol: water (1:1), was used as the mobile phase and the plate placed into the chamber until the solvent had reached 1 cm from the top of the plate (solvent front). The plate was left to air dry for 30min or heat applied with a hot air dryer to evaporate the solvent, and then the compounds were visualised under UV light at 254nm and 365nm. Any visualised bands were marked with a pencil, to allow R*f* values to be calculated.

The plate was also developed by spraying with anisaldehyde-H_2SO_4 spray reagent (0.5ml anisaldehyde in 10ml glacial acetic acid, 85 methanol and 5ml conc. sulphuric acid) to detect sugars, steroids and terpenes. Standards included aloin, glucose, fructose and mannose.

The R*f* values for each band were calculated using the following equation:

$$R_f \text{ values} = \frac{\text{Distance traveled by the band}}{\text{Distance traveled by the mobile phase}}$$

2.1.2. Flash Column Chromatography (FCC)

Freeze dried Aloe vera inner gel powder (5g) was dissolved in 8ml distilled water, stirred well and then a few drops of methanol added. Some heat was

applied using a hot air dryer to ensure that the powder had completely dissolved. The clear solution was then mixed with 10g of Bulk sorbent Isolute® HM-N. The mixture was kept in a fume hood to evaporate the solvent (around 48 hours) and the dried mixture was ground in a porcelain dish to obtain a fine powder. This was then directly injected onto the head of a dry pre-filled reverse phase flash column chromatography silica cartridge and packed using a plastic rod. The cartridge was closed with a special filter and the column fixed to the Flash Master Personal Apparatus. The elution was carried out under pressure, flow rate: 20ml/min, isocratic with solvent systems optimised for the best separation. Elution was carried out using methanol:water in a gradient elution starting with 100% water and gradually decreasing the water until 100% methanol (Table 2-1). The eluted fractions were collected in 1.5ml glass vials, labeled (1-100).

Table 2-1 Flash chromatography solvent system. (See table 3-1 page 46).

Solvent system (all volumes were 100ml)		**Fractions collected (≈ 20ml each vial)**	**Pooled fractions**
Water	Methanol		
95%	5%	1—3	1-3 = **A1**
90%	10%	4—8	4-10=**A2**
85%	15%	9—12	
80%	20%	13—16	11-20=**A3**
75%	25%	17-20	
70%	30%	21-24	21-25=**A4**
65%	35%	25-28	26-32=**A5**
60%	40%	29-32	
55%	45%	33-36	
50%	50%	37-40	33-40=**A6**
45%	55%	41-44	
40%	60%	45-49	41-54=**A7**
35%	65%	50-53	
30%	70%	54-57	
25%	75%	58-61	
20%	80%	62-65	
15%	85%	66-69	55-79=**A8**
10%	90%	70-73	
5%	95%	74-78	
0%	100%	79-100	80-100=**A9**

2.1.3. NMR Proton (^{1}H NMR) and COSY

All one dimensional (1D) samples were obtained using Delta™ NMR software. Known amounts of samples of both crude Aloe vera inner gel FD and SD and for any subsequent fractions (10-20 mg) were dissolved in Deuterium oxide (D_2O) in clean dry containers and transferred carefully to NMR tubes (thin walled NMR sample tubes to be measured.

The observed chemical shift δ values were obtained in ppm and the coupling constant (J) in Hz. Spectra were referenced to residual solvent samples. H^1 NMR spectra were measured at a magnetic field strength of 400.13 MHz using a JEOL Delta GX 400 MHz FT nuclear magnetic resonance (NMR) Spectrometer. ChemBioDraw Ultra, Version 11, was used to draw compound structures, and also to predict ^{1}H NMR data.

2.2. Biological Methods

2.2.1. Preparation of Complete medium (CM)

THP-1 cells were grown in complete RPMI medium (CM) prepared with 500 ml RPMI 1640 medium, supplemented with foetal calf serum (10%, v/v, FCS), Streptomycin/ Penicillin (1%, v/v), and L-glutamine (1%, v/v). CM was used in all immunoassays, cell culture and the dilution of test fractions in ELISA. All procedures were performed aseptically under a laminar flow system.

2.2.2. THP-1 cell culture

Cells were normally grown in 75cm^2 flasks with 50ml CM, incubated at 37°C, 5% CO_2 and 100% humidity. In order to maintain cells in log phase of growth, they were checked microscopically using a light microscope every 3-4 days and sub-cultured by transferring 5ml of cell suspension to a fresh flask with 45ml of CM. For bioassays, the cells from one flask were centrifuged 150g, 5min, and 4°C. The supernatant was discarded and the pellet re-suspended in 15ml CM. A cell count was taken using a Neubauer-Improved Haemocytometer and the cells diluted to the desired number for each particular assay. This was calculated using the equation:

Number of cells/ml=Total number counted $\times 10^4$

2.2.3. Resurrecting THP-1 cells

THP-1 cells were stored at -80°C or in liquid nitrogen. To resurrect the cells, 1 cryovial (1x10^6 cells/ml) was rapidly thawed at 37°C, and then added to 20ml of CM in a 50ml centrifuge tube. The cells were centrifuged at 150g for 5min at 4°C. The supernatant was discarded and the pellet re-suspended in 5ml CM and poured into a 25cm^2 cell culture flask.

2.2.4. Freezing THP-1 cells

THP-1 cells were frozen for long-term storage as follows; cells were centrifuged at 150g for 5 min at 4°C. The supernatant was discarded while the pellet was resuspended in 10ml freezing medium (CM, plus 10%, v/v FCS and 10%, v/v, DMSO). Cells (1x10^6 cells/ml) were then quickly aliquoted into sterile labeled

cryovials (1ml) and stored in a -80°C freezer temporarily. They were then transferred to liquid nitrogen storage.

2.2.5. Cell cytotoxicity

The cytotoxicity assay was designed to evaluate the toxicity of crude Aloe vera inner gel on THP-1 cells. The assay was performed using Alamarblue™ which is an indicator dye; in the presence of viable, metabolizing cells Alamarblue™ turns from blue to pink while wells with dead cells remain blue.

The assay was performed aseptically in a sterile flow hood, in a 96-well plate. A 100µl/well of Aloe vera (10mg/ml in complete medium) was added to the first column of rows A, B, C and G of a 96 well tissue culture. Row G acted as a background control for coloured material as it only contained Aloe vera and medium. If the tested plant material was dissolved in DMSO, 100µl DMSO was added to the first wells of rows D and E (solvent control). These rows were left blank if the plant material was water soluble. 100µl of medium was then added to all the remaining wells, and a 1:2 serial dilution carried out across the plate. The last 100µl was discarded into Virkon® solution. Then, 100µl of THP-1 cells ($1x10^6$ cells/ml) suspended in complete medium containing 10% (v/v) Alamarblue™) was plated out in all the wells except row G (background control). Row F contained cells in media only, which acted as a positive control, 100µl of medium plus 10% (v/v) Alamarblue™ was added. In row H the medium was carefully removed and replaced with 200µl of NaCl solution (300mg/ml) plus 10% (v/v) Alamarblue™ to lyse the cells and act as a negative control. The plate was then placed in an incubator overnight at 37°C, 5% CO_2 and 100% humidity.

The following day (Day 1), 100µl supernatant from each well was carefully transferred to a fresh plate and read on a SpectraMax M5 microplate reader at 570nm and 600nm.

Wells were replenished with fresh 100µl/well CM containing 10% (v/v) Alamarblue™. The plate was incubated overnight, and the next day (Day 2) the plate was read again. The equation used to calculate the percentage cell growth was:

$$\% \text{ Cell growth} = \frac{\text{Mean of (OD570 - OD600) of test agent}}{\text{Mean of (OD570 - OD600) of positive control}} \times 100$$

OD; Optical density

Percentage cell growth was plotted against the concentration of the test agent;
A percentage above 50% was considered non-toxic.

2.2.6. Stimulation of THP-1 cells with Lipopolysaccharide (LPS)

Plant extract (1mg) was dissolved in 1ml complete media (CM) in sterile 20 ml universal centrifuge tubes. Vigorous shaking using a vortex was employed to ensure dissolving of the tested fractions. They were filtered through a 0.22µm filter into sterile Bijoux.

Lipopolysaccharide (10mg) was dissolved in 1ml CM and aliquoted. The LPS was vigorously mixed by vortex before use. THP-1 cells (1×10^6 cells/ml, 1ml) were added to each well of a 24-well plate, together with 100µl/well of 1mg/ml plant extract with and without LPS (10µl/well final well concentration

0.05mg/ml). Wells were made up to a final volume of 1110µl with CM. Each treatment was performed in triplicate using the template Figure 2-1.

Plates were incubated for 24h at 37°C, 5% CO_2 and 100% humidity. Afterwards, the supernatants were transferred to 1.5ml sterile microfuge tubes and centrifuged in a micro centrifuge at 13,000rpm for 5 min at 4°C. The supernatants were transferred to fresh microfuge tubes and stored at -20°C until assayed for cytokine levels by ELISA.

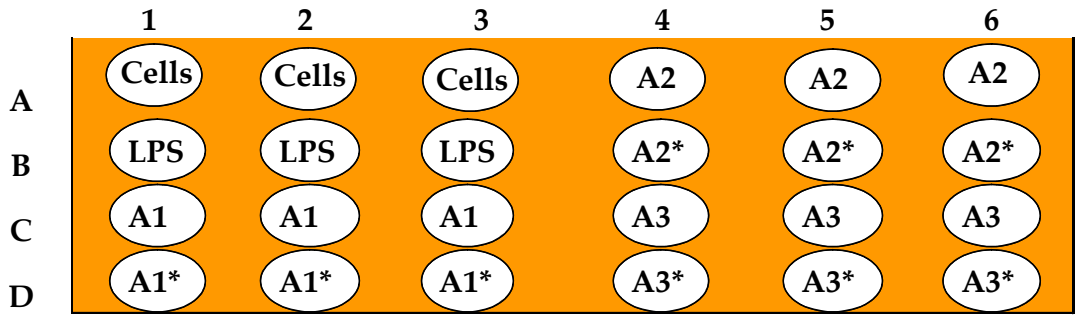


Figure 2-1 Typical layout of a THP-1 cell stimulation assay. These tests were performed on one 24-well plate. A1,-A3 represent three different samples (these could be crude aloe vera or fractions) and A1*-A3* indicates wells with plant material+ LPS.

2.2.7. Enzyme linked immunosorbent assay (ELISA)

A sandwich ELISA was carried out with supernatants (section 2.2.6) from the LPS stimulation assay to evaluate the production of tumour necrosis factor alpha (TNF-α) cytokine, using an ELISA READY-SET-GO! Kit. All procedures were carried out according to the manufacture's instructions, while wash steps were carried out using phosphate buffered saline (PBS) recipe pH 7.4 containing (v/v) Tween®.

F16 Maxisorp Loose Nunc-immuno modules were coated with 100 µl/well capture antibody (1:250 dilution) in coating buffer and incubated overnight at 4°C. Wells were washed three times in wash buffer and blocked with 200 µl/well assay diluent. The modules were incubated at room temperature for 1 hour and then washed.

The supplied TNF-α standard (5µl) was added to 10ml assay diluent to make a top standard concentration of 500pg/ml. 100µl/well was added in duplicate wells at the top of the modules and a 1:2 serial dilution was carried out down the modules. The bottom two wells contained assay diluent only (blank). For the samples, 100µl/well of cell supernatant was added in triplicate and modules incubated at room temperature for 2h.

The modules were then washed five times and 100 µl/well detection antibody in diluent (1:250 dilution) added. The modules were incubated at room temperature for 1h, followed by another five washes. Then, 100 µl/well of Avidin-HRP in diluent (1:250 dilution) was added and incubated for 30min at room temperature and the modules washed seven times. The wells were developed with 100µl/well substrate solution, and incubated for 15min at room

temperature. The enzyme reaction was stopped by adding 50µl/well 10% (v/v) sulphuric acid (H_2SO_4) and read at 450nm. The concentration of TNF-α produced in each sample was calculated from the slope equation of the standard curve. In the case of IL-1β, these steps were carried out using the same procedures as above, but substituting IL-1β specific reagents instead of TNF- α.

2.3. Statistics

All the data were analysed statistically on MINTAB 13 using an unpaired, 2-tailed student's t-Test. Data with p values less than 0.05 were considered significant.

3. Results

3.1. Analytical Results

3.1.1. Thin Layer Chromatography (TLC)

Crude Aloe vera inner gel and its fractions were screened by reverse phase TLC in order to establish the best solvent system for Flash chromatography. The two crude samples FD and SD of Aloe vera inner gel showed no differences in R*f* value (Figure 3-1) indicating that the same solvent system could be used for both samples.

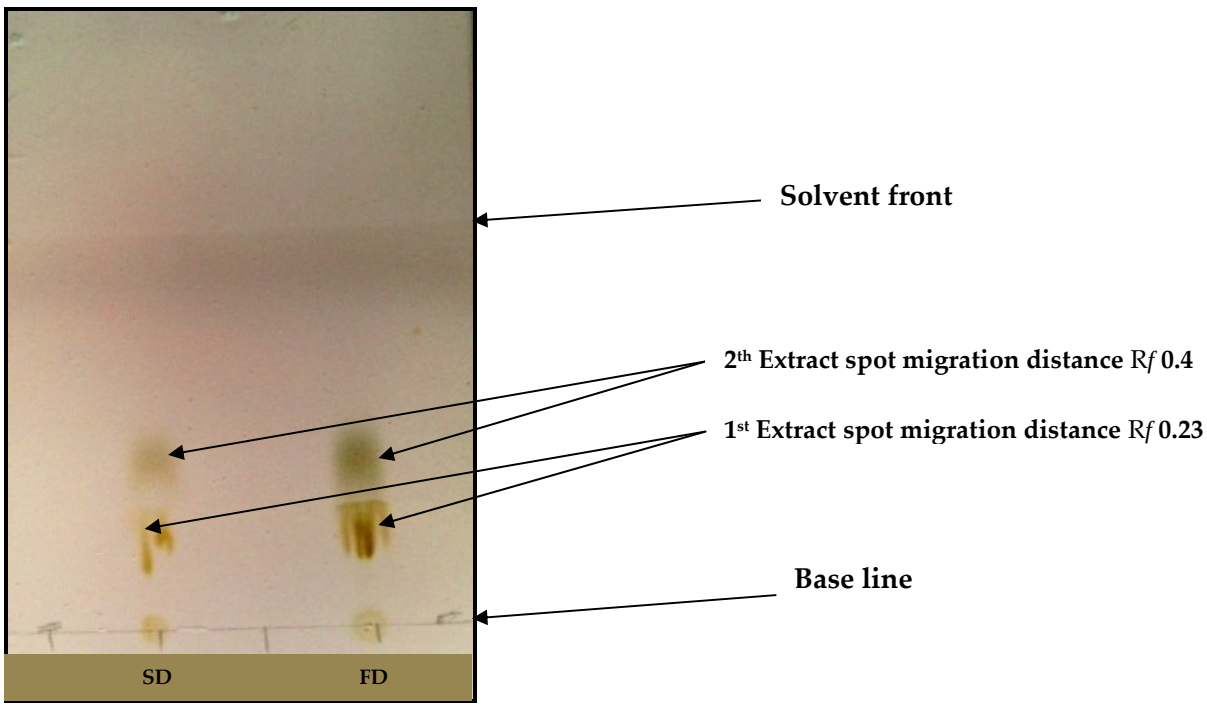


Figure 3-1 TLC of crude Aloe vera freeze-dried (FD) and spray-dried (SD) inner gel Reverse phase TLC Aluminium Sheets, silica gel 60 F_{254} were used. The solvent system was butanol:acetic acid:water (4:1:5), and the plate developed with annisaldehyde spray reagent.

3.1.2. Freeze-drying of Aloe vera inner gel and its fractions

100 vials were collected; TLC was carried out to identify the similar vials. These were pooled into nine vials labeled (A1-A9) table 2-1.

Freeze-drying or lyophilization is a convenient method for removing water from samples. Firstly all fractions obtained from flash chromatography were concentrated by rotary evaporation. Then all the collected fractions were frozen at low temperature (below -40°C). They were then placed in a freeze-drier (under vacuum) for 36-72h. Each fraction showed different physical appearances, and the colour varied from bright white, (vial 7), buff, (vial 6, 8 and 9), yellowish brown, (vial 4) to a dark brown colour, (vial 1 and 3) as shown in Figure 3-2. The texture of some fractions had a honey-like touch, (vial 3), while some were more sticky, (vial 1 and vial 3), while a few crystallised as granules (vial 4). Table 3-1 shows the yields for each fraction.

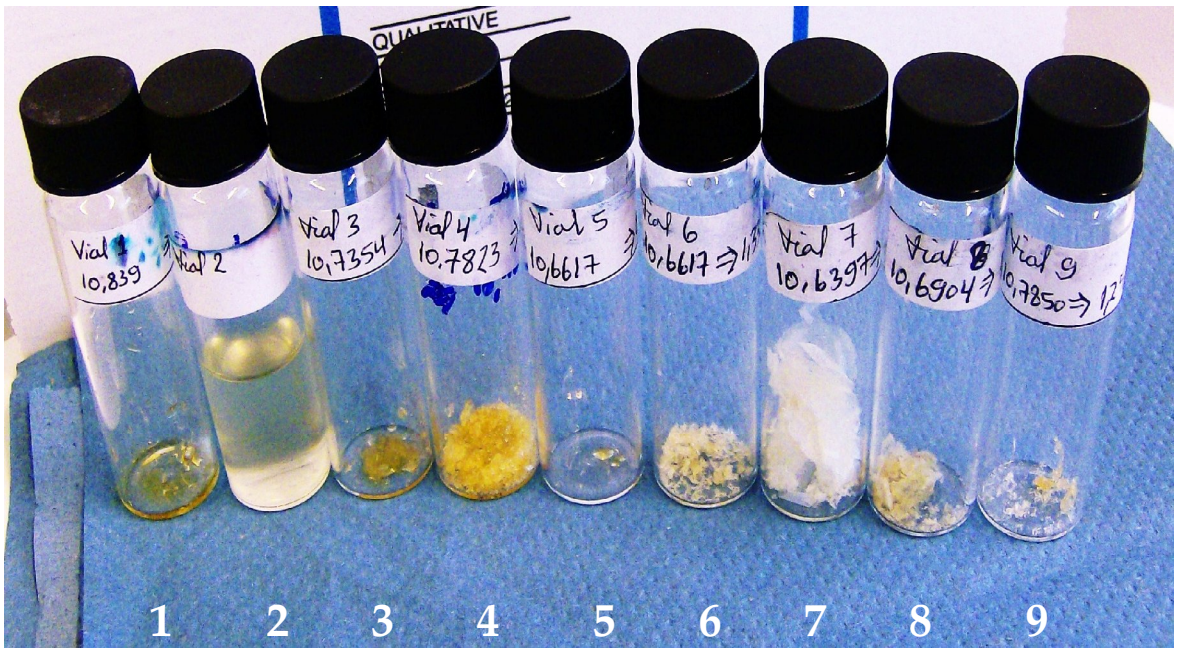


Figure 3-2 Appearances of the fractions of Aloe vera inner gel after freezing-drying.

Table 3-1 Percentage of yields of freeze-dried Aloe vera inner gel fractions obtained by Flash chromatography (See table 2-1 page 36).

Faction label	Fraction weight (mg)	Fraction yields %
A1	1.3	32.3
A2	0.59	14.7
A3	0.42	10.4
A4	0.24	6.0
A5	0.29	7.2
A6	0.29	7.2
A7	0.48	11.9
A8	0.27	6.7
A9	0.14	3.5

Total fraction weight was 4.02g from the original 5g

3.1.3. Proton ^{1}HNMR results of Aloe vera inner gel and its fractions

The two crude samples of Aloe vera inner gel (FD and SD) were investigated by NMR proton spectra to determine their chemical profiles. The results are detailed in the next sections.

3.1.3.1. Comparison of proton ^{1}HNMR spectra of crude FD and SD of Aloe vera inner gel

Generally, the chemical shifts of protons appeared mainly in the region of 0.5 to 6ppm. The FD and SD Aloe vera samples were quite similar in their chemical shift patterns (Figures 3-3 and 3-4 respectively). Comparison of ^{1}HNMR described by Bozzi *et al.*, (2007) (Figure 3-9) was made.

In terms of quality, the ^{1}HNMR for both samples of FD and SD Aloe vera inner gel indicated the presence of significant signals related to the presence of glucose, acemannan and malic acid as in Table 3-2. This was indicative of high quality Aloe vera.

In the present study both FD and SD Aloe vera samples showed small signals at δ 5.2 and δ 5.6 ppm which were on the water signal corresponding to maltodextrin (Figures 3-3 and 3-4). ^{1}HNMR showed the absence of signals corresponding to organic acids; lactic, succinic and acetic acid also indicating confirmation of the good quality and degree of freshness of both FD and SD Aloe vera samples. However, there were some significant differences in signals; some signals were detected in the FD sample only, while absent in the SD sample and *vice versa*.

Expansion of some areas of the NMR spectra enabled the identification of differences between SD as compared to FD (Figures 3-5 to 3-8). Small signals were not visible before expansion. Significant differences were detected as follows:

At position A (Figures 3-3 to 3-8) of FD, a signal was detected at δ1.08 and 1.07, which was absent in the SD sample. In addition, at position C, Figure 3-5 of FD there are signals noted at δ 1.8-1.9 which were absent in the SD sample spectra. Position D, points to two signals around δ 2.3-2.5 in the FD sample, absent in the SD sample. Moreover, at position E, a peak δ 2.98ppm in the SD sample has more components than the FD sample. In contrast at position F, many peaks appear at δ 3.22-3.28 in the SD sample and small peaks in the FD sample are not as clear. In position G, a significant signal appears at δ 3.55 and 3.56ppm which

is absent in the FD sample, also in position H, signals were shown around δ 3.56 and 3.60ppm which were absent in the SD sample. Finally, signals at position J, δ 5.19ppm are present in the SD sample only (Figure 3-8).

In summary, detected peaks from ^{1}HNMR spectra in the FD sample that were absent in the SD sample; were peaks at position δ 1.08, 1.07, 1.9, 1.9, 2.3-2.5 and 2.98ppm. On the other hand, the SD sample showed peaks at δ2.98, 3.22-3.28, 3.55, 3.56 and 5.19ppm which were absent from the FD sample.

The labels A-I on the spectra show positions with differences between both samples.

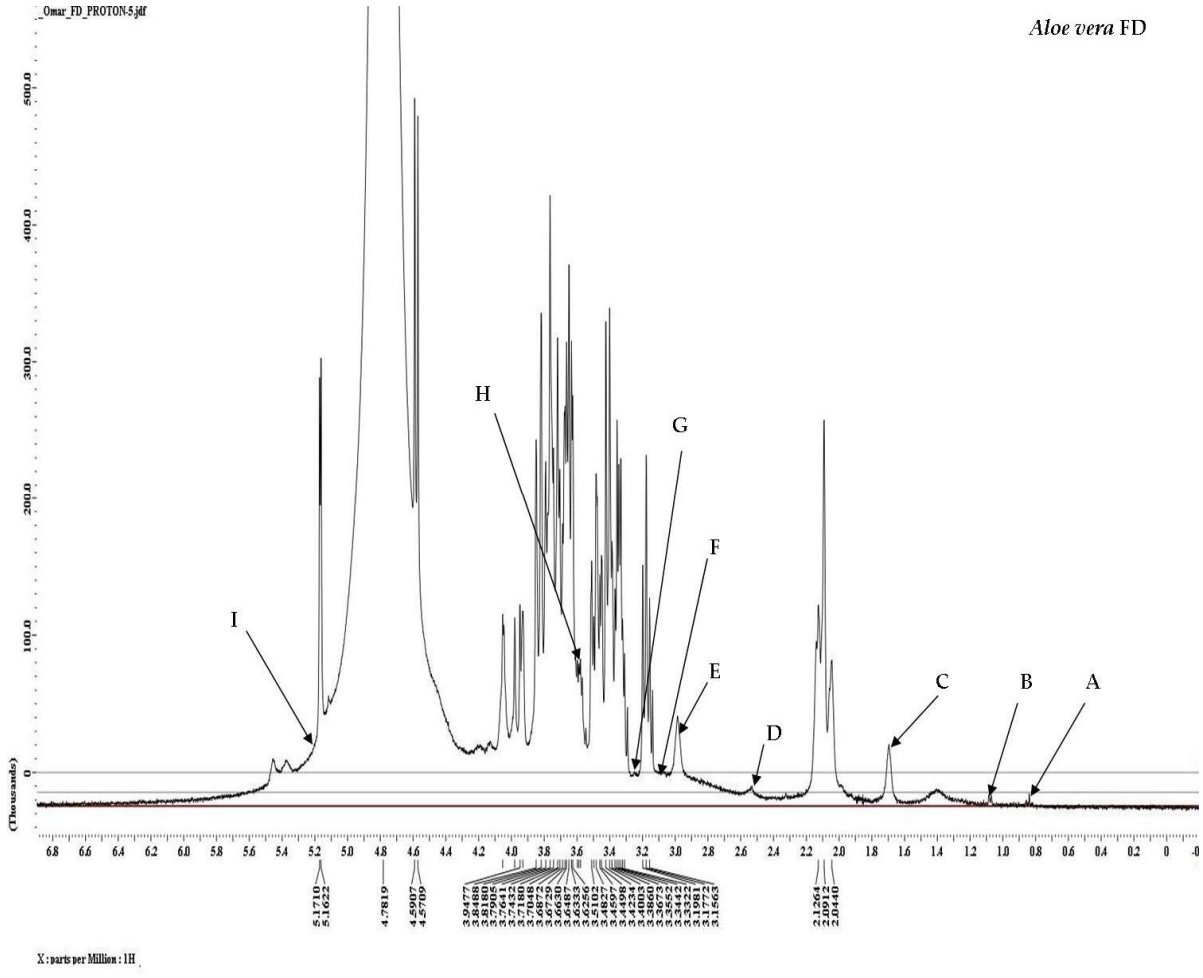


Figure 3-3 Full ^{1}H NMR spectra of crude FD Aloe vera inner gel in D_2O (0-7ppm) (JEOL Delta GX 400 MHz).

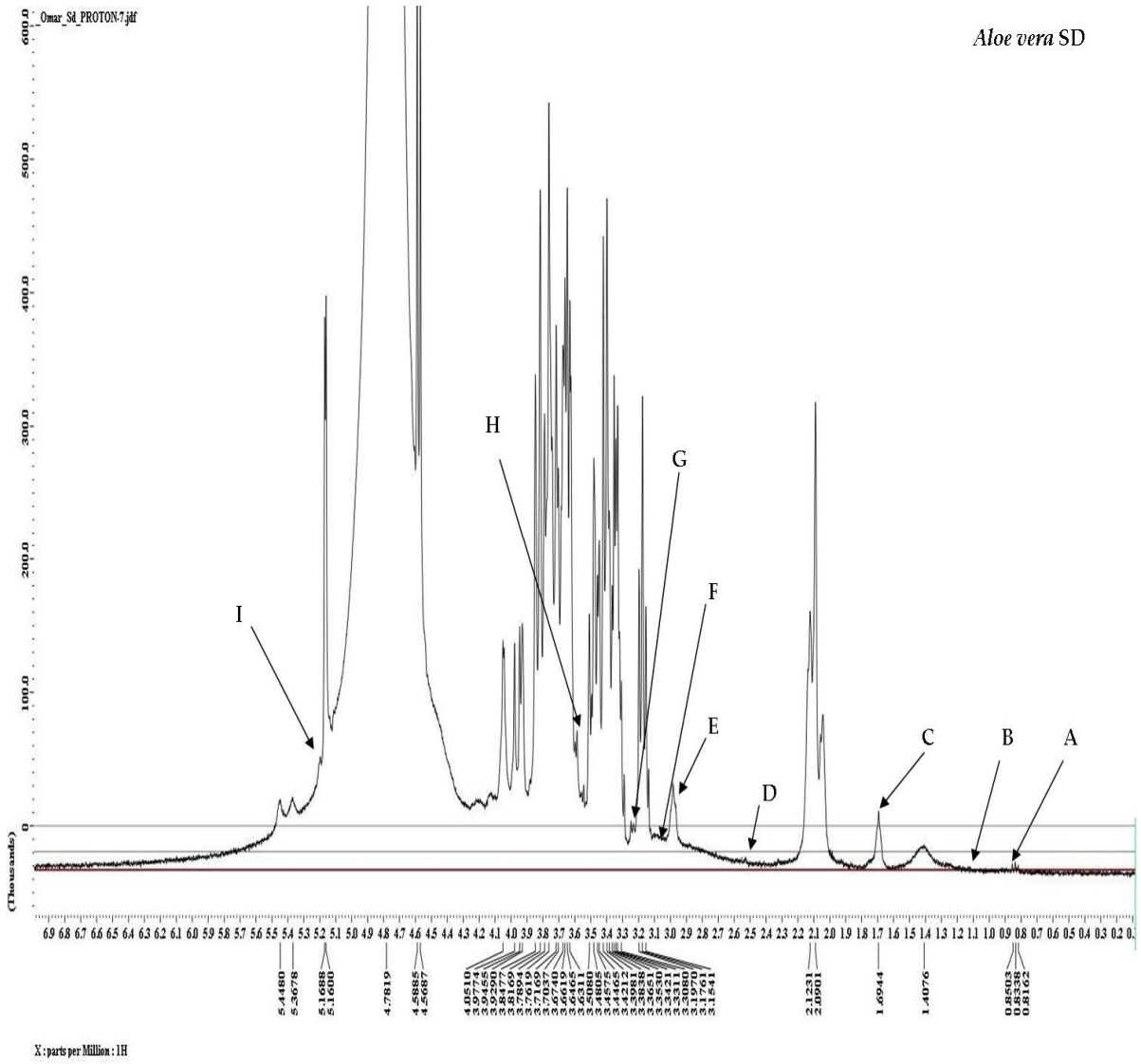


Figure 3-4 Full ^{1}H NMR spectra of crude SD Aloe vera inner gel in D_2O (0-7ppm) (JEOL Delta GX 400 MHz).

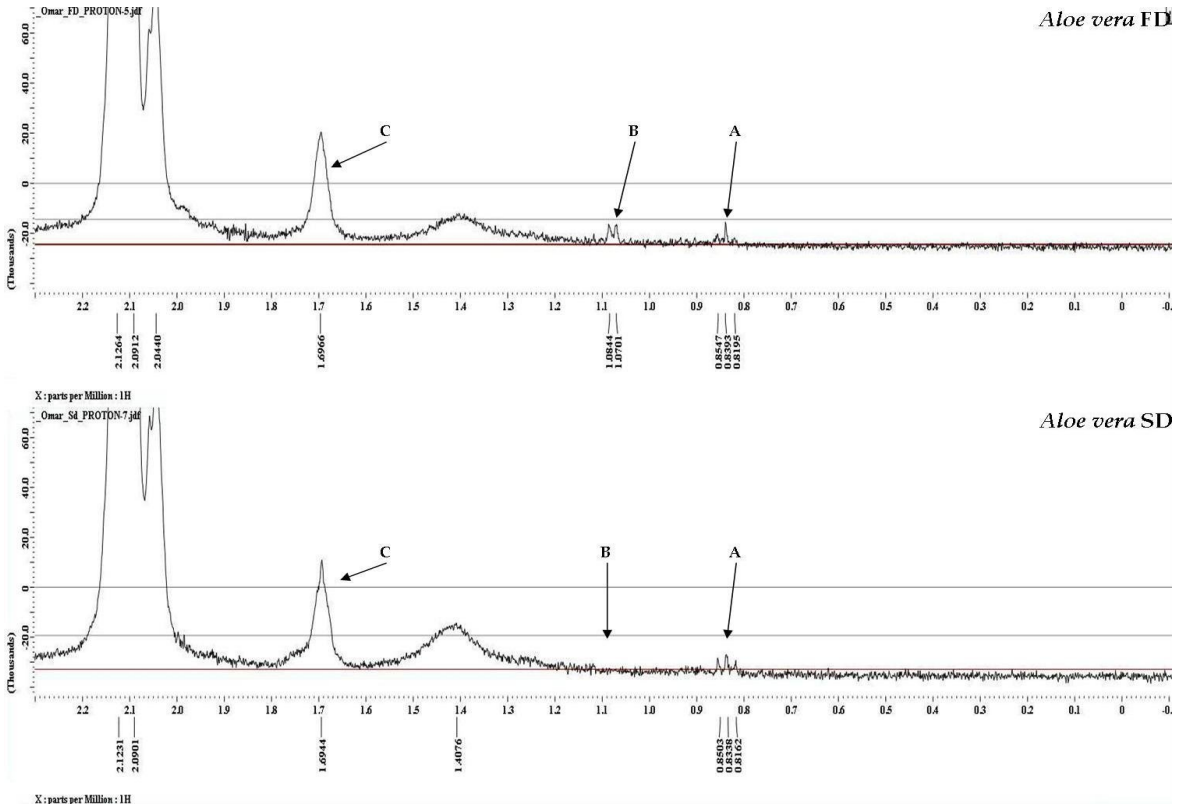


Figure 3-5 Expansion of ^{1}H NMR spectra of crude FD and SD Aloe vera inner gel in D_2O (0-2.3ppm) (JEOL Delta GX 400 MHz).

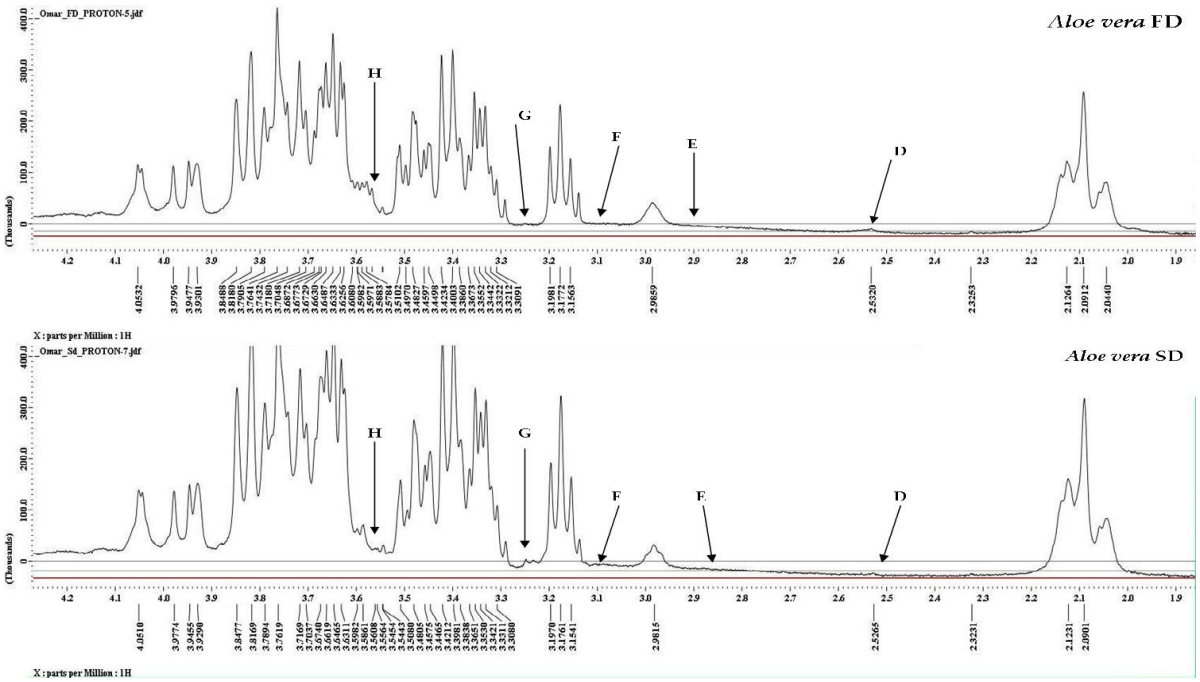


Figure 3-6 Expansion of ^{1}H NMR spectra of crude FD and SD Aloe vera inner gel in D_2O (1.9-4.2ppm) (JEOL Delta GX 400 MHz).

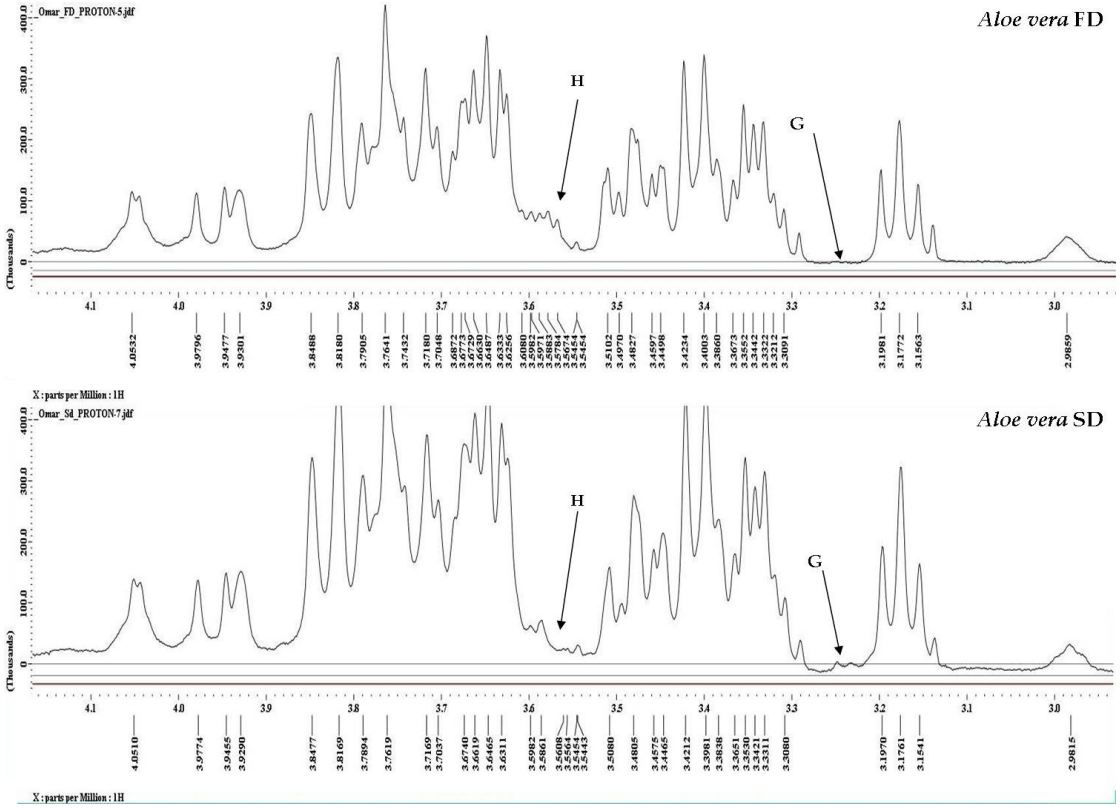


Figure 3-7 Expansion of ^{1}H NMR spectra of crude FD and SD Aloe vera inner gel in D_2O (3-4.1ppm) (JEOL Delta GX 400 MHz).

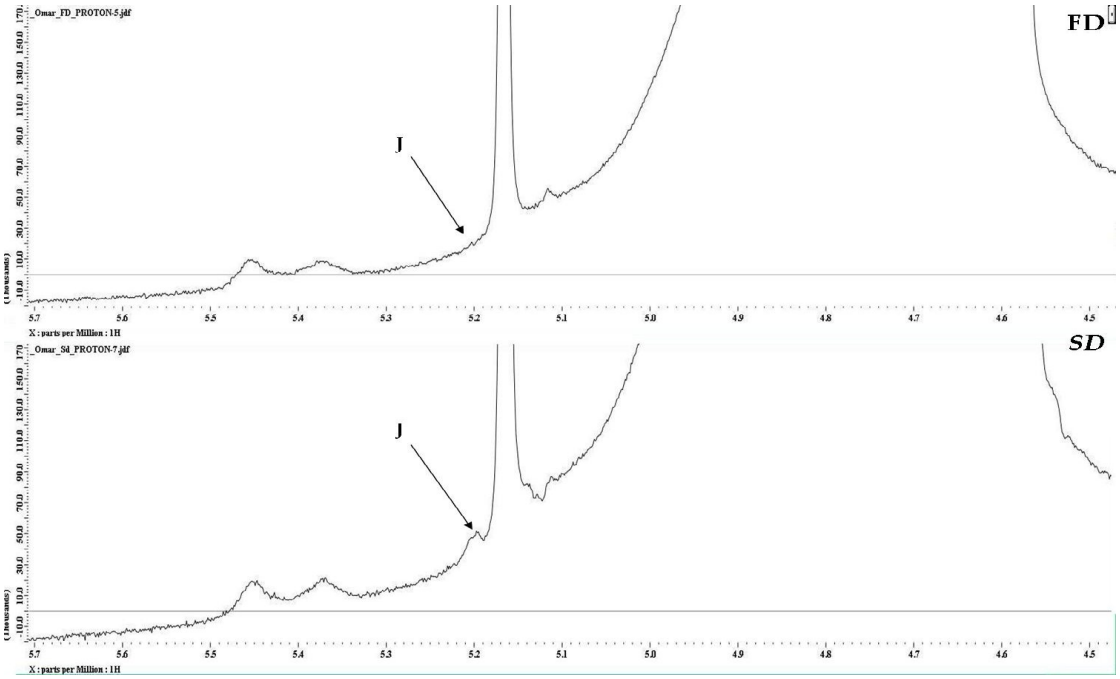


Figure 3-8 Expansion of ^{1}H NMR spectra of crude FD and SD Aloe vera inner gel in D_2O (4.5-5.7ppm) (JEOL Delta GX 400 MHz).

From the NMR spectra of FD and SD Aloe vera it was not possible to elucidate the structure of the components due to the samples being in crude form. Therefore, a reference in the literature as reported by Bozzi, *et al.* (2007), was used which recognised the chemical shift of some components of Aloe vera inner gel (Table 3-2). They compared different commercial samples of Aloe vera gel with one fresh Aloe vera inner gel sample to be used as a reference. ^{1}HNMR spectra of fresh Aloe vera inner gel (reference) are shown in Figure 3-9. The conditions used in this experiment were as follows; samples were dissolved in 0.7 ml of 99.9% D_2O using a Bruker DPX-360 spectrometer at 360.13 MHz, where these conditions are similar to the present study. These spectra were used as a reference guide in terms of identification of the present Aloe vera inner gel ^{1}HNMR spectra for both crude extracts (Figures 3-3 and 3-4), and for active fractions A7 and A9 in comparison with the crude FD sample (Figure 3-10).

Table 3-2 The prediction of ^{1}HNMR signals for suggested compounds in FD and SD Aloe vera inner gel as compared to the literature.

Suggested compound	Chemical shift (δ) (ppm)	FD	SD
Acemannan	2.00-2.26	✓	✓
Malic acid	2.5-2.7 and 4.7	✓	✓
Glucose	5.2 , 4.6	✓	✓

Adapted from Bozzi *et al.*, (2007).

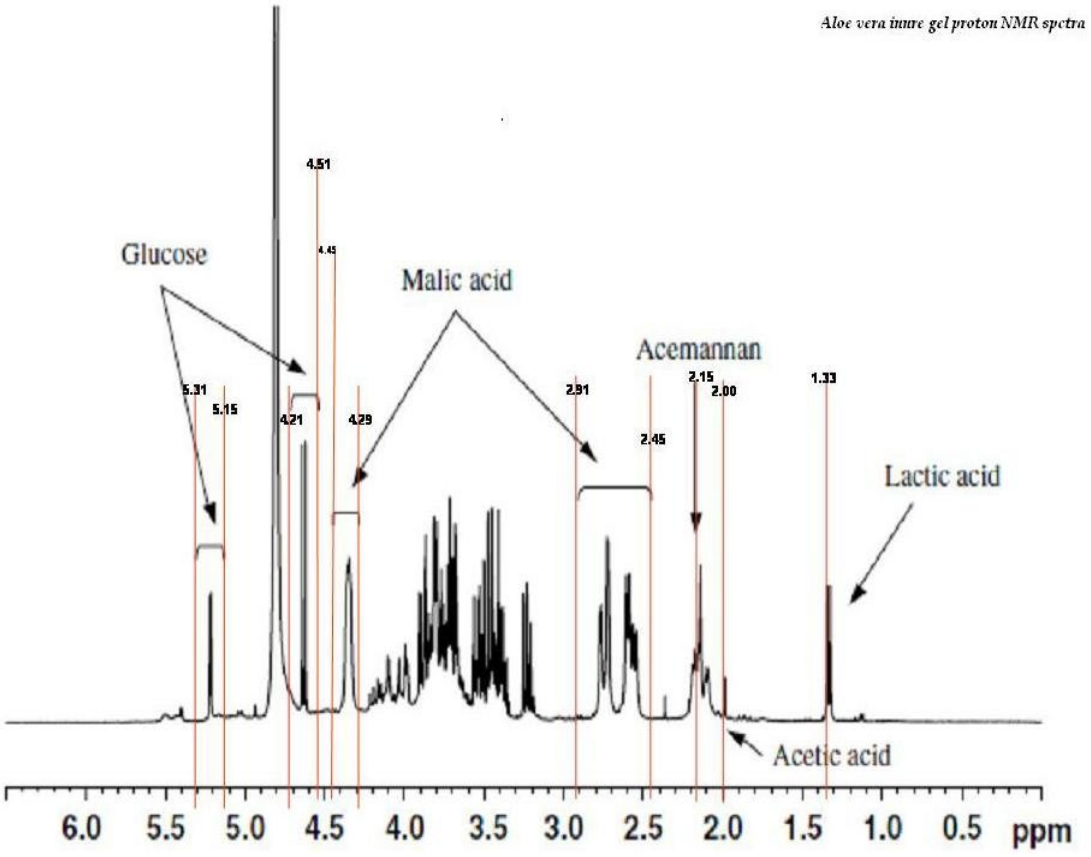


Figure 3-9 ^{1}H NMR spectra of crude fresh Aloe vera inner gel. The samples were dissolved in 0.7 ml of 99.9% D_2O (Euriso-Top, France) using (Bruker DPX-360 spectrometer) at (360.13 MHz) adapted from *Bozzi et al.*,(2007).

3.1.3.2. Proton NMR spectra of active fractions (A7) and (A9) compared with crude Aloe vera FD

The reason for choosing these two fractions A7 and A9 were based on the biological activity (section 3.2.4, Figure 3-15). ^{1}HNMR were run for fractions A7 and A9 in D_2O, in order to identify and attempt to determine the ingredients of those fractions, compared with the crude FD sample. The chemical shift of protons appeared in region 0.5 to 10ppm. There was some similarity in both spectra of A7 and A9, but they had some differences in comparison with crude FD.

The differences between fractions A7 and A9 (Figure 3-10), appeared in the aliphatic region in position I (Figure 3-11) at δ 0.8-2.0ppm. Also there were differences in the aromatic region at position II (Figure 3-12) at δ 7.35-7.85ppm; these additional signals were notably not found in the crude FD sample (Figure 3-12), which might be due to the concentration of active ingredients in these fractions while they were only present in small amounts in the crude FD.

Figures 3-11 and 3-12 show the expansions of ^{1}HNMR spectra of both fractions A7 and A9, respectively, compared to the crude FD sample, where the signals were not visible before the expansion. Significant differences were detected as follows;

At position I (Figures 3-11 and 3-12) signals were detected at δ0.83 and 0.89ppm, in both fractions A7 and A9 and appeared as small peaks in FD, and signals appeared in A9 at δ 0.91 and 0.93ppm while these were absent from A7 and FD. Doublet signals at δ 1.08 and 1.09ppm in A7 and FD were absent in F9, while another doublet signal at δ 1.13-1.15 was clearly seen in A7 and was not present in A9 and FD. Moreover, a sharp singlet signal at δ1.195ppm in A7 and A9 was not detected in the crude FD. Doublet signals at δ1.27 and 1.29ppm detected in A7 and A9 were not detected in crude FD. Additionally, a signal at δ1.69ppm clearly appeared in crude FD, but was absent in both fractions A7 and A9. In addition, a doublet signal at δ1.92 and 1.93ppm clearly showed up in A9 and was not detected in A7 and FD. A signal at δ 1.945ppm in A7 was detected as a minute peak in A9 but was not detected in FD.

Signals between δ 2.04 and 2.15ppm were clearly detected in all the samples (A7 and A9 and crude FD). Furthermore, a signal at δ 3.29ppm appeared in fractions A7 and A9 and was absent in crude FD (Figure 3-10).

At position II (Figure 3-12) a doublet signal was noted at δ 6.61-6.62ppm detected in A9 and was not detected in A7 and crude FD. Furthermore, there was a signal at δ 7.35-7.54 ppm in A7 and A9 which was absent in crude FD.

A doublet signal at δ 7.8 and 7.82 ppm in A7 was absent in A9 and crude FD. Another doublet signal at δ 7.35 and 7.54 ppm in A9 was absent in A9 and crude FD.

The labels I and II on the spectra emphasise positions of differences between fractions A7 and A9 as compared to crude FD.

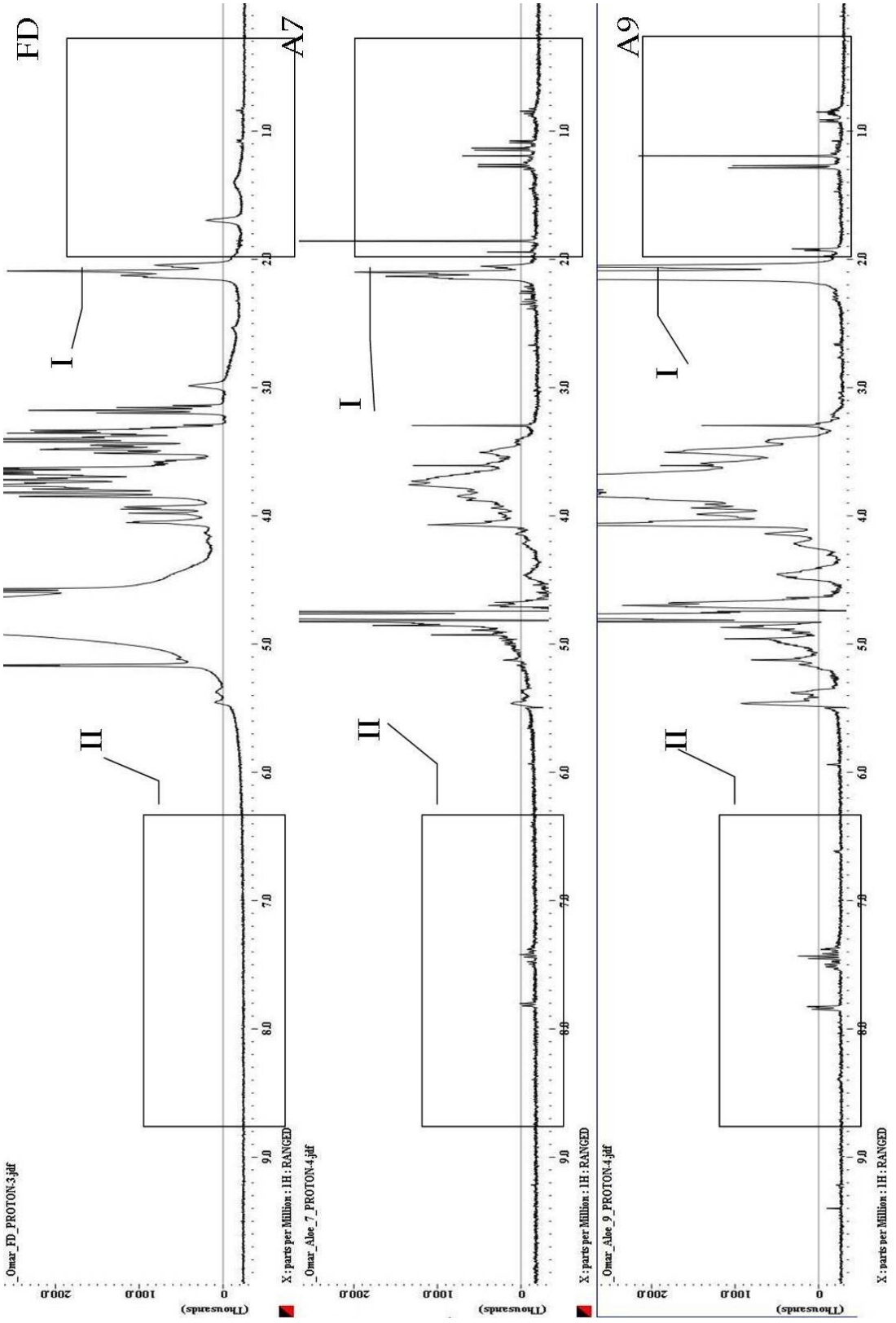


Figure 3-10 Full ^{1}H NMR spectra of crude Aloe vera FD and fractions A7 and A9 in D_2O (0-10ppm) (JEOL Delta GX 400 MHz).

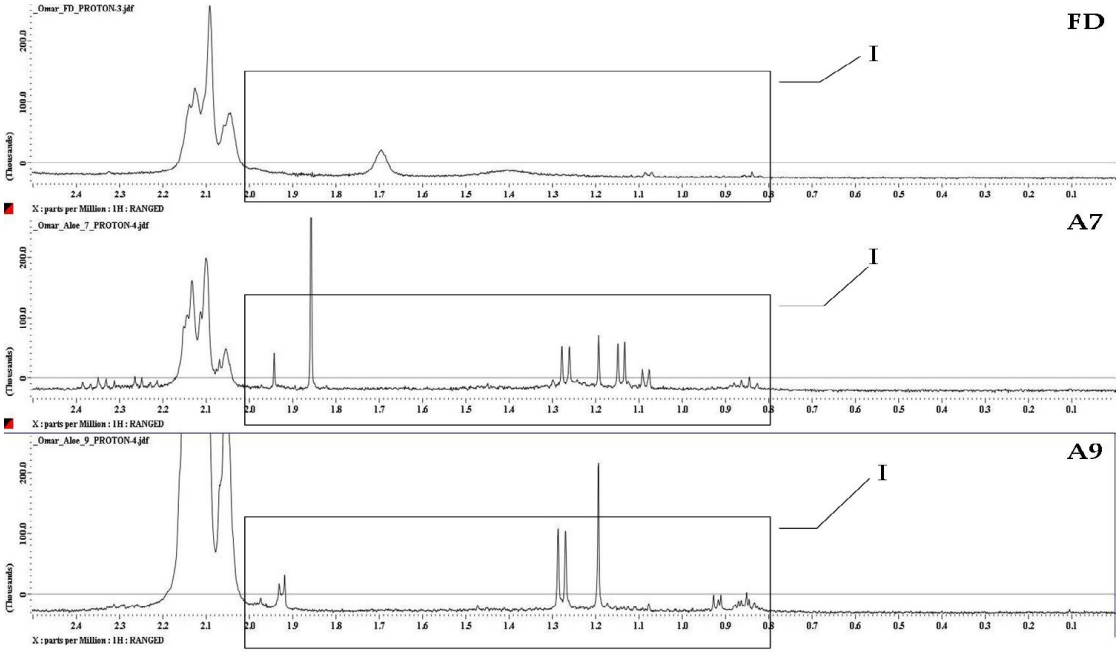


Figure 3-11 Expansion of ^{1}H NMR spectra of crude Aloe vera FD and fractions A7 and A9 in D_2O (0-2.5ppm) (JEOL Delta GX 400 MHz).

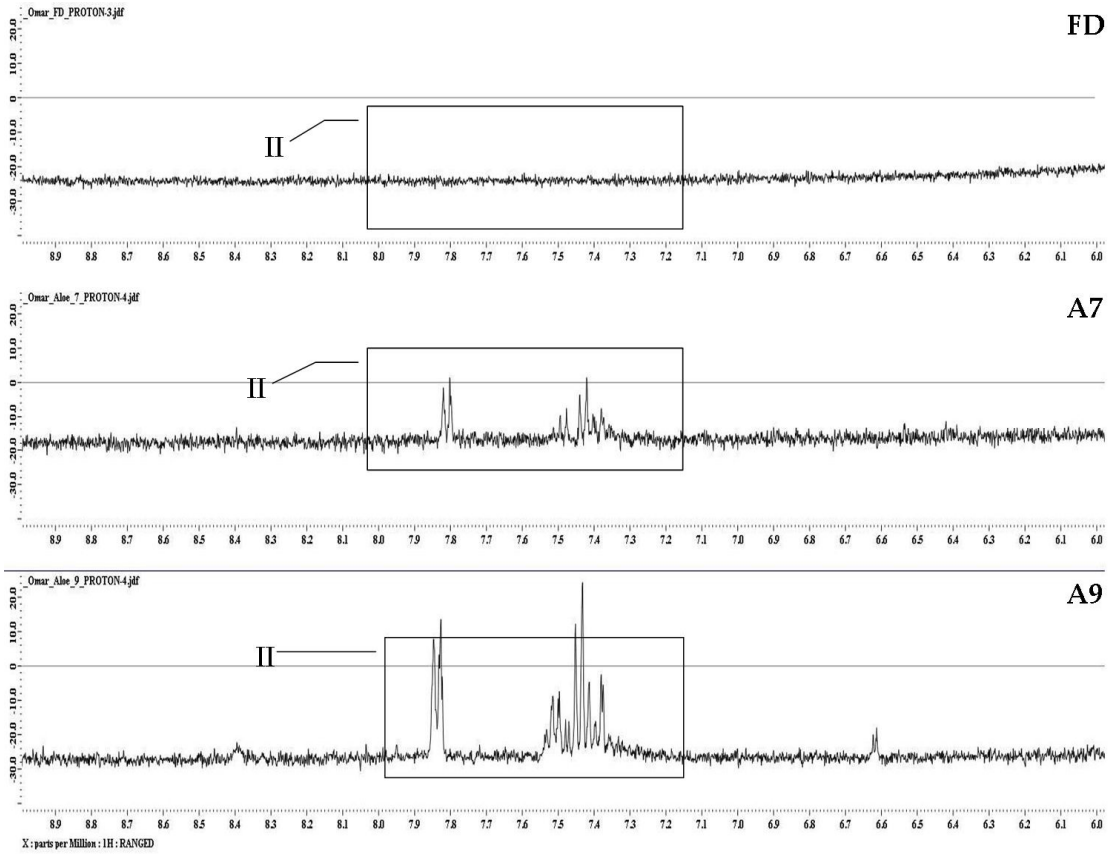


Figure 3-12 Expansion of ^{1}H NMR spectra of crude Aloe vera FD and fractions A7 and A9 in D_2O (6.0-8.9ppm) (JEOL Delta GX 400 MHz).

3.2. Biological Results

3.2.1. Cytotoxicity of Aloe vera

Crude FD did not show any toxic activity toward the THP-1 cells (results not shown).

3.2.2. Standard curve generated for of cytokine ELISA

A standard curve was set up for each experiment, which was used to calculate levels of cytokines in THP-1 cells in controls and treated cells. Figure 3-13 shows an example of a TNF-α standard curve used to determine the concentration of TNF-α produced by the THP-1 cells, stimulated by LPS. The curve was repeated for each experiment, and concentrations of TNF-α (pg/ml) were calculated from the regression curve.

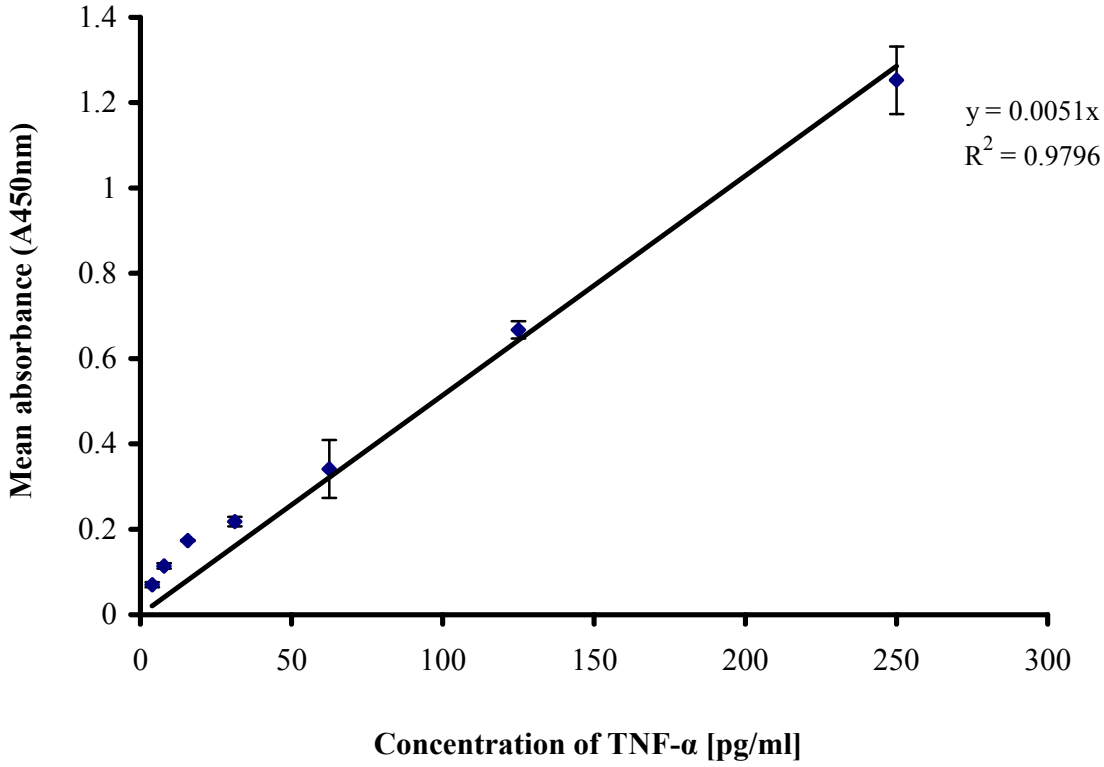


Figure 3-13 A typical standard curve of human TNF-α. Each point represents duplicate measurements of TNF-α standard read at 450 nm after subtracting the background reading at 570 nm ± S.D.

3.2.3. Comparison of the effect of crude FD and SD samples in TNF-α production from LPS stimulated THP-1 cells

Figure 3-14 shows the production of TNF-α by THP-1 cells stimulated with LPS in the presence and absence of crude Aloe vera inner gel (1mg/ml) FD and SD. FD and SD significantly ($p \leq 0.0001$) reduced the amount of TNF-α from 76±1.78pg/ml for cells with LPS to 58±3.24pg/ml and 54±2.9pg/mg, respectively. This indicates that Aloe vera inner gel with two different physical drying processes has similar anti-inflammatory activities.

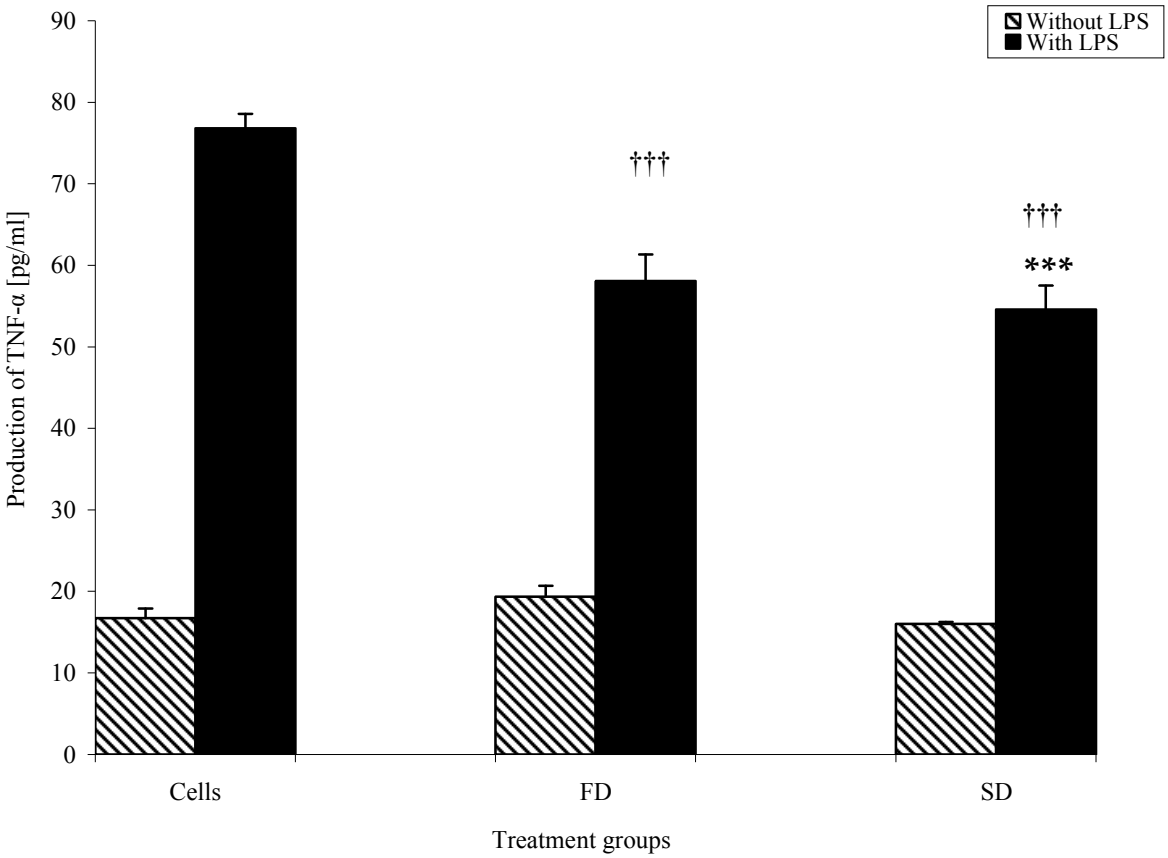


Figure 3-14 TNF-α production from THP-1 cells in the presences and absences of LPS and FD and SD Aloe vera. Each bar represents the mean of triplicate measurements ±SD and at least three different experiments were conducted. *** indicates significantly ($p \leq 0.001$) lower values compared with the cells+LPS control. ††† indicates significantly ($p \leq 0.0001$) higher values compared with the cells only control.

3.2.4. TNF-α production from LPS stimulated THP-1 cells in presence of crude FD and fractions A1-9

FD was separated by Flash chromatography into nine fractions A1-A9. Figure 3-15 shows the production of TNF-α by THP-1 cells stimulated with LPS in the presence and absence of crude FD (1mg/ml) and its fractions (A1-A9). The crude Aloe vera inner gel exhibited a significant ($p \leq 0.01$) decrease in the production of TNF-α, 893±56pg/ml as compared to THP-1 cells plus LPS, 1369±57pg/ml (Figure 3-15). Fractions A7 and A9 showed more significant reduction than all of the separated fractions (1008±12 and 1090±103pg/ml, $p \leq 0.01$ respectively), followed by fraction A2 (1099±12pg/ml, $p \leq 0.01$)

Fractions A3, A4 and A5 showed slight reduction in TNF-α from 1369±57 pg/ml to 1195±82.18, 1138±35.8, and 1275±3.75pg/ml, respectively. Fractions A4, A5 and A6 showed pro-inflammatory properties by increased TNF-α production compared with THP-1 cells alone from 56±3.44pg/ml to 415±26.56, 741±17.18 and 1893± 1.56pg/ml, $p \leq 0.001$, respectively.

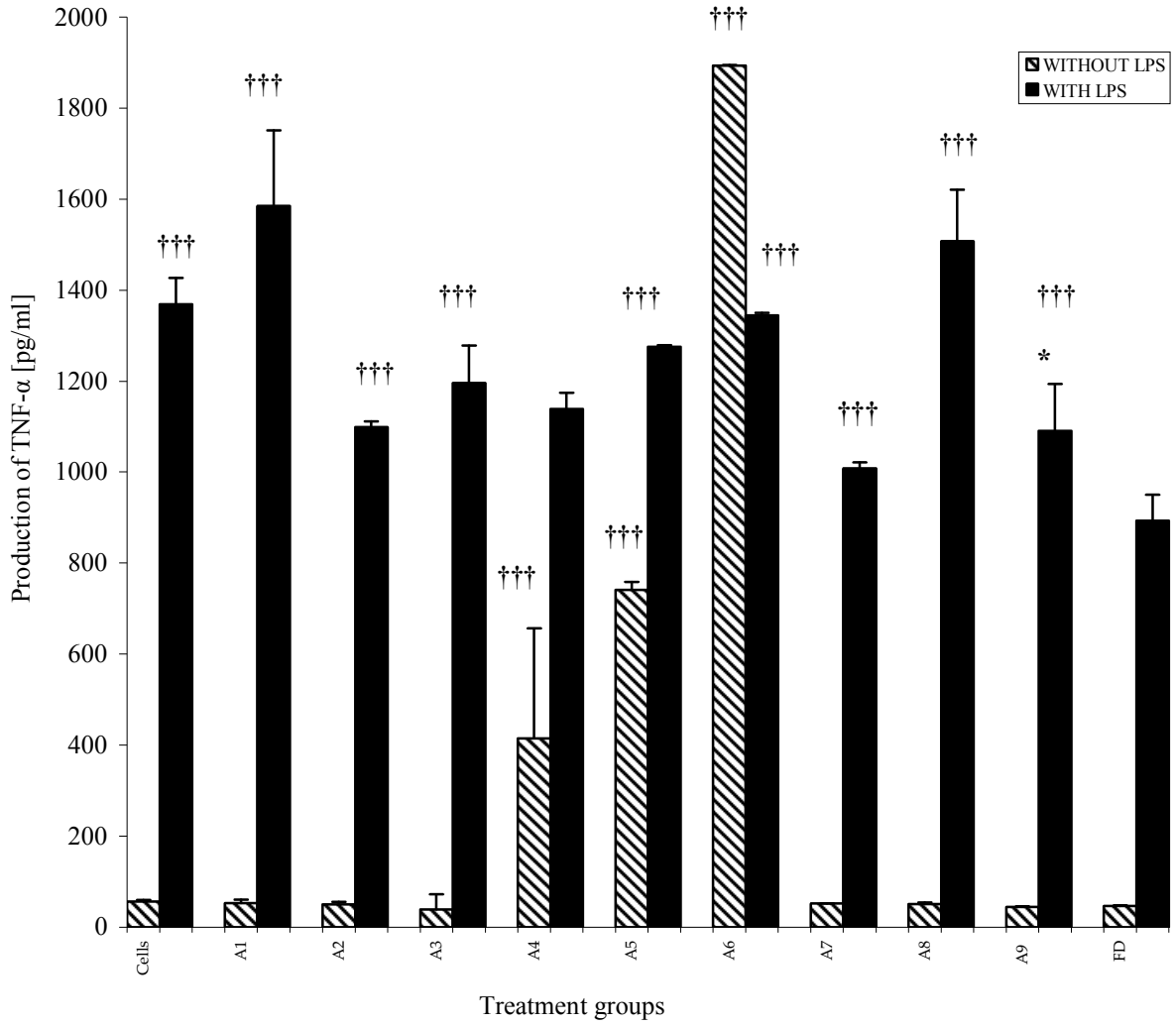


Figure 3-15 TNF-α production from THP-1 cells in the presences and absences of LPS and crude FD Aloe vera inner gel compared with fractions A1-A9. Each bar represents the mean of triplicate measurements ±SD and n=3, the concentration was then calculated from the standard curve. * indicates significantly ($p \leq 0.05$) lower values compared with the cells + LPS control. ††† indicates significantly ($p \leq 0.0001$) higher values compared with the cells only control.

3.2.5. IL-1β production from LPS stimulated THP-1 cells in presence of crude FD and fractions A1-9

Figure 3-16 shows the production of IL-1β by THP-1 cells stimulated with LPS in the presence and absence of crude Aloe vera inner gel (1mg/ml) and fractions A1 to A9. The crude FD exhibited a significant ($p \leq 0.0001$) decrease in the production of IL-1β 404.76±21.06pg/ml, as compared to THP-1 cells plus LPS 570.95±25pg/ml. Fractions A2, A4, A6, A7, A8 and A9 showed a more significant decrease in production of IL-1β than all the fractions (322.22±8.14, 420.95±9.37, 407.30±7.29, 317.3±46.94, 332.06±79.73 and 278.10±0.95pg/ml respectively, $p \leq 0.001$). Fraction A3 showed a slight significant reduction in IL-1β (433.81±47.23pg/ml, $p \leq 0.001$). Conversely, fractions A4, A5 and A6 showed pro-inflammatory properties with increased production of IL-1β compared to THP-1 cells alone from 43.65±2.2pg/ml to 165.87± 44.8, 242±55 and 343.02± 84.45pg/ml, $p \leq 0.001$, respectively.

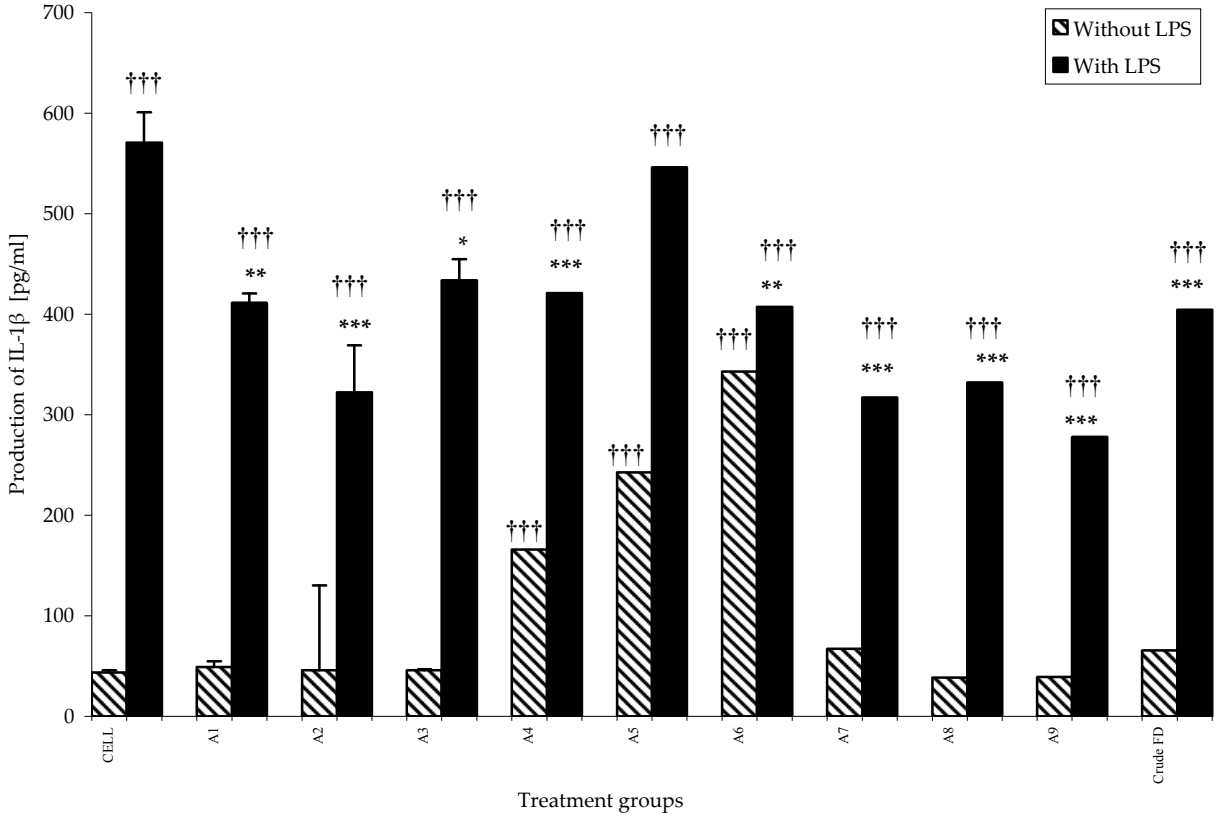


Figure 3-16 IL-1β production from THP-1 cells in the presences and absences of LPS and crude FD Aloe vera inner gel compared with fractions A1-A9. Each bar represents the mean of triplicate measurements ±SD and at least 3 different experiments were conducted. * indicate (p≤0.05), ** indicate (p≤0.01) *** indicate (p≤0.001) lower values compared with the cells +LPS control. ††† indicates significantly (p≤0.0001) higher values compared with the cells only control.

4. Discussion

Aloe vera inner gel was chosen in this study based on its folkloric uses and it has been reported in numerous *in vivo*, *in vitro* and clinical studies, for its anti-inflammatory activity (Reynolds and Dweck, 1999). These activities reflect the presence of several biologically active substances in the inner gel.

The main purpose of this study was carried out firstly to compare the chemical profiles of two preparations of Aloe vera inner gel FD and SD, to see if processing affects composition of compounds and secondly to relate this to therapeutic activity. Evaluation of the anti-inflammatory activity of crude freeze-dried and spray-dried of inner gel was carried out but due to time limits of this study only crude FD was fractioned to isolate compounds responsible for anti-inflammatory activity.

Aloe vera inner gel is commonly prepared by spray-drying and freeze-drying processes. It is conceived that the spray-drying process uses high heat exposure that can change some potentially valuable ingredients. The freeze-drying process or lyophilization uses cold temperature and vacuum. This leads to evaporation and sublimation of only the water in aloe juice. The benefit of this technique is that heat induced changes are avoided (Meadows, 1983). Therefore, the main purpose of this study was to determine if there was a difference between the FD and SD processes in terms of chemical and biological activity. Firstly to compare the chemical profiles of two preparations of Aloe vera inner gel FD and SD, to see if processing affects composition of compounds and secondly to relate this to therapeutic activity.

The study set out to look at the role of these Aloe vera extracts on the production of the pro-inflammatory cytokines TNF-α and IL-1β. They were chosen due to their importance in inflammation and because they are produced in large amounts by THP-1 cells when stimulated with LPS, as also do macrophages *in vivo (*Habeeb *et al.*, *2007)*; TNF-α promotes inflammation through a number of different pathways including NF-κB leading to the production of other pro-inflammatory cytokines such as IL-1β and IFN-γ.

In the assays, if TNF-α or IL-1β production increased in the absence of LPS, the plant sample was classed as pro-inflammatory and if TNF-α production was inhibited in presence of LPS, the plant samples was anti-inflammatory.

In the present study prior to testing the anti-inflammatory effects of crude FD, an AlamarBlue™ cytotoxicity assay was carried out to assess any cytotoxic properties of Aloe vera. Crude Aloe vera inner gel was non-toxic to THP-1 cells and this finding is in agreement with previous work by Stables, (2004), who had used the same assay to determine cytotoxicity and reported, that the whole Aloe vera inner gel, aqueous and methanol extracts were not cytotoxic at any concentration (Stables, 2004) . However, Winters *et al.*, (1981) demonstrated that a lectin-like substance in Aloe vera gel fractions from commercial products were cytotoxic to both normal human and tumor cells (Boudreau and Beland, 2006) although it is unknown at what concentration.

4.1. Chemical comparison of crude of FD and SD of Aloe vera inner gel.

TLC was carried out prior to fractionation by Flash chromatography. TLC analysis of FD and SD samples was carried out using reverse phase TLC plates (Figure 3-1). The three bands observed for both samples were virtually identical by R*f* values. Therefore it was concluded that the same solvent system could be used.

Proton NMR spectra of crude samples showed significant signals which indicate the differences between FD and SD Aloe vera (Figures 3-3 and 3-4, respectively). In general the chemical shift was similar and had the same patterns, but there were some variations in these spectra as indicated by some signals appearing in SD and absent in FD, a possible explanation for these variations might be related to chemical changes.

In the current study, a glucose signal was found at δ5.16 and δ4.57ppm, acetyl groups of acemannan generate a characteristic set of signals around 2.00–2.26ppm and malic acid δ2.5-2.8 and δ3-4.4 (Figures 3-3, 3-4 and Table 3-2) as compared with the Bozzi, *et al*. (2007) spectra (Figure 3-9). According to Bozzi, *et al*. (2007) the presence of glucose, acemannan and malic acid was used as an indication of the quality of Aloe vera inner gel samples. Since acemannan is the major polysaccharide present in Aloe vera gel powders, it can be considered as the fingerprint of the plant (Diehl and Teichmüller, 1998)

Since these samples were still in crude form, a clear and sharp signal in ^{1}HNMR spectra could not be seen to elucidate the chemical structures. Similarly, COSY

NMR spectra was carried out but produced noisy signals (results not shown), therefore further purification using advanced chromatographic techniques are required starting with FD.

4.2. Biological comparison of crude freeze-dried and spray-dried Aloe vera inner gel

TNF-α ELISA carried out for both FD and SD showed significant reduction of TNF-α. There was little difference between both samples which was significant. Since there was little difference in biological activity, it remained that the chemical composition of the two samples should be further investigated and this was began with FD fractionation. There are variations were observed in all the ELISA results. In addition, different repeats of the same experiment at different times during the year showed some variation such as (figures 3-14 and 3-15). Variations in experimental controls might be attributed to a decrease either in the activity of LPS in stimulating THP-1 cells to release the cytokines or even THP-1 cell variation of different passages and/or prolonged sub-culturing. Nevertheless, we attempted to overcome this by using fresh ELISA kits, LPS and THP-1 cells and so some differences were minimized.

4.3. Fractionation of crude FD Aloe vera inner gel

TLC was carried out prior to fractionation by Flash chromatography. Fractionation was carried out using flash chromatography using mixtures of methanol/water. In addition, TLC screening of the extracted fractions was used to monitor the progress of elution from the column during the separation process (Figure 3-2). 100 fractions were obtained; these fractions were collected and similar ones combined together.

The pooled fractions resulted in nine new fractions labeled A1-A9. All these fractions were collected and pooled and the solvents dried off, and any remaining water was lyophilized. During this freeze drying step, all these fractions exhibited variations in yields and notable differences in physical appearance were attributed to differences in the chemical ingredients of each of them. A precipitate formed in different fractions were labeled A1-A9 which was used in further bioassays and ^{1}HNMR investigation. In terms of fraction yields obtained there were significant differences in percentage of fractions yields (Table 3-1). These variations might be attributed to the existence of various components in different ratios.

Further ^{1}HNMR investigation was done on these fractions to elucidate the identity of its compounds. Fractions A7 and A9 were examined as they had anti-inflammatory activities. Non active fractions or pro-inflammatory fractions were not investigated due to shortage of time, but they were kept for further investigation.

According to the NMR spectra of these fractions they showed no sharp signals, which indicate that they might be not pure ingredient therefore it is recommended that further sub-fractionation or use of another suitable chromatography technique is required in order to determine the components attributed to the biological activities.

^{1}HNMR spectra of fractions A7 and A9 (Figure 3-10), showed differences compared with crude FD Aloe vera, particularly in the aliphatic region at position (I) and aromatic region at position (II) (Figures 3-11 and 3-12

respectively). Peaks appeared in fractions A7 and A9 that were absent in the crude extract especially in the aromatic region at position II (Figure 3-12).

It is difficult to explain this result, but it might be related to the compounds responsible for enhancing the anti-inflammatory activity of these fractions. In addition the presence of other compounds exerting an additional activity or in synergism with those ones, could lead to the overall significant biological activity of these fractions compared to the crude Aloe vera FD inner gel (Reynolds, 2004).

Based on ^{1}H NMR spectra, no sharp signals were appeared which could be related to the nature or the ingredients of these fractions as they might not be in single or pure form. Further NMR investigation using ^{13}C NMR and COSY for fraction A7 showed nose spectra which indicate that they have many components as shown in Appendix 5.1 and 5.2. Subsequently, further purification and sub-fractionation of these fractions is required before re-testing in a bioassay. In addition, these variations might correspond to variable concentrations of active substances in these fractions after Flash chromatography extraction, compared to crude FD, which may lead to concentration of the active ingredients in these fractions compared with its presence in minute amounts in crude FD. This is in agreement with Atherton (1998) as he reported that Aloe vera is comprised of approximately 99% water, all of these chemicals are contained in the remaining 1% of the plant. He also claimed that there are a small percentage of individual ingredients, although there are many ingredients.

4.4. Biological activity of the fractions of crude FD

Crude FD and fractions (A1-A9) were evaluated for release or suppression of TNF-α and IL-1β from THP-1 cells stimulated by LPS. The results, showed fractions A7, and A9 with the highest decrease in production of TNF-α compared with FD crude and its fractions. On the other hand fractions A2, A3, A7, A8 and A9 exhibit significant decrease in IL-1β compared with crude FD and the other fractions.

Fractions A4, A5 and A6 showed significant increase in the level of both TNF-α and IL-1β compared with the cells only control. This pro-inflammatory effect could be related to the immune-stimulatory activity of Aloe vera. In agreement with Stables, (2004) Aloe vera inner gel possesses compounds that are both pro-inflammatory and anti-inflammatory, by affecting the NF–κB pathway. Acemannan has been shown to promote maturation in chicken macrophages (Djeraba and Quere, 2000) and maturation of dendritic cells (Lee *et al.*, 2001). Pugh *et al.*, (2001) isolated pure Aloe vera components called aloeride, high molecular weight polysaccharides which possess an immunostimulatory activity and were shown to increase NF-kappa B in THP-1 cells to high levels compared with those of LPS. Aloeride induced the expression of the mRNAs encoding IL-1β and TNF-α to levels equal to those observed in cells maximally activated by LPS and notably it was more immunostimulatory than acemannan (Pugh *et al.*, 2001). Leung *et al.*, (2004) demonstrated that three different polysaccharide fractions stimulated peritoneal macrophages, splenic T- and B-cells proliferation, and activated them to secrete TNF-α, IL-1β, INF-γ, IL-2 and IL-6 (Leung *et al.*, 2004).

This controversial effect of Aloe vera as anti or pro-inflammatory, was also shown by (Davis, 1991), whose work showed that Aloe vera inner gel had inhibitory and stimulatory activity at the same time. The variation of anti-inflammatory activity among those fractions may be correlated to differences in the presence of active agents in the inner gel (Reynolds and Dweck, 1999). However, this difference in reducing production of TNF-α and IL-1β may be attributed to variation in chemical compositions and anti-inflammatory properties. This suggests that the inhibitory affects of the fractions are not the same. Since fractions A7 and A9 demonstrated the most potent anti-inflammatory properties, therefore they should be investigated in more detail using another suitable chromatography techniques might be possible in order to finding pure active components and to determine related anti-inflammatory mechanism of action.

In this study differences in ^{1}HNMR spectra of both crude FD and SD are shown (Figures 3-3 and 3-4). Thereby these differences might be attributed to loss or degradation hydrolysis of some ingredients during the processing steps of the plant especially with the SD Aloe vera, in particular due to heat treatment during the drying process or upon storage.
In the present study using data of Bozzi, *et al.* (2007), as a reference guide similar profiles were obtained.

Nevertheless, it was not possible to detect and elucidate the chemical structures of all the fractions of Aloe vera. In order to determine the components, ChemBioDraw Ultra was used to draw all the possible chemical components and to predict ^{1}HNMR spectra for these components. These components were known to be present in Aloe vera (Dagne et al., 2000; Hamman, 2008; Shelton,

1991; Waller et al., 1978). From the literature these was compared with the original 1HNMR spectrum and are shown in Appendix 1-4.

Aloe vera has been analysed using several chromatographical techniques for fractionation, purification and size determination of its components. These include thin layer chromatography, high performance liquid chromatography (HPLC), mass spectrometry (MS) and liquid chromatography mass spectrometry (LCMS) and gas chromatography mass spectroscopy (GCMS) and nuclear magnetic resonance (NMR) spectroscopy.

Nine phenolic constituents of aloe vera (aloesin, 2'-O feruloylaloesin, aloeresin A, barbaloin, isobarbaloin, aloenin, aloe-emodin, 8-C-glucosyl-7-O-methyl-(S)-aloesol, isoaloeresin D and aloeresin E.), were elucidated by a reverse phase high-performance liquid chromatography (Okamura *et al.*, 1996). In addition, an other study by Dagne *et. al.*, (2000) demonstrated that reverse phase HPLC of the methanol extract of leaf exudate is the best method for establishing chemical profiles in aloe (Dagne *et al.*, 2000). Membrane fractionation and gel filtration HPLC were used to purified three different molecular weight polysaccharide fractions of aloe vera (Leung *et al.*, 2004). In addition, phenolic components were isolated from a methanolic extract of Aloe vera using a combination of column and TLC further by HPLC and electron-impact ionisation (EI) mass spectra (MS), were indicated that have potent antioxidant activity (Lee *et al.*, 2000b).

HPLC analysis using size exclusion chromatographic (SEC), has shown widely differing levels of mucopolysaccharides of a number of commercial preparations of aloe products (Ross *et al.*, 1997). Fractionation of methanolic extracts of aloe vera gel, were analyzed and further fractionated by reverse phase-HPLC (RP-

HPLC). Five fractions were identified using electrospray ionization (ESI) mass spectroscopy and shown to have antimicrobial activity (Cock, 2008).

Esua and Rauwald, (2006) used NMR spectroscopy, ESIMS, MALDITOF-MS and capillary electrophoresis to characterize three different novel maloyl glucans from Aloe vera gel. (veracylglucan A, veracylglucan B and veracylglucan C). Veracylglucan B was demonstrated to have potent anti-inflammatory and anti-proliferative effect (Esua and Rauwald, 2006). Five phytosterol components were isolated and identified from aloe vera gel using silica gel column chromatography; identified as lophenol, 24-methyl-lophenol, 24-ethyl-lophenol, cycloartanol, and 24-methylene-cycloartanol *(*Tanaka *et al.*, 2006).

Recently, Lawrence *et al.*, (2009) using silica gel thin layer and column chromatography isolated components from methanolic and ethanolic extracts of Aloe vera were identified on the basis of gas chromatography mass spectrometry (GC-MS) (Lawrence *et al.*, 2009). So there could be use in future investigations

5. Conclusion

The present study was designed to compare chemical and anti-inflammatory activity of freeze-dried and spray-dried Aloe vera inner gel and observe if there is any relationship between applying the heat treatment during drying process and biological activity. Only FD Aloe vera was fractionated due to shortage of time, but to complete the study of SD requires similar treatment, but using further chromatographic analysis to help in elucidation of the individual components.

This study has demonstrated that Aloe vera possesses anti-inflammatory activity. Additionally, it was also shown that some of the fractions possess pro-inflammatory effects.

The actual compound structures could not be identified so it was not possible to say which compound is responsible for the activity. If in the future it is possible to identify the compounds and the difference between FD and SD, then the compounds can be studied further in *in vivo* and clinical studies.

6. References

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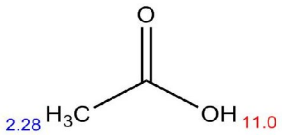
Appendices

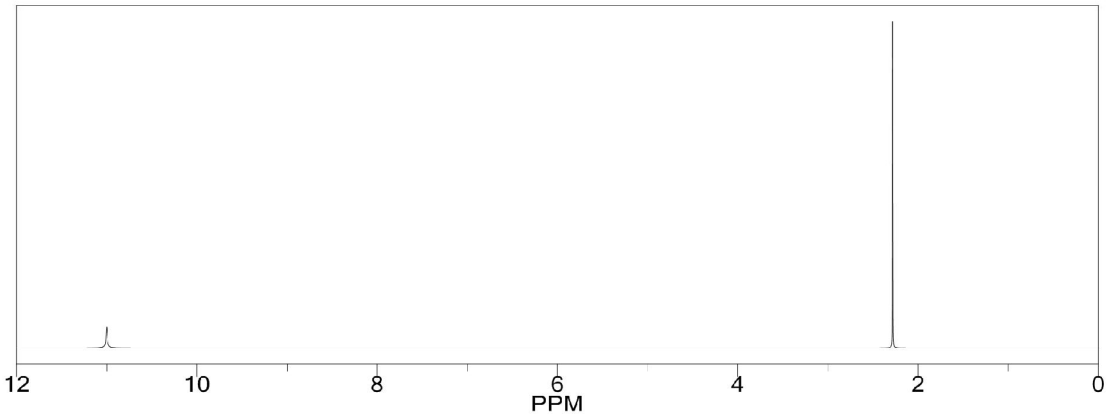
Components present in *Aloe vera* inner gel according to the literature (Dagne et al., 2000; Hamman, 2008; Shelton, 1991; Waller et al., 1978).

Appendix 1. Organic acid

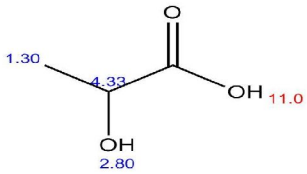
1. ^{1}HNMR of acetic acid simulation using **chemBioDraw** v*ersion* 11.0 (2008)



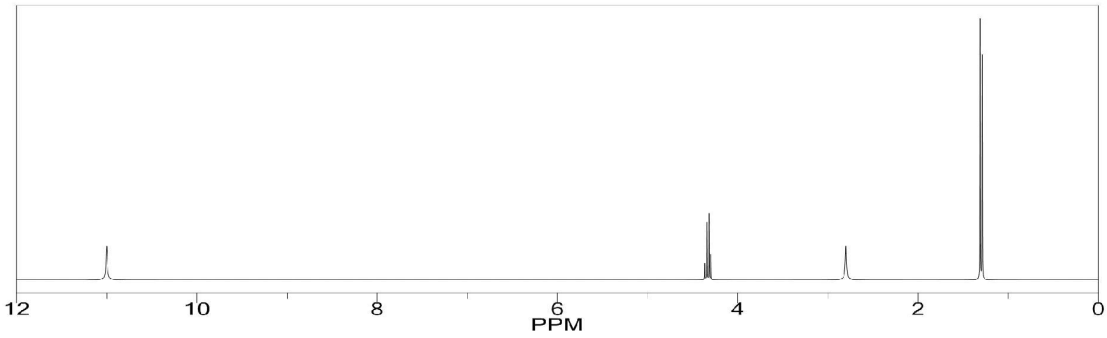
Acetic acid



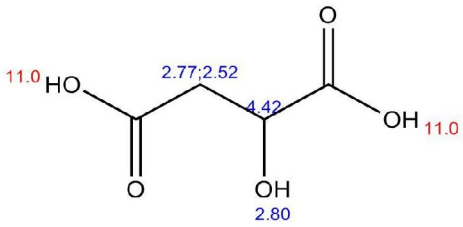
2. [1]HNMR of lactic acid simulation using **chemBioDraw** v*ersion* 11.0 (2008)



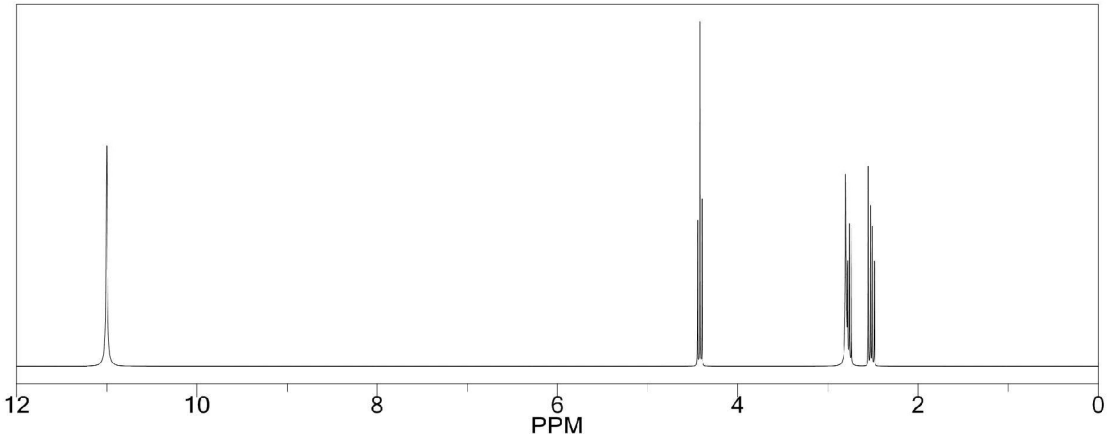
Lactic acid



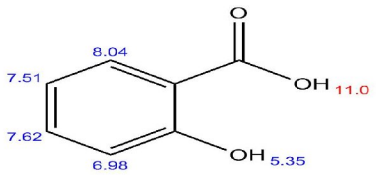
3. [1]HNMR of malic acid simulation using **chemBioDraw** v*ersion* 11.0 (2008)



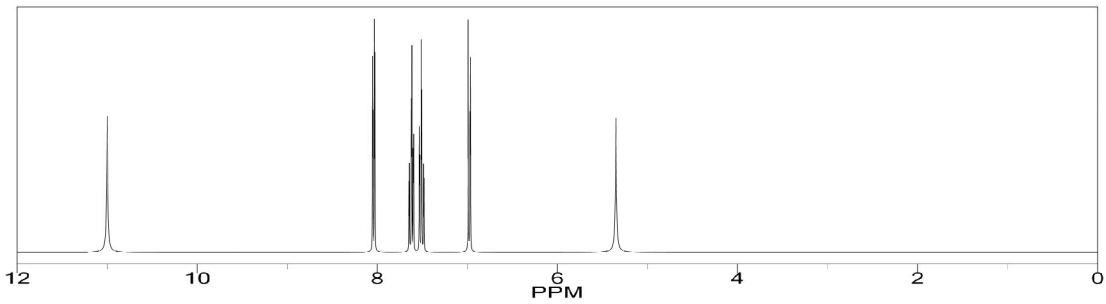
Malic acid



4. [1]HNMR of salicylic acid simulation using **chemBioDraw** v*ersion* 11.0 (2008)



Salicylic acid



Appendix 2. Amino acids

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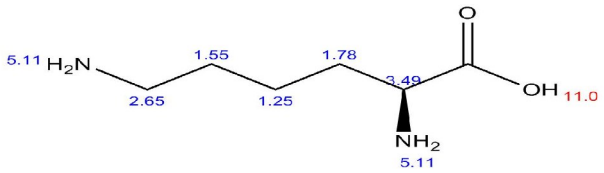
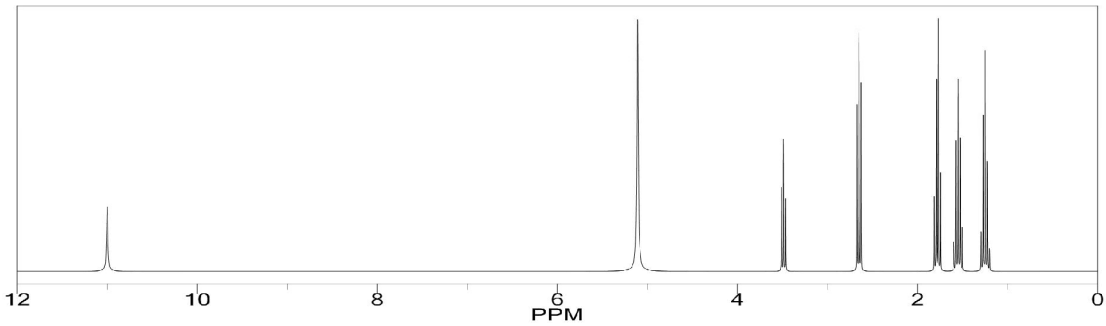


Lysine



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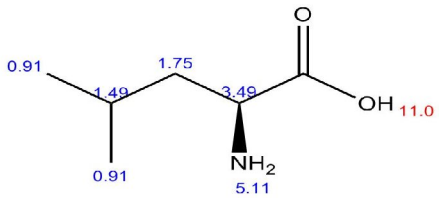
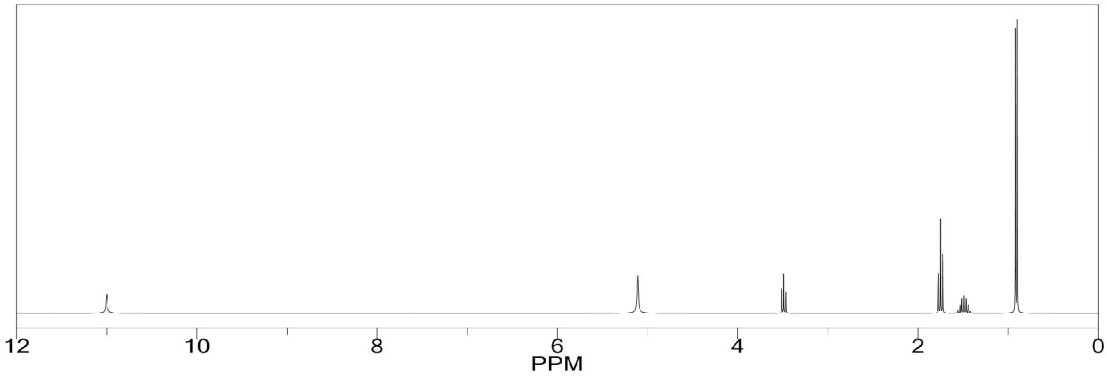


Leucine



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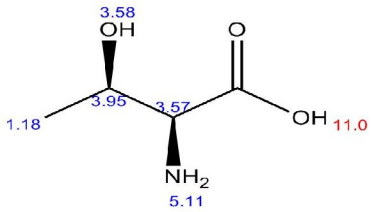
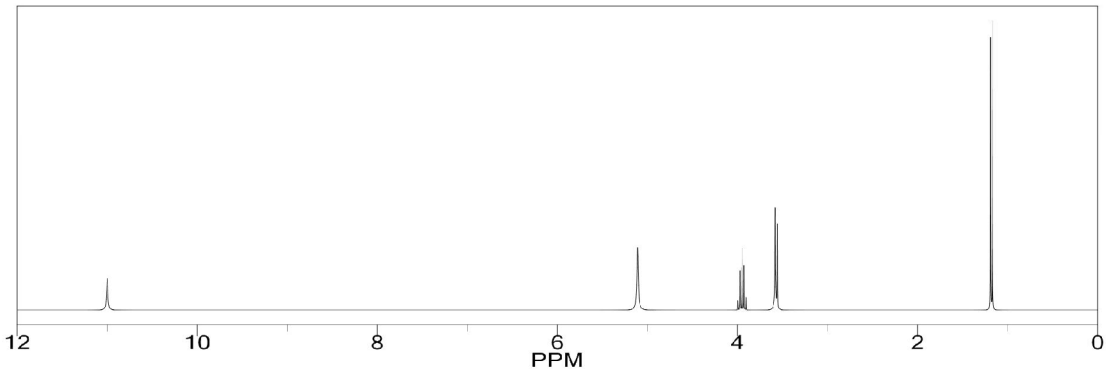


Threonine



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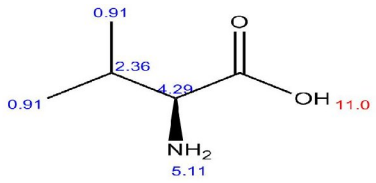
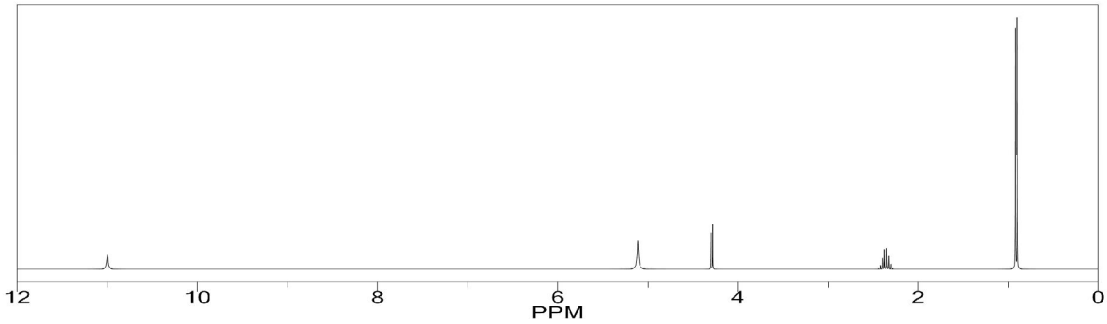


Valine



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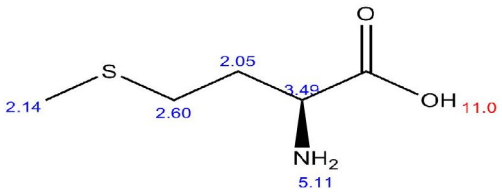
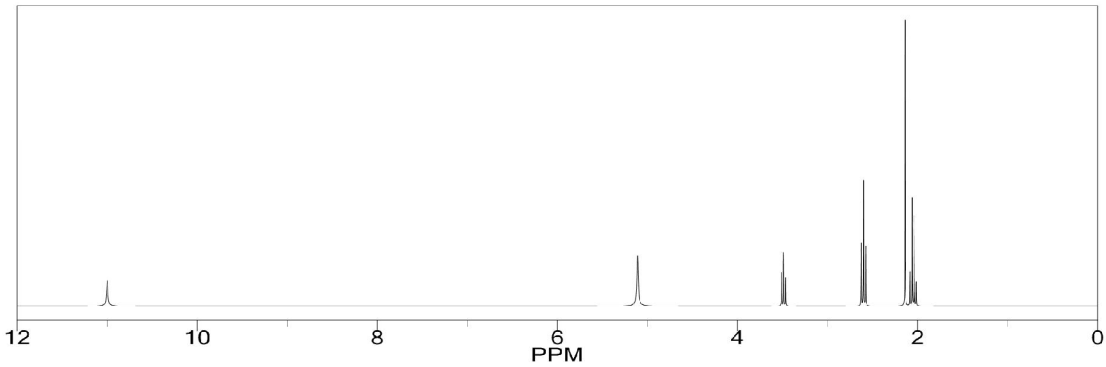
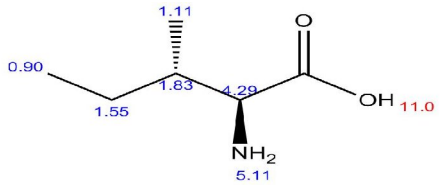


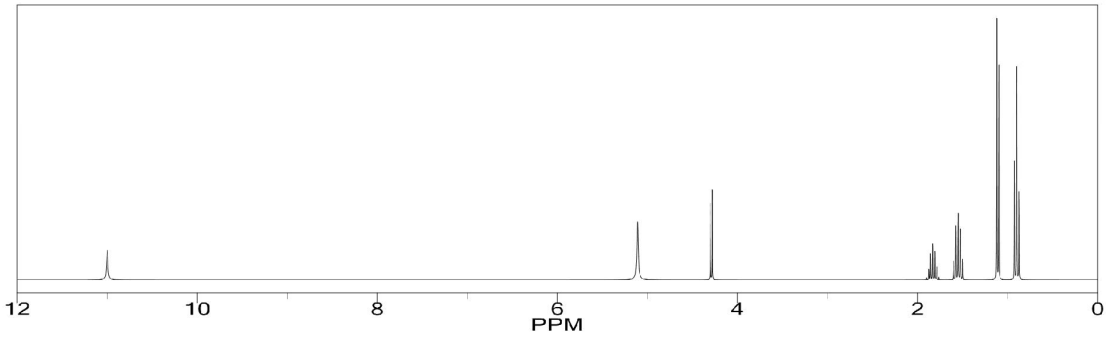
Methionine



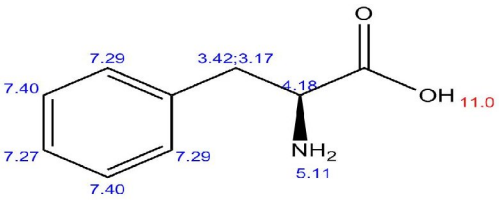
6. [1]HNMR of isoleucine simulation using **chemBioDraw** *version* 11.0 (2008)



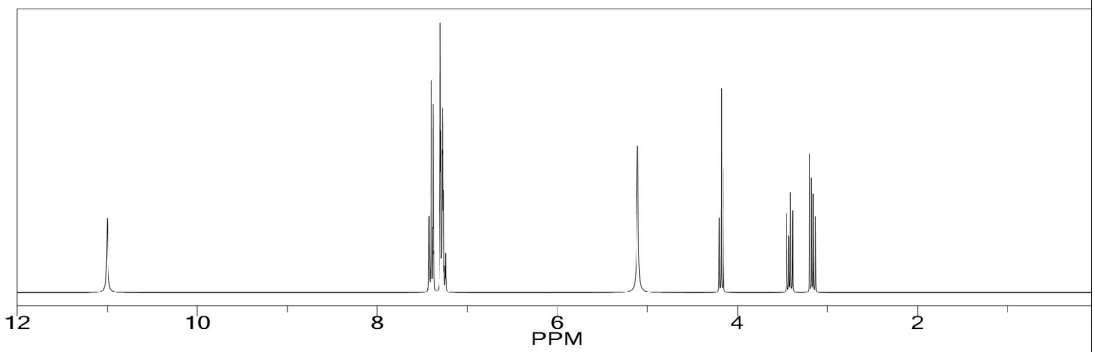
Isoleucine



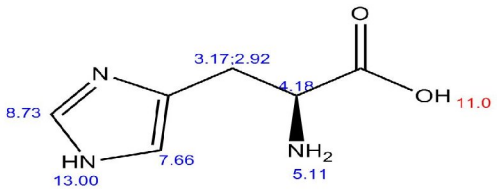
7. [1]HNMR of phenylalanine simulation using **chemBioDraw** *version* 11.0 (2008)



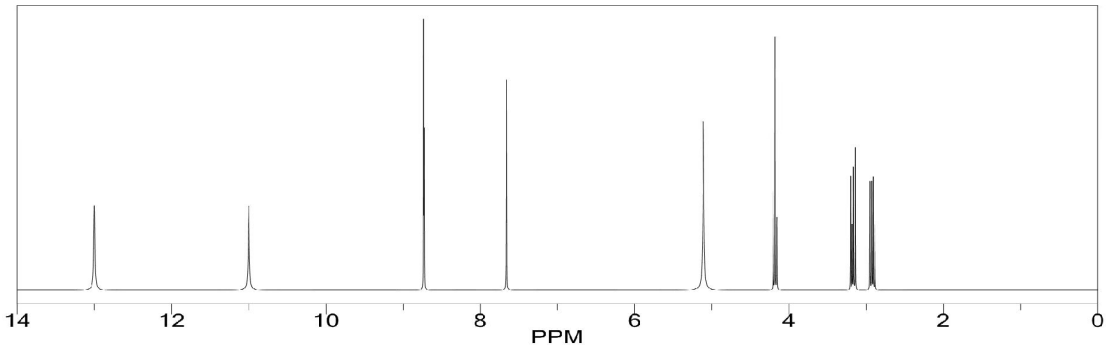
Phenylalanine



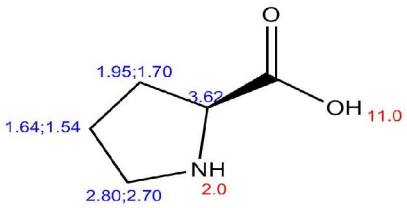
8. [1]HNMR of histidine simulation using **chemBioDraw** *version* 11.0 (2008)



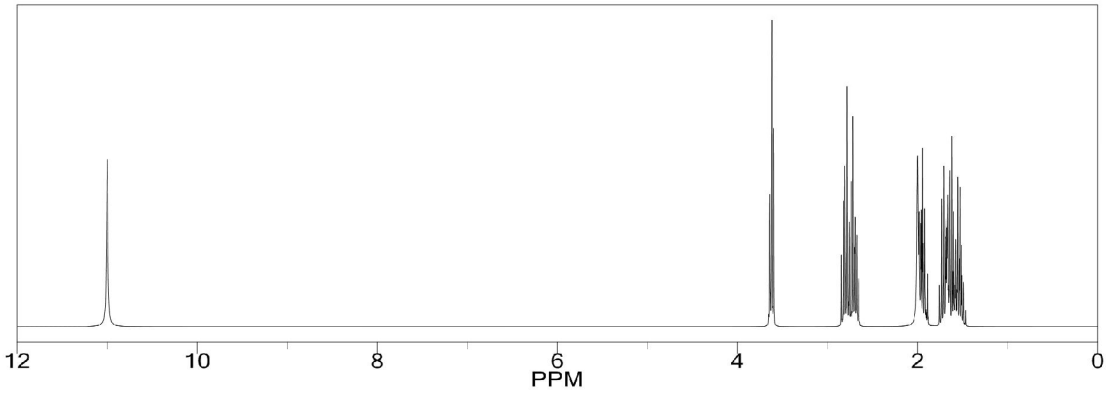
Histidine



9. [1]HNMR of proline simulation using **chemBioDraw** *version* 11.0 (2008)



Proline



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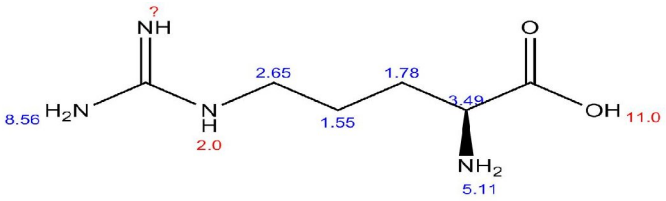
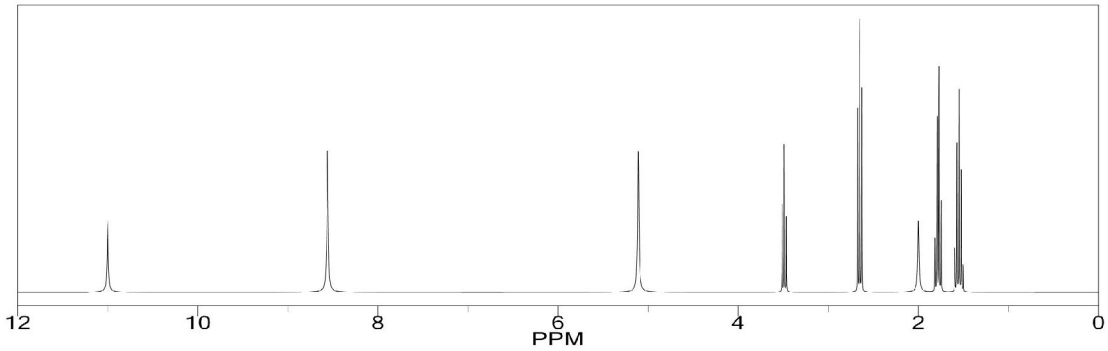
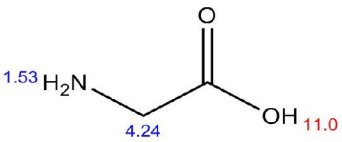


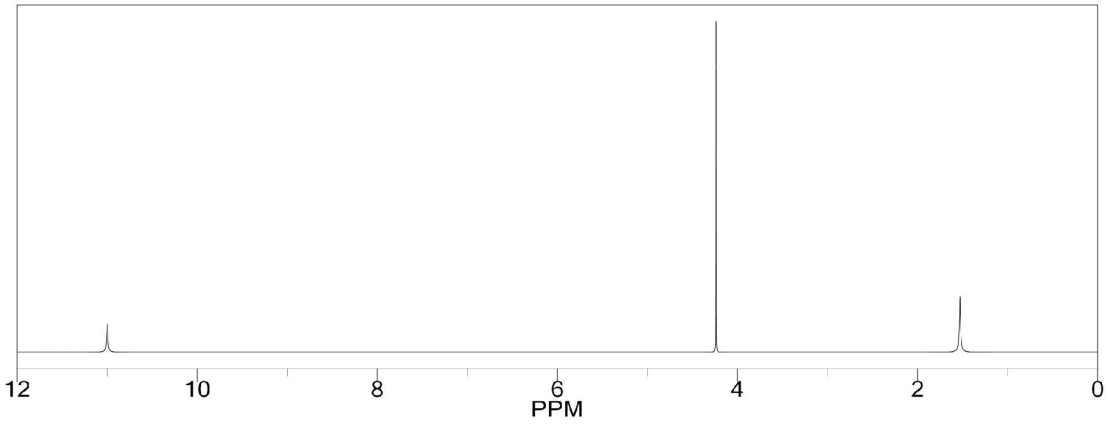
Arginine



11. ^{1}HNMR of glycerine simulation using **chemBioDraw** v*ersion* 11.0 (2008)



Glycine



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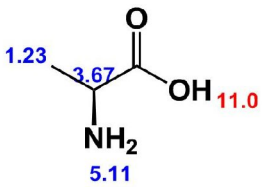
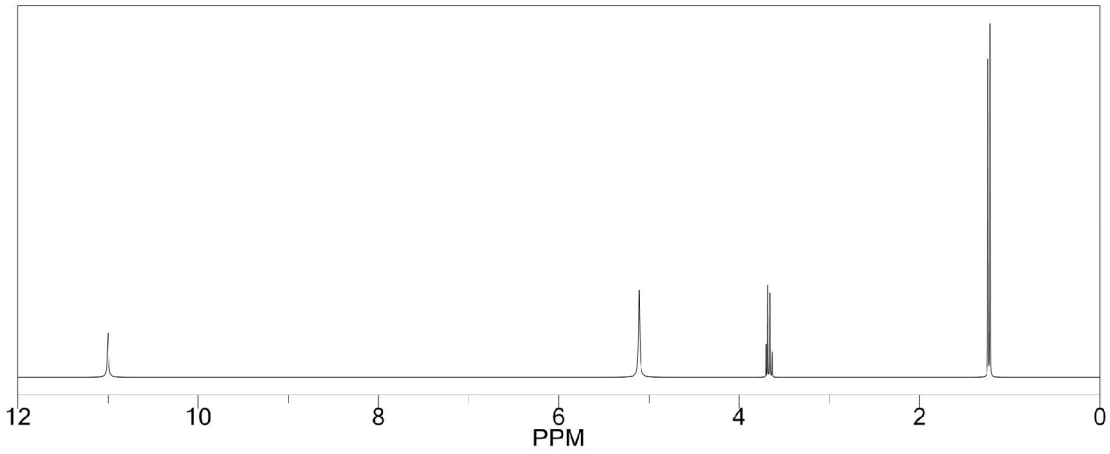


Alanine



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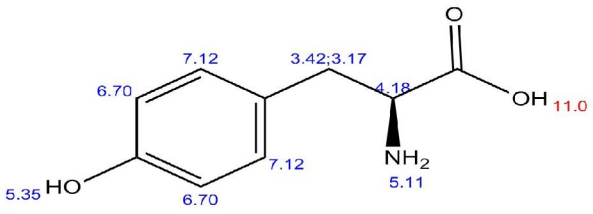
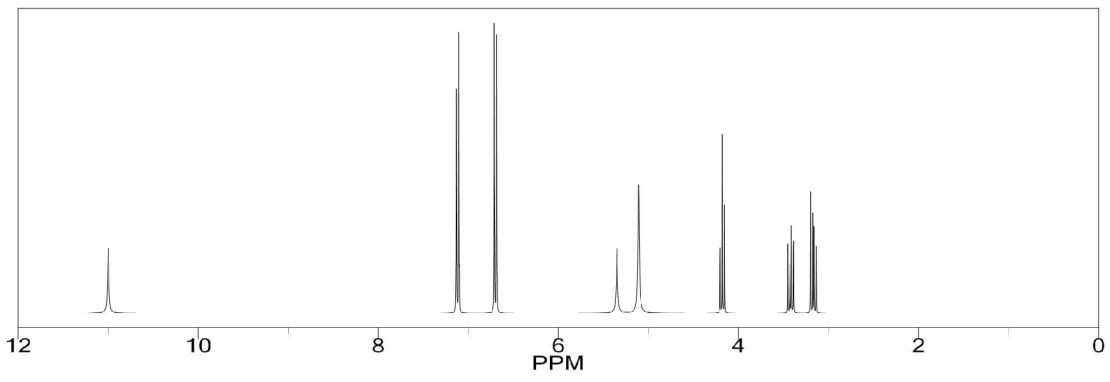
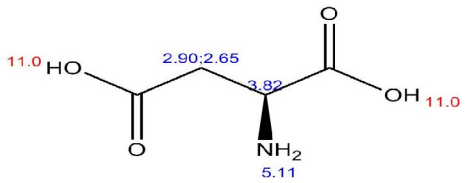


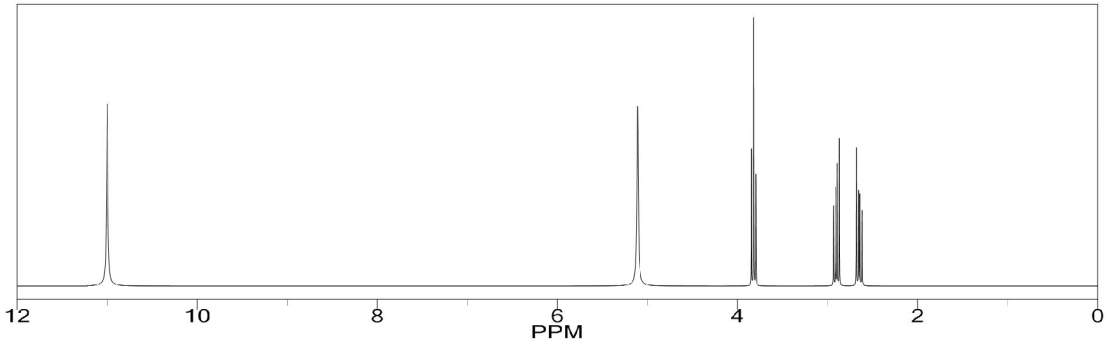
Tyrosine



14. [1]HNMR of aspartic acid simulation using **chemBioDraw** v*ersion* 11.0 (2008



Aspartic acid



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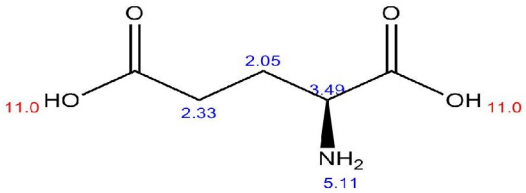
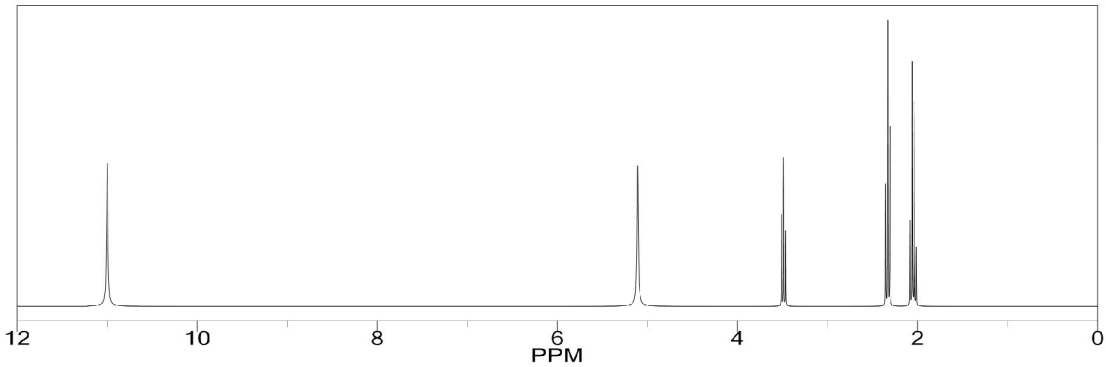
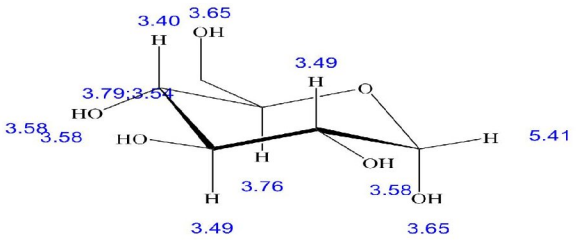


Glutamic acid

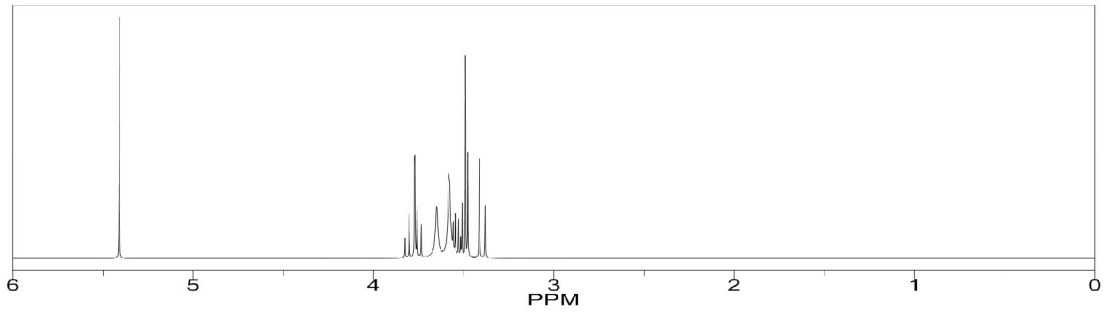


Appendix 3. Sugars

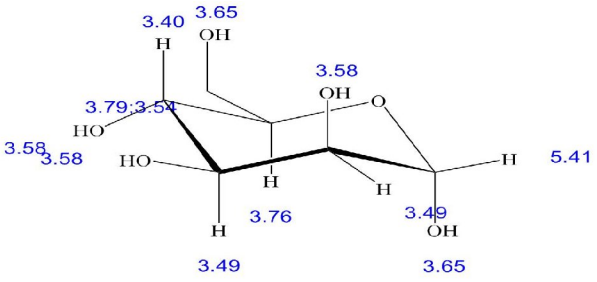
1. [1]HNMR of glucose simulation using chemBioDraw v*ersion* 11.0 (2008)



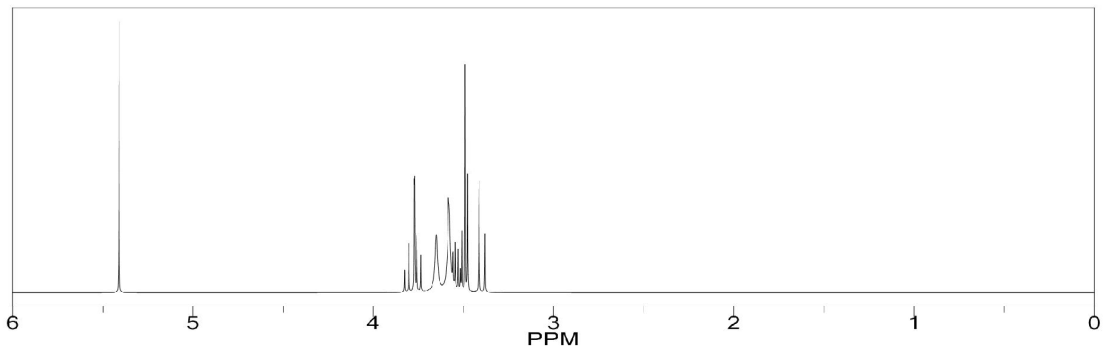
Glucose



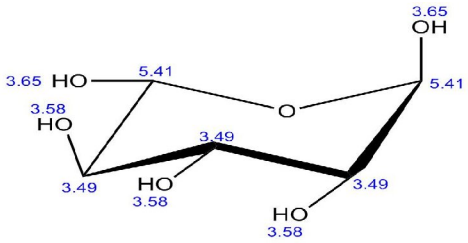
2. [1]HNMR of mannose simulation using **chemBioDraw** v*ersion* 11.0 (2008)



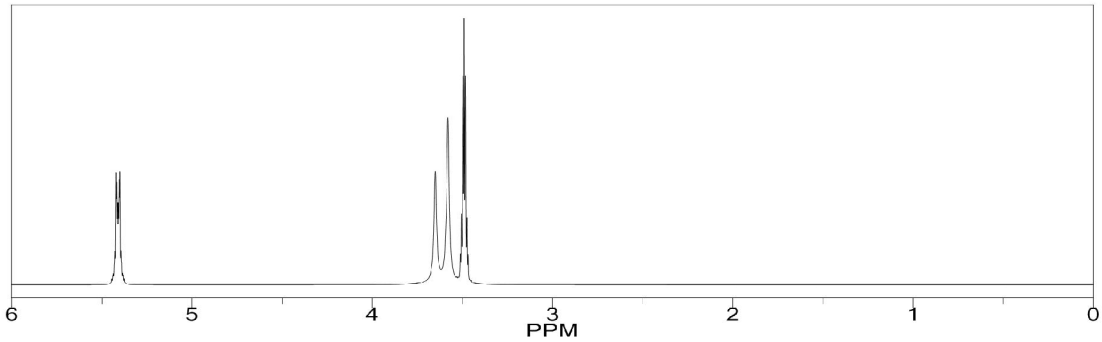
Mannose



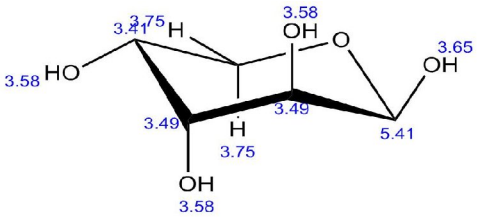
3. [1]HNMR of rhamnose simulation using **chemBioDraw** v*ersion* 11.0 (2008)



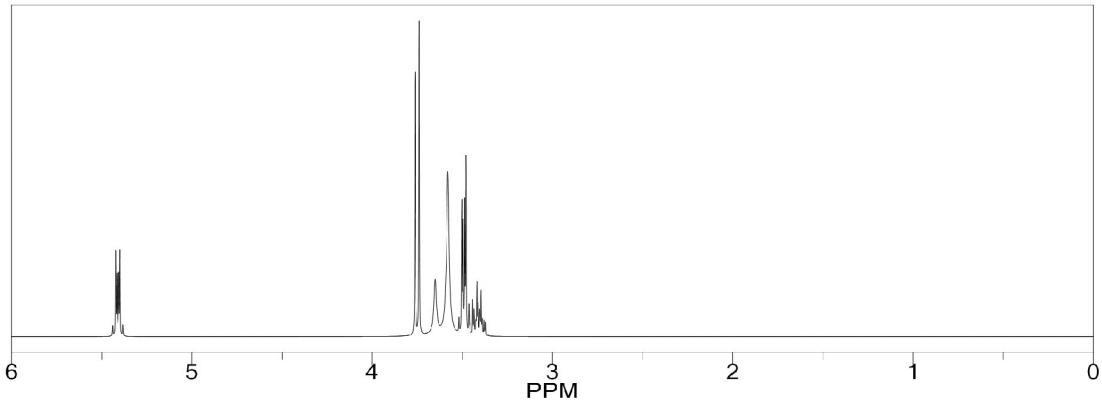
Rhamnose



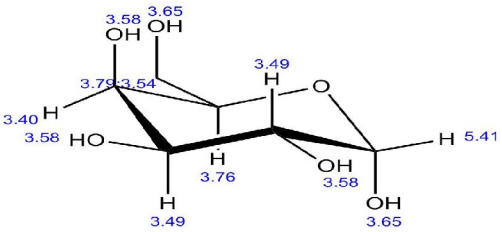
4. [1]HNMR of arabinose simulation using **chemBioDraw** v*ersion* 11.0 (2008)



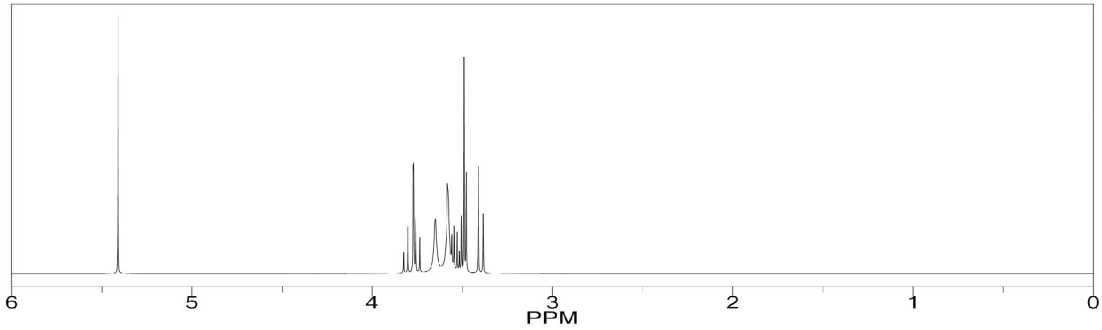
Arabinose



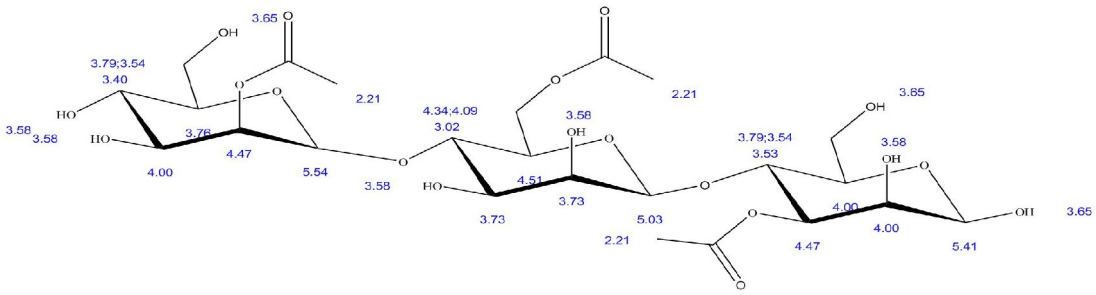
5. [1]HNMR of galactose simulation using **chemBioDraw** v*ersion* 11.0 (2008)



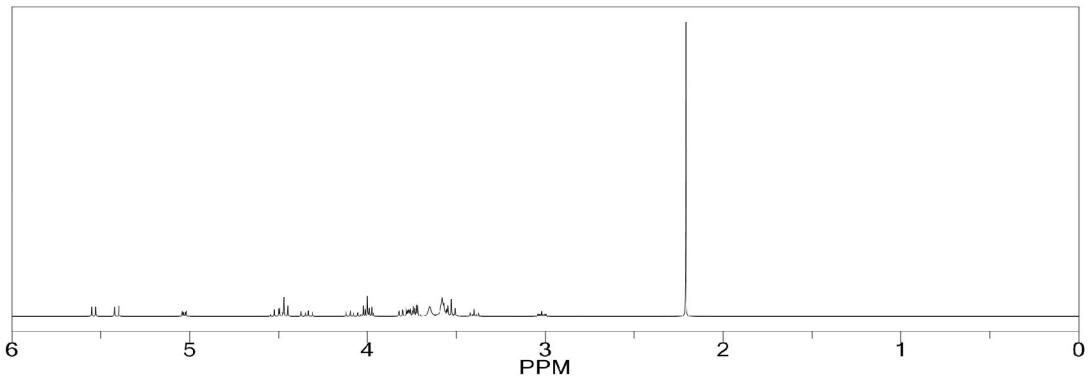
Galactose



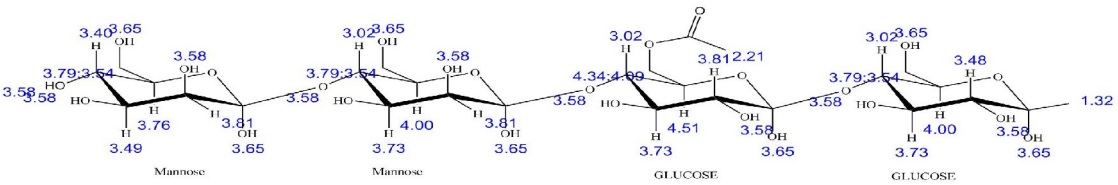
6. [1]HNMR of acemannan simulation using **chemBioDraw** v*ersion* 11.0 (2008)



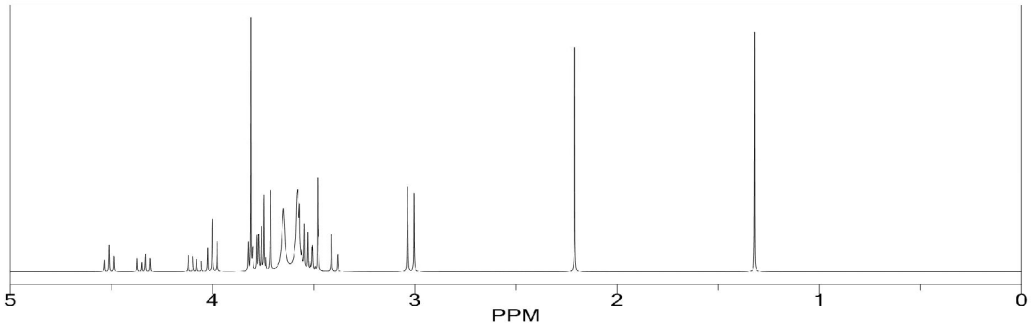
Acemannan



7. [1]HNMR of glucomannan simulation using **chemBioDraw** v*ersion* 11.0 (2008)

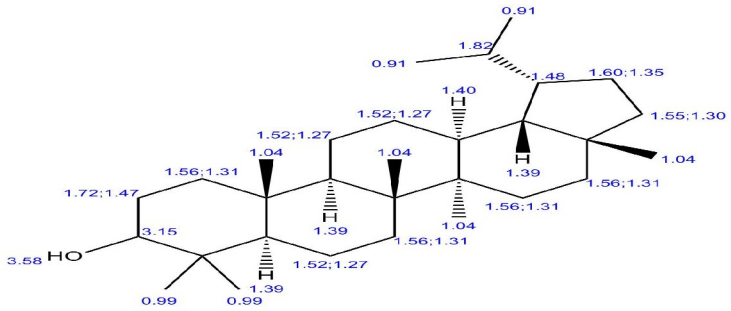


Glucomannan

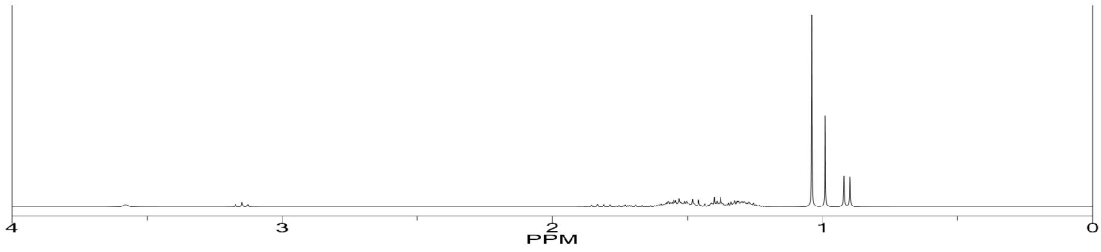


Appendix 4. Sterols and triterpenoids

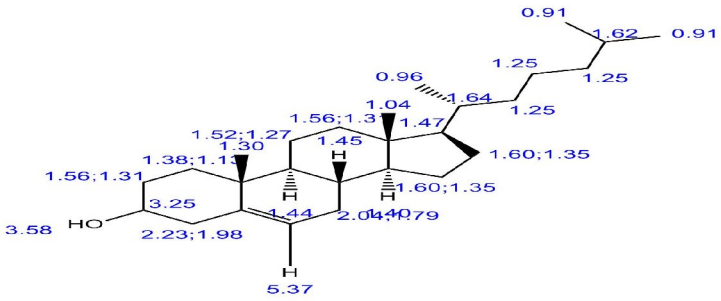
1. [1]HNMR of lupeol simulation using **chemBioDraw** *version* 11.0 (2008)



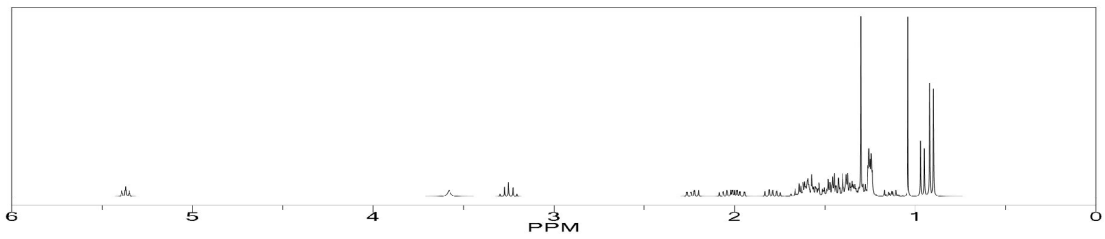
Lupeol



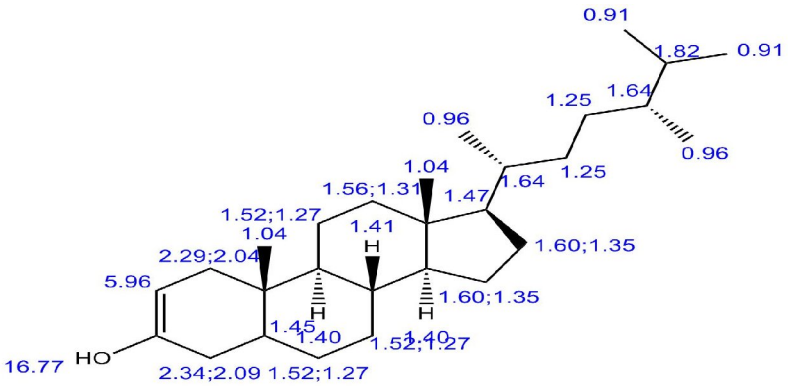
2. [1]HNMR of cholesterol simulation using **chemBioDraw** *version* 11.0 (2008)



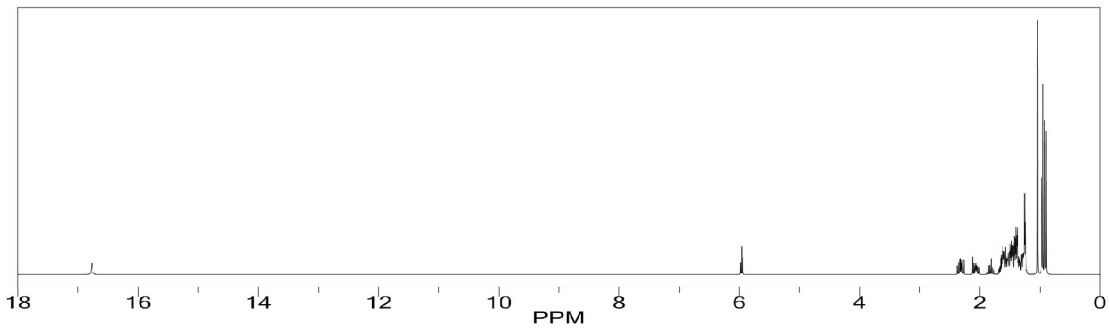
Cholesterol



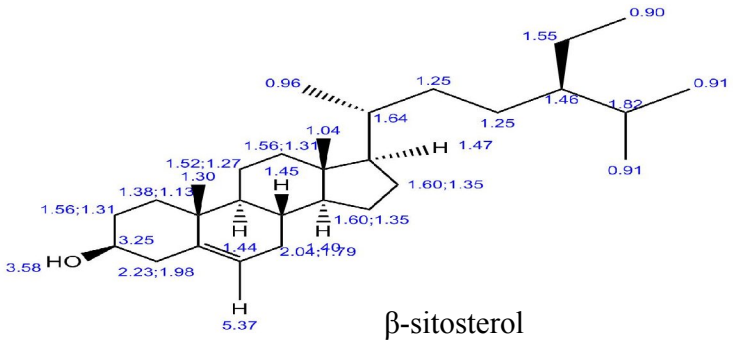
3. [1]HNMR of campesterol simulation using **chemBioDraw** v*ersion* 11.0 (2008)



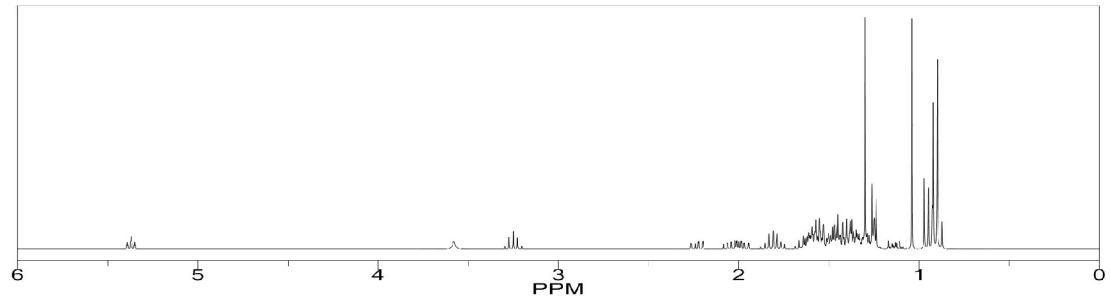
Campesterol



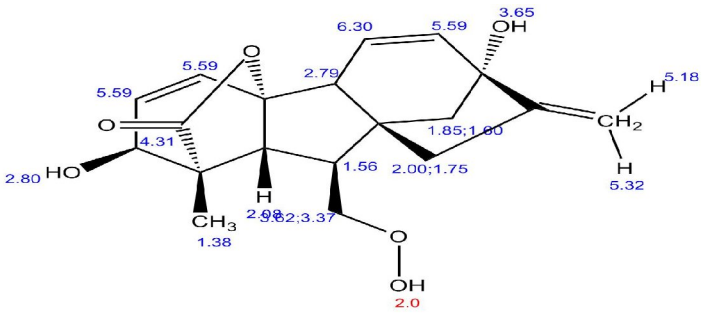
4. [1]HNMR of β-sitosterol simulation using **chemBioDraw** v*ersion* 11.0 (2008)



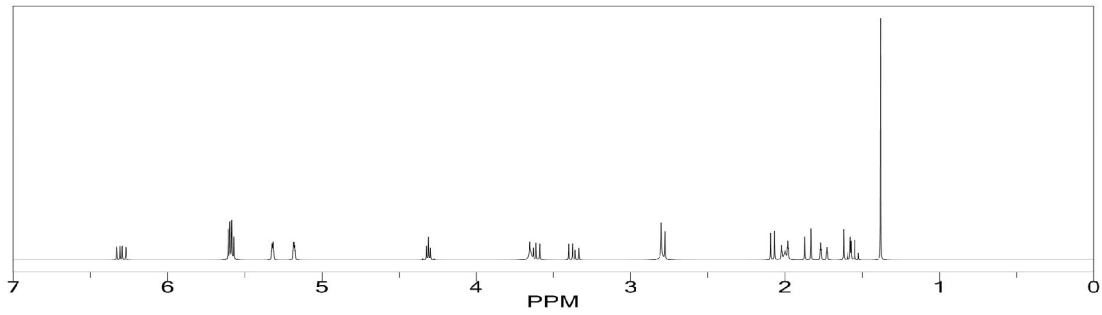
β-sitosterol



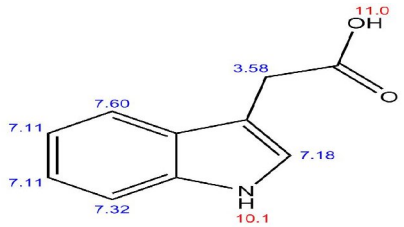
5. [1]HNMR of gibberellin simulation using **chemBioDraw** *version* 11.0 (2008)



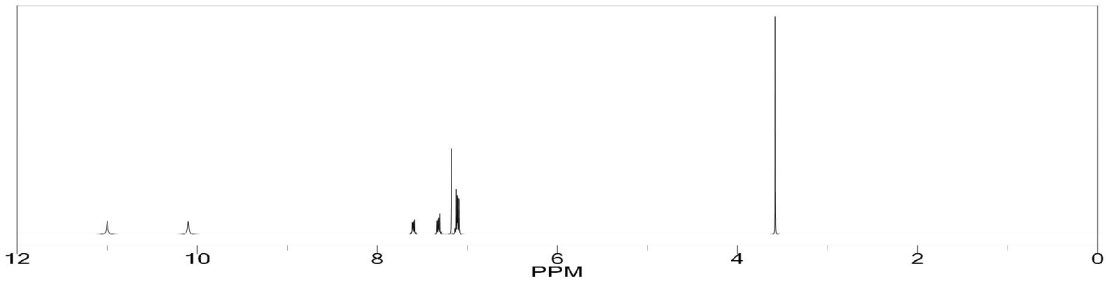
Gibberellin



6. [1]HNMR of auxins Simulation using **chemBioDraw** *version* 11.0 (2008)

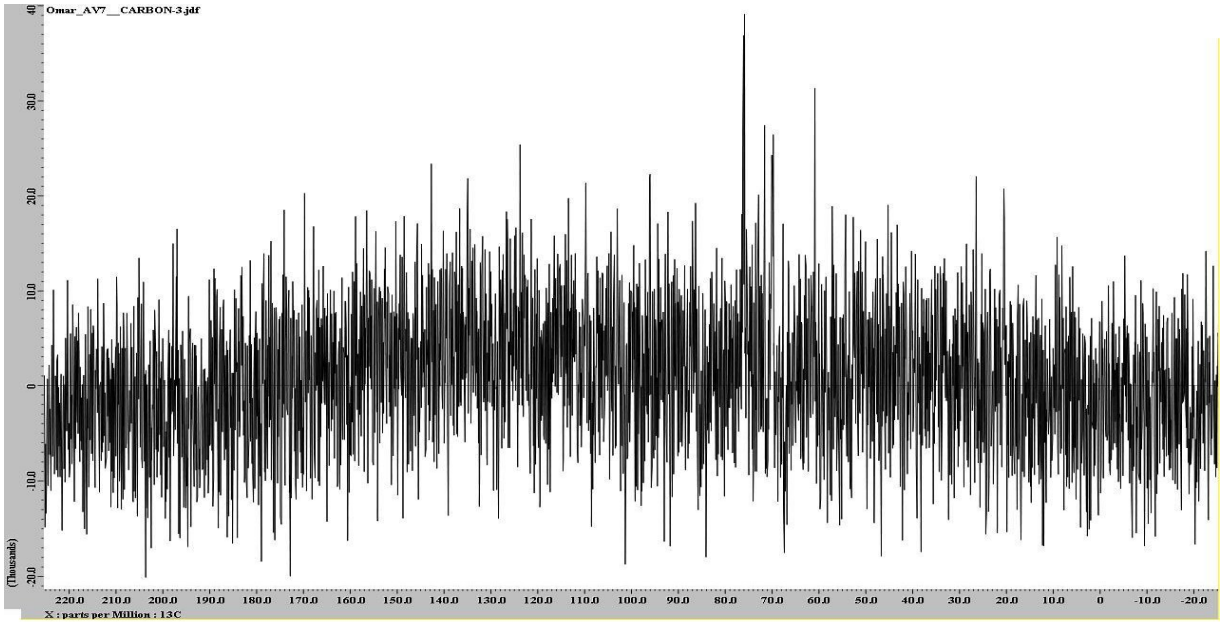


Auxins



Appendix 5. Fraction A7

1. ^{13}C NMR spectrum of fraction A7 in D_2O (JEOL Delta GX 400 MHz).



2. ^{1}H-^{1}H COSY NMR spectrum of fraction A7 in D_2O (JEOL Delta GX 400 MHz).