Nematicidal and antimicrobial evaluation of extracts, nanosized materials, and fractions, of selected plants, and the identification of the bioactive phytochemicals

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Erklärung

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Saarbruecken, Datum

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Dedicated to My

Beloved Family

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Abbreviations

AACT	Acetoacetyl-CoA thiolase
αAS	α-Amyrin synthase
AcAc-CoA	acetoacetyl-CoA
AP	Aerial parts
βAS	β-Amyrin synthase
BaX	Apoptosis regulator (Bcl-2 gene family member)
Bcl-2	Apoptosis regulator proteins
Caspase	Cysteine Aspartic Acid Specific Protease
CS	Cycloartenol synthase
DCM	Dichloromethane
DMAPP	Dimethylallyl diphosphate
DMSO	Dimethyl sulfoxide
DS	Dammarenediol synthase
DXP	Deoxyxylulose 5-phosphate
E. coli	*Escherichia coli*
ESI	Electrospray ionization
EtOAc	Ethylacetate

GS1	Ion source gas
h	Hours
Hep3B	Human hepatoma cells
HMG-CoA	(*S*)-3-hydroxy-3-methylglutaryl-CoA
HPH	High Pressure Homogenization
HSS	High Speed Stirring
IC_{50}	The half maximal inhibitory concent
IPP	Isopentenyl diphosphate
LB	Luria broth; Lysogeny broth
LD	Laser Diffraction
LS	lanosterol synthase
LuS	lupeol synthase
m/z	Mass by charge ratio
MeOH	Methanol
MIC	Minimum inhibitory concentration
MVA	Mevalonate pathway
NMR	Nuclear Magnetic Resonance
PCS	Photon Correlation Spectroscopy

PE	Petroleum ether
R*f*	Retention factor
S. carnosus	*Staphylococcus carnosus*
S. cerevisiae	*Saccharomyces cerevisiae*
TLC	Thin layer chromatography
TNFR	Tumor Necrosis Factor receptor
TPP	Thiamine pyrophosphate
TTC	Triphenyltetrazolium chloride
UV	Ultraviolet
YPD	Yeast Extract-Peptone-Dextrose

Summary

The methanol extracts of leaves, aerial parts, fruits, and resins of 17 plants used in the Arabian Peninsula were screened for, nematicidal and antimicrobial activities.

*Solanum incanum*was studied further in a different way by "nanosizing of original plant materials" to explore another alternative way to extensive extraction and isolation procedures. This plant has further been milled to more or less uniform particles of microscopic and nanoscopic size. These particles have been tested against model nematodes *(Steinernemafeltiae*) and bacteria (*Escherichia coli*). They exhibited activity against*Steinernemafeltiae*, which is comparable to the one seen for processed extracts of the same respective plant.

Dendrosicyos socotranus (Cucumber plant) was also studied as part of this thesis. This plant has been phytochemically screened and partitioned for identification of phytochemicals present affecting the above mentioned worms and microorganisms according to its traditional use.Thefractionation process was performed and several fractions of themethanolic extract from*D. socotranus*leaveswere obtained and tested against*S. feltiae, Staphylococcus carnosus,E. coli*and*Saccharomyces cerevisiae*. The fractionation procedure was performed by solvent-solvent partitioning and thereafter the compounds were isolated by preparative TLC and characterized by using mass spectroscopic analytical tools.

Zusammenfassung

Methanolextrakte von Blättern, oberirdischen Pflanzenteilen, Früchten und Harz von 17 auf der arabischen Halbinsel genutzten Pflanzen wurden hinsichtlich ihrer antinematodischen und antimikrobiellen Aktivität gescreent.

„*Solanum incanum*“ wurde untersucht, um dadurch einen alternativen Weg der Extraktions- und Isolationsmethode zu entwickeln. Diese Pflanze wurde in mehr oder weniger gleichmäßige mikro- und nanoskopisch kleine Partikel gemahlen. Die Partikel wurden auf ihre Wirkung gegen die Modelorganismen *Steinernema feltiae* (Nematoden) und *Escherichia coli* (Bakterien) untersucht. Sie zeigten eine Wirkung gegen *Steinernema feltiae*, welche vergleichbar mit der des Extraktes derselben Pflanze ist.

Dendrosicyos socotranus (Gurkenpflanze) wurde ebenfalls als Teil dieser Doktorarbeit untersucht. Diese Pflanze wurde phytochemisch gescreent und zur Identifizierung von sekundären Pflanzenstoffen, welche die oben genannten Würmer und Mikroorganismen, in Übereinstimmung mit ihrem traditionellem Nutzen, beeinflussen, aufgetrennt. Der Fraktionierungsprozess wurde durchgeführt und die verschiedenen erhaltenen Fraktionen des methanolischen Extraktes von *D. socotranus* gegen *S. feltiae, Staphylococcus carnosus, E. coli* und *Saccharomyces cerevisiae* getestet. Der Fraktionierungsprozess wurde mit verschiedenen Lösungsmitteln durchgeführt und die Verbindungen anschließend mit Hilfe einer preparativen TLC isoliert und unter Verwendung eines Massenspektrometers analysiert.

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Introduction

Medicinal plants encompass different types of plants used in herbal medicine and possessing disease curing or health improvement activities. They are the "base" of traditional medicine, which means thousands of people in the developing countries use medicinal plants frequently. Moreover, plants have been used by tribals and local people to cure various diseases, hence, several difficult diseases related to vitality, diabetes, memory loss, could still be treated effectively by the use of herbal medicine, which is generally not possible by the mainstream medicine. Therefore, the search for new antimicrobials, nematicidal, anti-diabetes, anti-inflammatory those of plant origin must be emphasized in the developed world where people are still using plants. Countries such as Yemen and Arabian Peninsula which possessa great biodiversity could be the starting point for such researches. Indeed, a large number of plants have been used for diverse purposes and tested throughout the globe for hundreds of years by different populations living in this area. Since infectious diseases represent an important cause of morbidity and mortality among the general population, particularly in developing countries, therefore, pharmaceutical companies have been motivated to develop new antimicrobial drugs in recent years, especially due to the constant rise of microorganisms resistant to conventional antimicrobials. Seemingly, bacterial species show the genetic ability to get and transmit resistance against currently available antibacterials as there are frequent reports on the isolation of bacteria that are known to be sensitive to routinely used drugs and became multiresistant to other medications available on the market [1, 2]. Therefore, common strategies embraced by pharmaceutical companies to meet the drug demands with new antimicrobial drugs include

changing the molecular structure of the existing medicines in order to make them more effective or improve the activity lost caused by bacterial resistance mechanisms [3].

In the light of the above, this study was first aimed to investigate the nematicidal and antimicrobial properties of methanolic extracts of different plants used in ethnopharmacology and ethnomedicine around the tropics and sub-tropics, and mainly in Saudi Arabia and Yemen.

Secondly, we turned our attention to mill the crude plant materials of *S. incanum* with readily available and economical (*i.e*., cheaper) methods, to render these materials into a useful form, in an attempt to explore and find another alternative way to extensive extraction and isolation procedures.

Thirdly, our goal was to isolate the bioactive compounds from the plant leaves of *Dendrosicyos socotranus* according to its uniqueness and its broad activity against bacteria, nematodes and yeast.

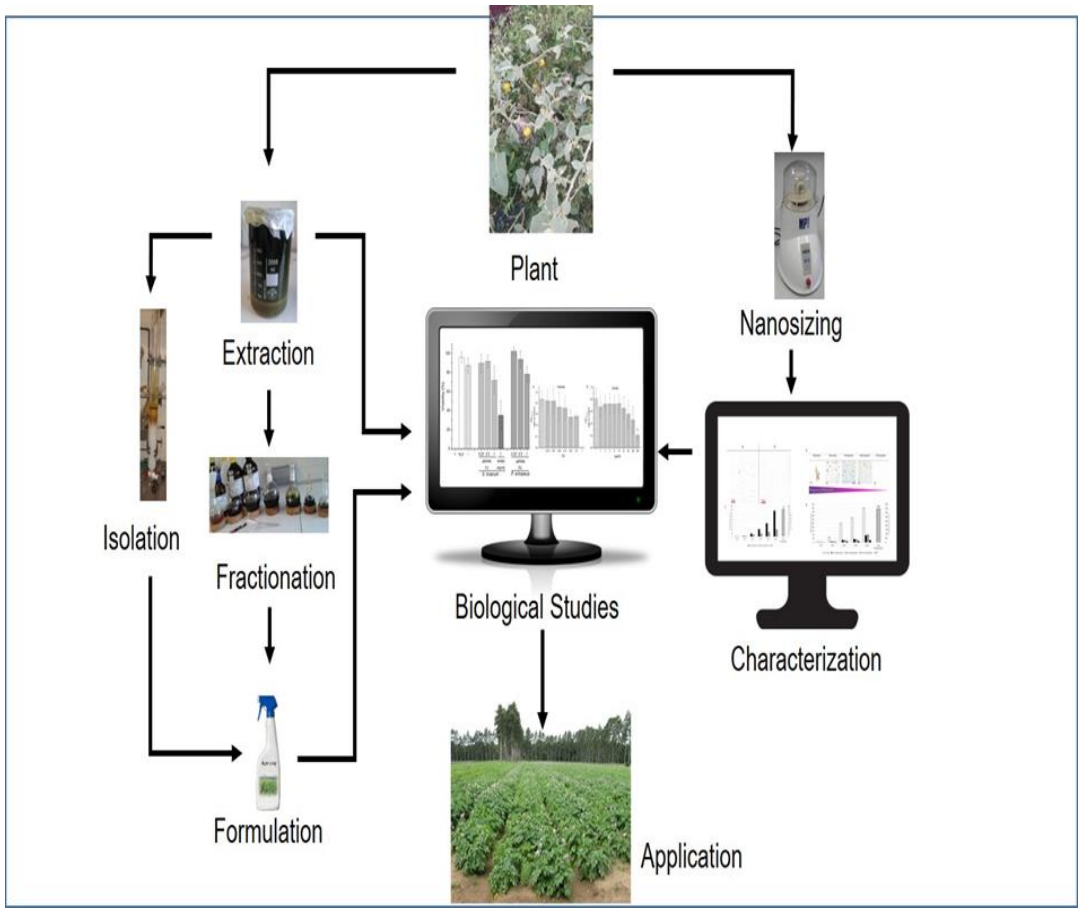


Figure 0. Schematic comparison of the more conventional methods to unlock the biological potential of natural materials via extraction, isolation, refinement and formulation on the left versus nanosizing of the crude material on the right (nanosizing may contain several steps, yet the methods are closely related). The major benefits and draw-backs associated with both avenues as far as we are currently aware of are highlighted. There are also various critical questions raised by nanosizing which ultimately deserve our attention.

CHAPTER I

Background of Traditional Medicine and Medicinal Plants of Yemen and Socotra Island

1.1 Overview of Yemen and Socotra Island

The Republic of Yemen locates in the southwestern edge of the Arabian Peninsula. It is surrounded by Saudi Arabia to the North, the Arabian Sea and the Gulf of Aden to the South, Oman to the East and the Red Sea to the West. Many islands in the Indian Ocean and the Red Sea, including Socotra Island, which is recognised as the biggest island in the Arabian Sea, belong to the Republic of Yemen. As 1st of January 2016, the population of Yemen was estimated to be 27,189,200 people compared to the population of 26,507,946 the year before according to the Yemen Population clock (http://countrymeters.info/en/Yemen). The Yemeni land area covers about 527,970 square kilometres. Though Yemen has a natural system of protected areas, very little forest is encompassed in these areas. Socotra Island is famous for its high level of indigenous plant species and has featured as a biosphere reserve. The use of plants for medicinal purposes is common, however, the extent of their commercial exploitation is limited.

Figure 1.1. Map of Yemen depicting its borders and its islands

1.2 Traditional Medicine of Yemen

Folk medicine is widespread in Yemen. Though, its application is particularly famous in provincial areas where it is utilised to manage several minor diseases. In rural areas of Yemen, sometimes the village people opt for traditional healing in the first instance, before going to orthodox medicine practitioners, because of the poor economic conditions of the citizens. Moreover, in those rural areas, traditional healers are usually consulted for their perceived knowledge and experience in treating different diseases [4, 5]. Alternative medicine practices such as cauterising, cupping, and bloodletting are still utilised for the treatment of several ailments in Yemen. Until today, there is no state pharmacopoeia in Yemen [6].

1.3 Medicinal Plant Resources of Yemen

Therapeutic, edible and fragrant plants, weeds and oregano used in Yemen and the areas around it date back for many years and constitute an essential aspect of its civilisation. Despite the fact that many herbs and spices involved have been neglected, medicinal herbs still play an essential role in public health. Some interesting medicinal plants and herbs (which have been studied here), their traditional uses and local names, are summarized in (Table 2.1, chapter 2).

Throughout history, medicinal herbs and plants have played invaluable roles for folks of Yemen as local drugs to treat infections and diseases, as well as beautifiers, flavourings, colourants, and additives. In Yemen, there is a huge and substantial plant diversity. Yemen has more than 3,000 plant species and around 10% of them have been identified as indigenous. Of the 3,000 plants, around 850 plant species are found on Socotra alone, 30% of them indigenous to Socotra. In this

study, different plants from Yemen and neighbouring areas have been studied, which have shown interesting activities against nematodes and different species of bacteria and fungi [7].

1.3.1 *Solanum incanum*

S. incanum is a small perennial herb that grows up to 2 m in height. Its stems and branches are covered with dense hairs and stout thorns. The leaves are 2.5–12 cm long and 2.5–8 cm wide, oval, elliptic, and grayish-green with a few spikes on the surfaces, and sinuate margins. The flowers are purple-blue corolla, 2.5 cm across with a prickly calyx and are permanent in fruit. The fruit (a berry) is round, approximately 3 cm in diameter, yellow when ripe with a lot of compressed-ovoid seeds [11-13]. The plant flowers and fruits almost throughout the year [14].

Figure 1.2. *S. incanum L.* ripe and unripe fruits (berries)

Synonyms of *Solanum incanum* L. are: *Solanum sanctum* L., *Solanum esculentum* Drege, *Solanum subexarmatum* Dunal, *Solanum delagoense* Dunal, *Solanum beniense* De Wild. Family — Solanaceae. It should be noted that solanaceae include valuable food crops such as potato (*S. tuberosum* L.), tomato (*S. lycopersicum* L.), aubergine (*S. melongena* L.), and chilli pepper (*Capsicum spp*.) in addition to many extensively used narcotic plants such as tobacco (*Nicotiana tabacum* L.), and Deadly Nightshade (*Atropa belladonna* L), the source of atropine. The huge

genus of *Solanum* L. with almost 1,500 species has become one of the largest genera of flowering plants [14].

S. incanum is locally named **as** ain Al baqar, 'arsam, 'arsan, hadaq (Saudi Arabia); mazg, mozj (Oman): helkem (Dhofari Arabic); nuqum (Yemen) [15, 16].

S. incanum has a wide range of ethnomedical applications. The plant parts are smashed together in water to produce a paste and are applied as a dressing on topical injuries. The berries, leaves and roots are boiled together in water and used as a drink against indigestion and dyspepsia. Moreover, the fruits are also boiled in oil and used as eardrops for otalgia. The dry fruits are burnt and the smoke emanating from it is used for treating haemorrhoids. In the Sultanate of Oman, a small hole is made in the fruit and an ulcerated or infected finger is put into it to for healing. Dried fruits are also heated slightly over naked flames and then eaten for the management of flatulence. In some areas of Oman (Dhofar) and in Yemen, the seeds of the fruit are burnt and the smoke inhaled to relieve toothaches [16-18]. Similarly, in some areas of Pakistan, the plant is used to treat toothaches. In contrast, goats and camels do not eat the plant and its presence in the field usually indicates overgrazing. The pollen from the flowers is nonetheless acceptable to honey bees [19].

Besides, in the other parts of the African continent, *S. incanum* is applied in Eastern and Southern Africa for treating skin diseases, genital infections and as a medication for gastric pains, dyspepsia, indigestion and fever. Beyond the use of the plant in traditional human medicine, the fruit is employed as well in traditional veterinary medicine. The juice from the berry is added to sheep’s nostrils for treating respiratory diseases. Also, the plant is employed as snakebite

medication, a remedy for liver and spleen ailments, and as a remedy for tooth and ear pains. In the northern parts of Nigeria, the roots and the fruits are sometimes employed as an important component in the formulation of arrow poisons. Unlike some its relatives, such as tomatoes or aubergines, the plant is considered in most regions of the Arab peninsula and most parts of West Africa as toxic, and most experts advise that caution is exercised when it is used internally [13].

Phytochemistry

Phytochemical studies and compound isolations have been performed on the *S. incanum* shrub which revealed the presence of a huge number of phytochemicals classified as medicinally significant. Most of those phytochemicals are steroidal alkaloids. Others include substances such as glycoalkaloids, flavonoids, and saponins [20]. Several members of the genus *Solanum* contain solasodine which is commercially important in the preparation of certain hormonal steroids. Other compounds isolated are steroidal sapogenin, diosgenin, and flavone glycosides [21].

Pharmacological Activity

According to the previous studies, the pharmacological activity of solanine and related steroidal alkaloids includes antifungal, antibacterial, analgesic, nematicidal and cytotoxic properties. In the southern part of Africa, it is reported that the plant has a therapeutic effect against multiple external benign tumours in animals [7, 22]. The poisonous alkaloid compound, solasodine, of the *Solanaceae* family is currently used commercially as a precursor for the production of complex steroidal compounds, predominantly as part of contraceptive tablets [23].

Nematicidal and Parasitic Activity

Another study, conducted on other two species of the Solanaceae family has shown that the extracts of *S. sisymbriifolium* and *S. nigrum* induce morphological changes in the body structure of the root-lesion nematode, affect its motility and cause mortality [24]. The *S. incanum* leaf extracts also show an interesting antiparasitic activity against the *Leishmania amazonensis* strain with an IC_{50} between < 12.5-26.9 µg/mL and acceptable selectivity indices of 8-5 [25]. Another study on aqueous fruit extract of *S. incanum* assessed its efficacy against cattle ticks. The results, in general, showed that *S. incanum* has had some acaricidal effect [26].

Antimicrobial and Antifungal Effects

An earlier study showed that the therapeutic activity of the berries of *S. incanum* against cutaneous mycotic infections and other pathological conditions was due to their content of solanine and related glycoalkaloids, such as saponins and cytostatic poisons [22, 27, 28]. Moreover, another study revealed that a more influential antimicrobial substance, with a phosphorylated structure similar to the purine adenine, could be isolated from the berries. The crystals of that compound were effective inhibitors of the growth of Gram-positive and negative bacteria, yeasts, dermatophytes, and some pathogens affecting agricultural produce [29]. Besides, in another study, it was suggested that ethanol extracts of this shrub are a great potential source of antibacterial compounds that could be used in the formulation of new antimicrobial drugs [30]. Likewise, *S. incanum* showed activity against Gram-negative and Gram-positive isolates but was more effective against Gram-negative organisms [31].

Anticancer Activity

It is important to mention other activities of the *S. incanum* on different microbes, cells, micro- and macro-organisms because of it medicinal significance. A very interesting secondary metabolite, named Solamargine, has been isolated from *S. incanum* a has been identified as steroidal alkaloid glycoside. Solamargine has been described to possess anticancer activity on human hepatoma cells (Hep3B) via triggering apoptosis in addition to elevating the level of TNFR-1 and 2 on the hepatoma cells [32]. Moreover, an earlier study, conducted in China, isolated a new steroidal alkaloid glycoside from the fresh berries of *S. incanum.* The said glycoside was named incanumine, and characterized as *O*-(3)-{beta-*D*-xylopyranosyl-(1-3glu)-[beta-*D*-xylopyranosyl-(1-4rha)-alpha-*L*-rhamnopyranosyl-(1-4)]-beta-*D*-glucopyranosyl}-solasodine. Solamargine, solasodine, ursolic acid, and ursolic acid derivatives (3-*O*-palmitoyl ursolic acid, 3-*O*-crotonyl ursolic acid, 3-*O*-propionyl ursolic acid) exhibit significant cytotoxic effects against human PLC/PRF/5 cells *in vitro* [33]. Besides, another study isolated the same steroidal glycoalkaloid solamargine which exhibits cytotoxic activity through the disruption of phosphatidylcholine/cholesterol liposomes at a concentration > 50 μM whereas the normally co-occurring glycoalkaloid solasonine is ineffective at up to 150 μM [34]. These two biologically active glycosidal alkaloids solasonine and solamargine were isolated earlier as well from the fresh ripe fruit of *S. incanum* by two countercurrent chromatographic methods [35]. Another result showed that Solamargine effectively inhibited hepatoma cell proliferation and increased apoptosis. This compound resulted in cell cycle arrest at the G2/M phase in the two cell lines. Furthermore, Solamargine down-regulated the levels of proliferation-associated (Ki67 and pcna) and anti-apoptotic (Bcl-2) proteins, and promoted the activity of apoptosis-associated proteins

(Bax, caspase-3 and caspase-9). Hence, the activation of the Bcl-2/Bax and caspase signalling pathways may be involved in the solamargine-induced apoptosis of hepatoma cells [36].

S. incanum Toxicity

It is important to note here that this herb (*S. incanum*) is considered as toxic and is avoided in many areas of the world. For human beings, it is used as a local remedy for several ailments mentioned earlier. It has been reported that the leaves of *S. incanum* have high quantities of alkaloids [37]. The fruits of *S. incanum* contain dimethylnitrosamine, a semi-volatile organic chemical in certain food stuffs. This organic chemical is toxic to the liver and other organs and may lead to several cases of cancer in some areas where the fruit juice is used to coagulate milk. Furthermore, skin cancer cases in animals have been noticed to be due to the contact with *S. incanum* berry. Also, these unripe berries of *S. incanum* were found to exhibit toxic effects in goats and allowing animals further to feed on this plant which could lead to harmful effects on their health [38]. Nevertheless, several concentrations (up to 15,000 mg/kg body weight) of *S. incanum* extract did not exhibit signs of toxicity when administered orally to mice [39].

1.3.2 *Dendrosicyos socotranus*

Dendrosicyos socotranus is a monotypic species of the Cucurbitaceae plant family. This species is indigenous to Socotra Island, Yemen Republic, and is the only species in the Cucurbitaceae family which grows as a tree form. The species name is essentially expressed as *D. socotranus* [40]. The species was first described by Isaac Bayley Balfour in 1882, therefore, the plant named as *D. socotranus* Balf.f [41] . The English name of *D. socotranus* is 'cucumber tree of Socotra'.

***D. socotranus* Taxonomy**

Kingdom	Phylum	Family	Genus	Species	Binomial name
Plantae	Cucurbitales	Cucurbitaceae	*Dendrosicyos*	*D. socotranus*	*Dendrosicyos socotranus* Balf.f.

Figure 1.3. *Dendrosicyos socotranus* leaves. This figure depicts the plant of *Dendrosicyos socotranus* from left (A) in the autumn, (B) and (C) in the summer.

Habitat and Ecology

The plant grows in depleted soils, containing quite a lot of a hard sedimentary rock, with a little water. It can grow to around six meters in height and around one meter in diameter. The flowers are yellow, and there are both male and female flowers on each plant. It is abundant in the dry parts of the island of Socotra, always associated with the plant *Croton socotranus* in the plains, and on limestone soils upto 500 m in elevation. It is widely spread, though irregularly, in several habitats of Socotra. The plant is endemic in Socotra island and perhaps scantily available in the

island of Samha but not in the nearby islands such as sponge Luffariellasponge, Luffariella Darsa or Abd al-Kuri [41].

Figure 1.4. Socotra island and the islands nearby.

D. socotranum is considered at risk of extinction due to the drop in quality of its environment because of dehydration in some areas and competition from other species more adapted to dryness. On the plains, seedlings develop and grow protected from livestock under cover of dense shrubs provided by spines such as *Lycium sokotranum* or by succulent plants such as *Cissus subaphylla*. These two plants have a big role in protecting the seedlings of *D.socotranus*. Therefore, along the southern region of Socotra and on the island of Samhah the regrowth of *Dendrosicyos* after dry spells is associated with the presence of colonies of Cissus, as they nearly grow together.

Nematicidal, Antimalarial and Antileishmanial Activity of *D. socotranus*

Some studies have shown that the extracts of *D. socotranus* have anthelmintic and antimalarial properties. Moreover, the same plant extract has also shown an interesting antiplasmodial activity at a concentration (IC_{50} = 2.3 µg/mL) which suggests that the active constituents in the extract may have been cytotoxic to *P. falciparum* trophozoites, thereby inhibiting their development to the schizont stage [42]. Besides, extracts of *D. socotranus* exhibited the protoscolicidal activity, significantly reducing and/or stopping protoscolex viability at concentrations of 5 mg and 10 mg/mL. Also oral and intraperitoneal administration of the extracts in white mice invoked noticeable inhibitory effects on the *in vivo* development of secondary hydatid cysts [43]. Additionally, the extracts of *D. socotranus* showed inhibitory activity against malarial plasmodes and leishmania parasites at 8.4 and < 0.25 µg/mL. The authors of this report, however, considered it non-specific because of high cytotoxicity at 0.7 µg/mL [44].

Anticancer Potential

According to the criteria of the American National Cancer Institute, the maximum IC_{50} limit to consider a crude extract promising for further purification and isolation is 30 μg/mL [45]. Thus, the methanolic extracts of *D. socotranus* can be considered as a highly promising source of anticancer compounds, as the activity was registered at less than 30 μg/mL [46].

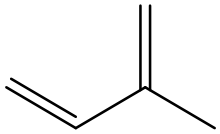
Phytochemistry

A new isocucurbitacin (Dendrocyin) with unusual cyclization in the side chain, which in numbers namely, 24 beta-ethoxy-20-25-epoxy-3-alpha, 16 alpha-dihydroxy-9-methyl-19-norlanost-5(6)-ene-2,11,22-trione has been isolated alongside isocucurbitacin R. The structural configuration was established by conventional spectroscopic (^{1}H NMR, ^{13}C NMR and DEPT) and two-dimensional NMR techniques (COSY, ^{1}H-^{13}C HMBC and ^{1}H-^{13}CHMQC) [47].

1.4 Terpenoids

Since this plant (*D.socotrana*) is full of terpenoids and saponins, we have compiled a short review on terpenoids and their potential benefits for human health and treatment of disease.

Terpenoids are naturally occurring plant products which comprise of one or more units of isoprene $(C_5H_8)n$ [48].



Isoprene unit

1.4.1 Classification of Terpenes

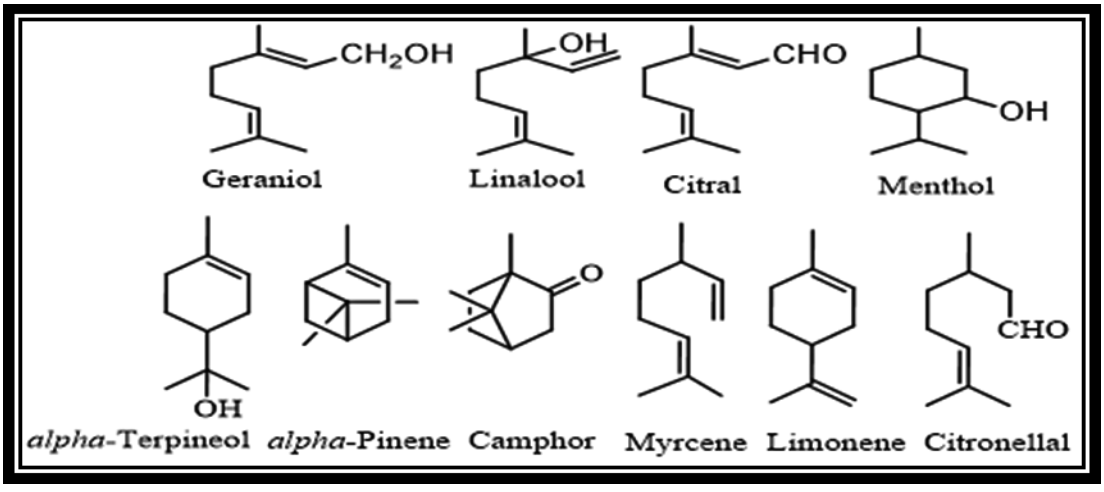
Terpenes are classified into many groups based on the number of carbon atoms and isoprene units present in their structure. They are classified as monoterpenes, sesquiterpenes, diterpenes, triterpenes, tetraterpenes, polyterpenes etc. The prefix is related to the number of isoprene units present in the molecule [49].

1.4.2 Monoterpenes

Monoterpenes form a class of terpenes that consist of two isoprene units and have the molecular formula $C_{10}H_{16}$. They may be acyclic (linear) or cyclic (containing rings). Biochemical modifications such as oxidation or rearrangement produce a variety of open chain and cyclised

monoterpenoids. They are low molecular weight volatile compounds, and they may be recognised by their distinctive odors.

Monoterpenoids give rise to a structurally varied group of compounds which may be grouped into nearly 35 differing structural analogues. Yet, the most regularly occurring structural variations are of the following types, namely: geraniol, linalool, citral, menthol, iridodial, terpineol, camphor, α-pinene, myrcene, limonene and citronella. They consist of two isoprene units and have the molecular formula $C_{10}H_{16}$. They are volatile natural products found in higher plants as essential oils and are broadly used in perfumery and flavouring agents. For instance, geraniol is the main constituent of geranium oil of *Pelargonium graveolens* and its isomer linalool is found in the oil of a garden herb, *Clary sage*. Citral, a lemon oil ingredient, is extracted from lemon grass oil (*Cymbopogon flexuousus*). Menthol is isolated from *Mentha arvensis*. It has a notable financial value and is widely used to flavour sweets, tobacco and toothpaste. It is also used for local analgesia and for its refreshing effects [50].



This figure shows the most regularly occurring structural variations of monoterpenes.

The pine oil (turpentine) contains two monoterpenes *viz*. terpineol and α-pinene. Camphor can be extracted from the camphor tree, *Cinnanomum camphora*. It is often applied to protect textiles from mites. α-pinene is a crude element for the industrial synthesis of camphor. Myrcene, limonene and citronellal are other types of monoterpenes which are used occasionally.

1.4.3 Sesquiterpenes

The sesquiterpenoids are widely present in nature and are the most widespread group of terpenoids. They are also commonly collected from the essential oils but at higher boiling points. Sesquiterpenoids contain three isoprene units and have the molecular formula $C_{15}H_{24}$.

Caryophyllene is obtained from clove oils, humulene from hop oil [51], cedrene from cedar wood oil [52] and longifolene from Indian turpentine oil (*Pinus ponderosa*). Some common examples of this group of terpenes are shown below.

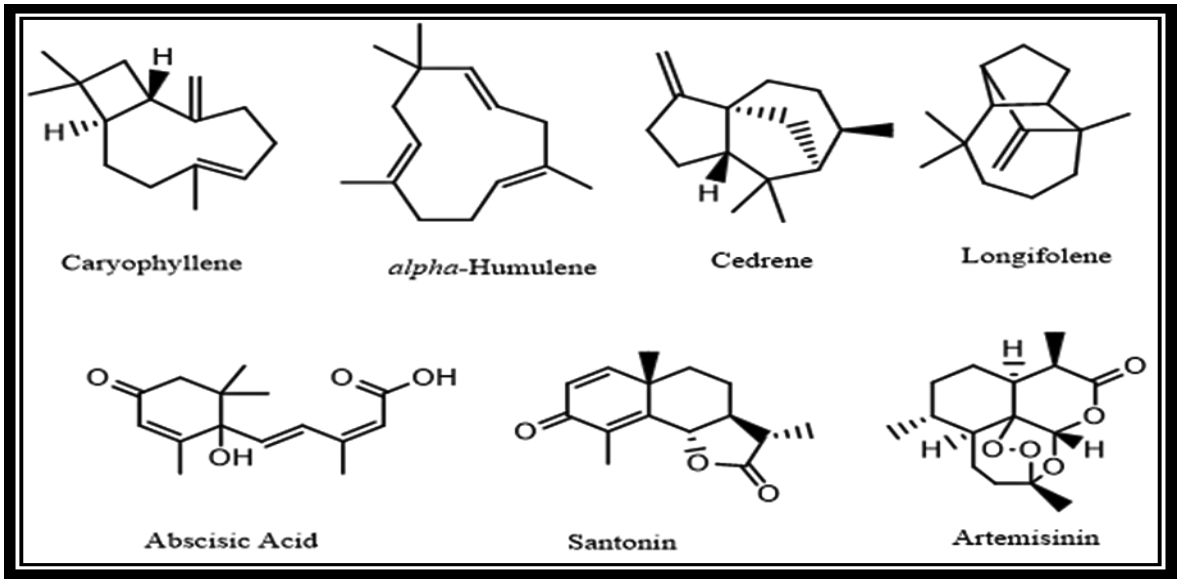
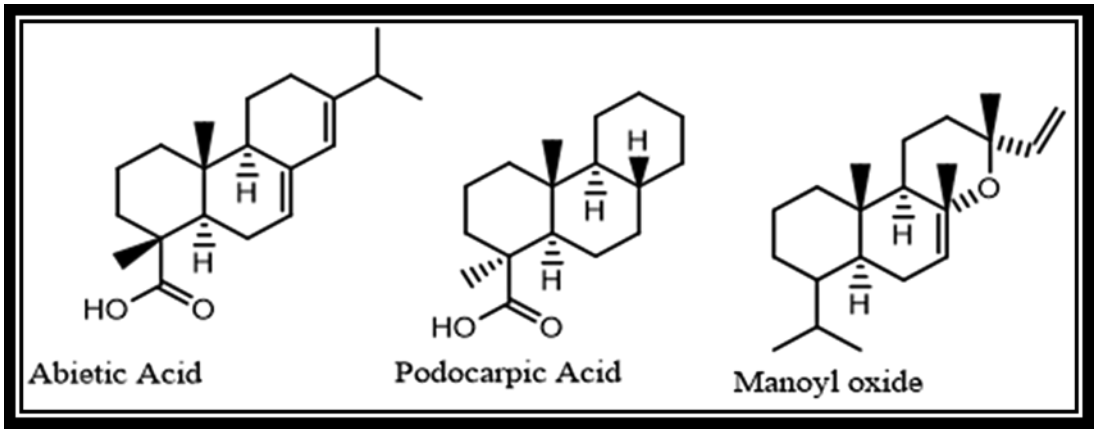


Figure 6. This figure depicts the most commonly structural variations of sesquiterpenoids.

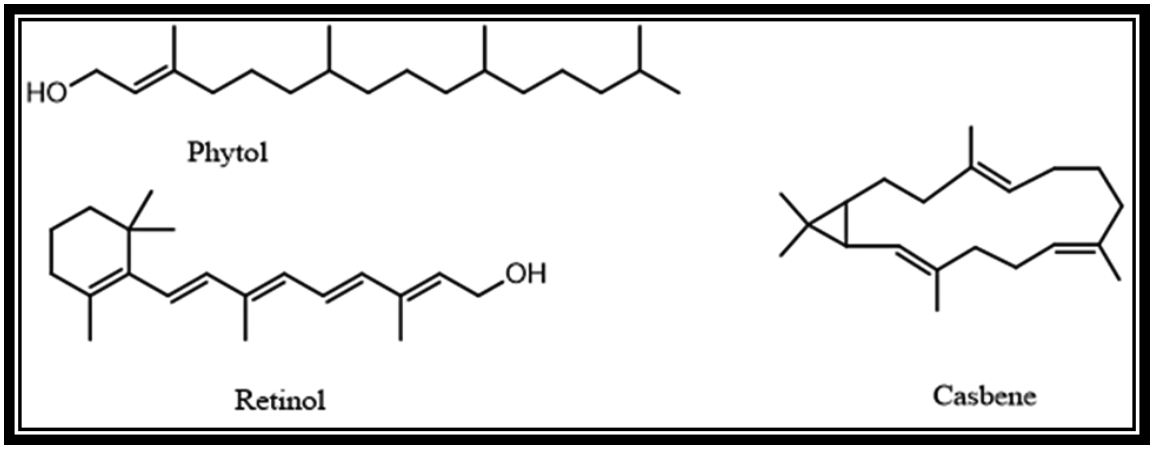
Sesquiterpenoid lactones such as santonin from *Artemisia maritima* (warm wood) and artemisinin obtained from *Artemisia annua* are commonly employed as medications. Abscisic acid, a plant hormone is also an example of a sesquiterpenoid. It suppresses growth of buds and promotes leaf senescence.

1.4.4 Diterpenes

Usually diterpenoids describe a broad group of non-volatile C20 compounds that have been essentially derived from geranyl pyrophosphate. Diterpenes are composed of four isoprene units of the general molecular formula $C_{20}H_{32}$. A few of the diterpenes are wood resin products. They include abietic acid from Pinus and Abies species [53], podocarpic acid from *Podocarpus cupressinum* and the neutral resin manoyl oxide.



The bioactive compounds such as phytol, retinol (vitamin A) and casbene (a phytoalexin) are also considered as diterpenes.



Taxol is also a diterpenoid, which was first separated from the phloem of the Pacific yew, *Taxus brevifolia* [54]. It is employed extensively for the therapy of breast and ovarian cancer.

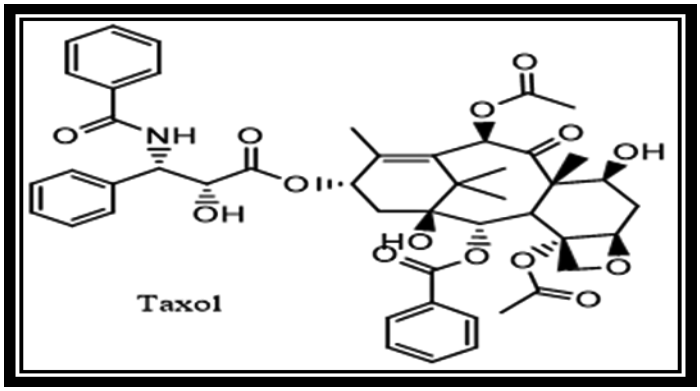
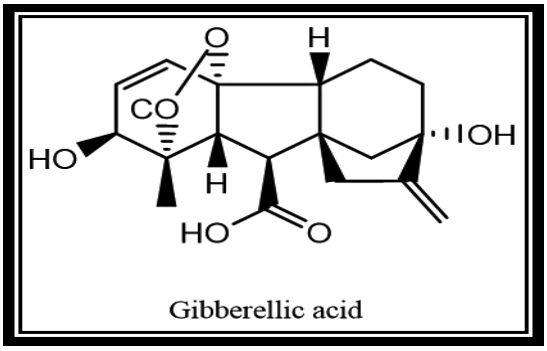


Figure 7. These figures highlight the most frequently structural variations of diterpenoid.

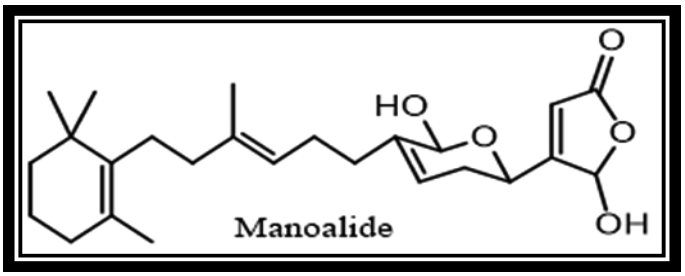
The plant hormone gibberellic acid is another diterpenoid which is synthesised as a phytotoxin by the fungus *Gibberella fujikuroi* [55]. It is applied in the malting step in beer production to increase α-amylase production and also for increasing berry size of "Emperatriz" seedless grape [56].



Gibberellic acid

1.4.5 Sesterterpenes

They have five isoprene units and a molecular formula $C_{25}H_{40}$. Sesterterpenes are available in copious amounts in marine sponges. They have excellent potentials as anti-inflammatory compounds. The sesterterpene manoalide [57], which has been isolated for the first time in the early 1980s from the sponge *Luffariella variabilis* by Scheuer *et al*. [58], represents the first marine natural product reported as a phospholipase A2 inhibitor, and it remains, to date, the most extensively investigated marine phospholipase A2 antagonist.

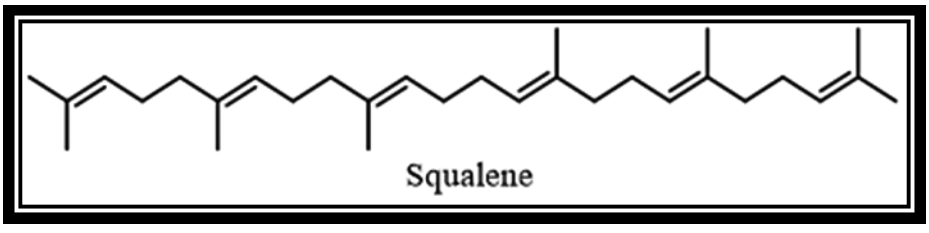


Manoalide

1.4.6 Triterpenes

Triterpenes are composed of six isoprene units with molecular formula $C_{30}H_{48}$. Squalene is a triterpene with the formula $C_{30}H_{50}$, a precursor for the biosynthesis of phytosterol or cholesterol in plants or animals, respectively. It is widespread in the animal and plant kingdoms. Scientists have discovered that, at the moment life appeared on Earth, microorganisms, and the cell membranes of higher organisms (later in the Precambrian era), contained large quantities of squalene, a substance likely to be essential to their survival in that hostile environment free of oxygen.

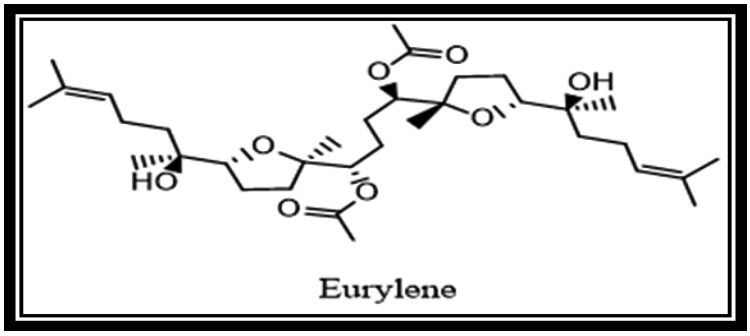
Squalene was discovered in 1906 by the Japanese researcher Dr. Mitsumaru Tsujimoto, an expert in oils and fats at Tokyo Industrial Testing Station. He separated the unsaponifiable fraction from the shark liver oil "kuroko-zame" and discovered the existence of a highly unsaturated hydrocarbon [59]. Ten years later, Tsujimoto succeeded to obtain by fractional vacuum of the liver oil from two deep-sea shark species an unsaturated hydrocarbon, with the chemical formula $C_{30}H_{50}$, which he named "squalene" [60]. The name came from the denomination of the sharks' family: *Squalidae*.



Triterpene structure (Squalene), the precursor for the biosynthesis of phytosterol or cholesterol in plants or animals.

1.4.6.1 Eurylene

Eurylene is a squalene-type triterpene bioactive compound which has been identified recently in *D.socotrana*. This compound was first isolated from the woods of *E. longifoliu* (Simaroubaceae) in 1991 by Hideji Itokawa *et al* [61]. Eurylene appears as colorless needles mp 146-148 °C, molecular formula, $C_{34}H_{58}O_{13}$.



Eurylene

((1*S*,4*R*)-4-acetyloxy-1-{(2*R*,5*R*)-5-[(2*S*)-2-hydroxy-6-methylhept-5-en-2-yl]-2-methyloxolan-2-yl}-4-{(2*S*,5*R*)-5-[(2*S*)-2-hydroxy-6-methylhept-5-en-2-yl]-2-methyloxolan-2-yl}butyl)acetate

In contrast to squalene and Eurylene, Steroids, such as cholesterol and the steroid hormones, are characterised by a carbon skeleton with four fused rings. They are distinguished by the functional groups attached to the rings.

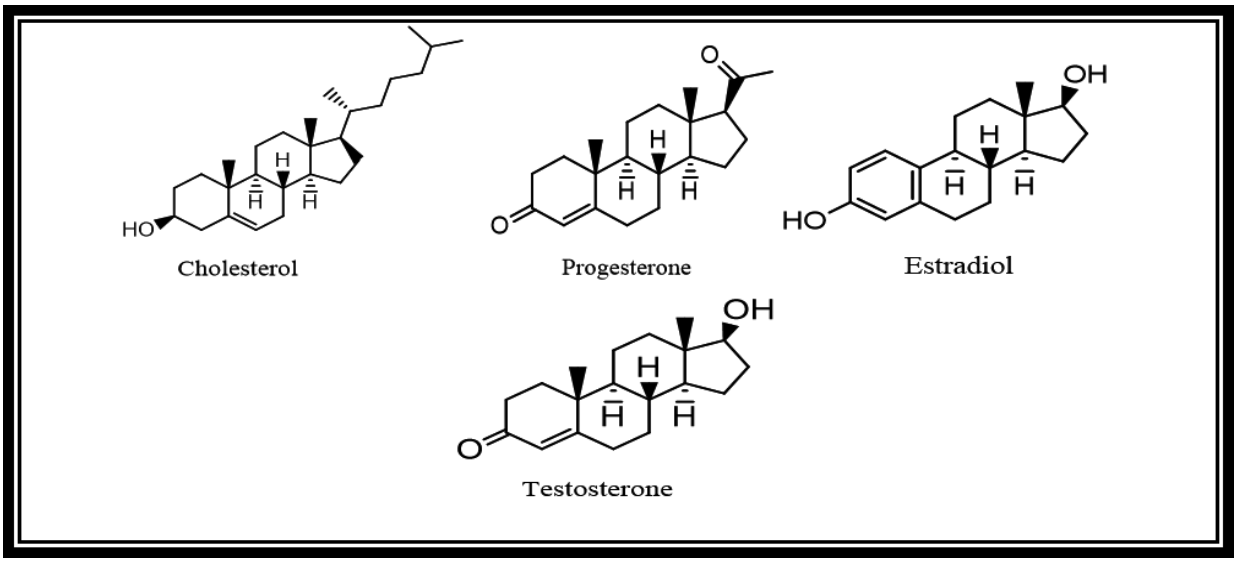
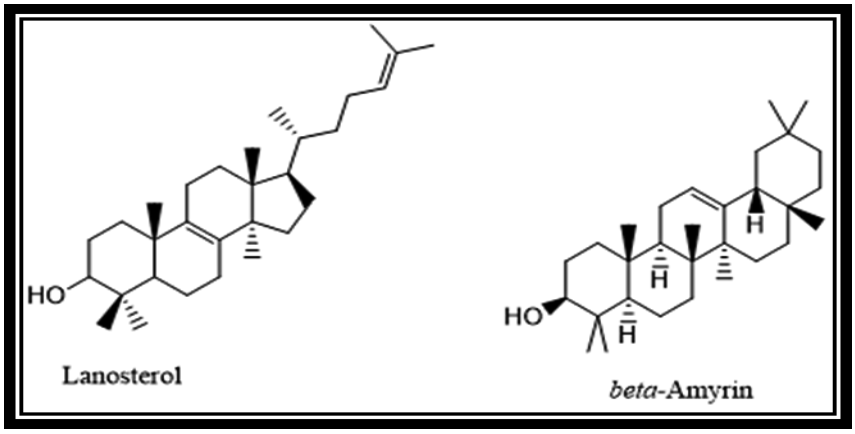


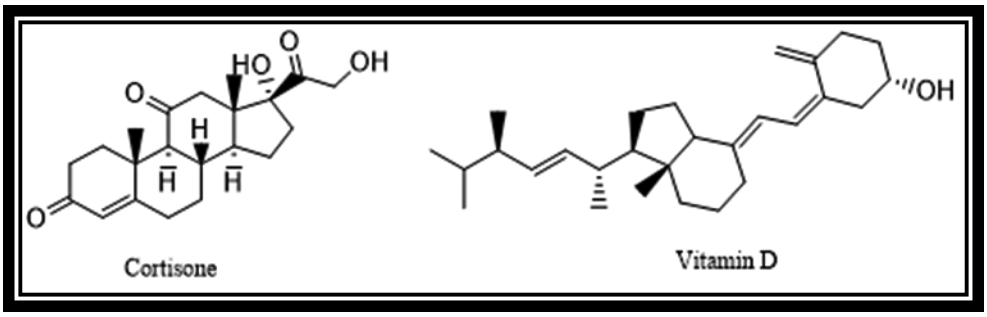
Figure 8. These figures of steroidal tetracyclic triterpene structures reflect the structural diversity of triterpenes as a cyclic and long chains structures.

Lanosterol is a tetracyclic triterpene present in wool fat. It is secreted by the sebaceous glands of wool-bearing animals. Most lanolin used by humans obtained from domestic sheep breeds that are raised specifically for their wool. Historically, many pharmacopoeias have referred to lanolin as wool fat (adeps lanae); however, as lanolin lacks glycerides (glycerol esters), it is not a true fat [62, 63]. Pentacyclic triterpenes such as α- and β-amyrin are ubiquitously distributed throughout the plant kingdom, in a free form as aglycones or in combined forms, and have long been known to exhibit a number of biological effects.



Here is another example the *tetra*- and pentacylic of triterpenes

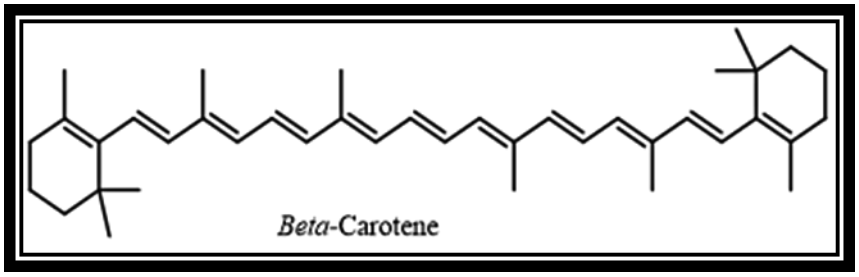
Another class of triterpenes is formed by the corticosteroids (*e.g*. cortisone) that are produced in the adrenal cortex of vertebrates, as well as the synthetic analogues of these hormones. These compounds are involved in a wide range of physiological processes, including stress response, immune response, and regulation of inflammation, carbohydrate metabolism, protein catabolism, blood electrolyte levels, and behaviour [64]. Studies have indicated that Vitamin D, a steroid triterpene, helps in the absorption of calcium and phosphate from the gastrointestinal tract [65, 66].



These two are steroidal triterpenes, a nother class of triterpenes which reflect the huge group of triterpenes and terpenenoid all together.

1.4.7 Tetraterpenes

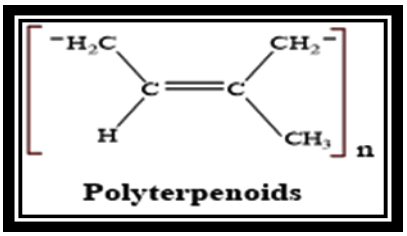
Tetraterpenes are terpenes consisting of eight isoprene units and have the molecular formula $C_{40}H_{64}$. Carotenoids are examples of tetraterpenes. They form a group of phytochemicals that are responsible for different colours of the foods and are also known to play important roles in the prevention of human diseases and maintenance of good health. In addition to being potent antioxidants, some carotenoids also contribute to dietary vitamin A.



Tetraterpene β-Carotene is the most common form of Carotenoids in plants and fruits.

1.4.8 Polyterpenes

The polyterpenes are terpenes containing more than eight isoprene units and are joined in a head-to-tail manner. The natural rubber, Indian rubber or caoutchouc, as produced initially, consists of polymers of the organic compound isoprene, with minor impurities of other organic compounds plus water, and is a fine example of a polyterpene [67].



1.4.9 Terpenoid Biosynthesis

Isopentenyl diphosphate (IPP) is the main intermediate in the biosynthesis of isoprenoids in all organisms. In nature, there are two different routes of IPP biosynthesis: the mevalonate (MVA) pathway and the deoxyxylulose 5-phosphate (DXP) pathway. The (MVA) pathway, the enzymes of which are confined to the cytosolic compartment, provides the precursor of triterpenes (sterols) and certain sesquiterpenes [68]; in plastids, the deoxyxylulose 5-phosphate (DXP) pathway works to supply IPP for the synthesis of monoterpenes and diterpenes [69, 70], several sesquiterpenes [71], tetraterpenes (carotenoids), and the prenyl side chains of chlorophyll and plastoquinone [72].

Therefore terpenoid biosynthesis can be divided into four phases. The first phase involves the origin of the isoprene unit, isopentenyl pyrophosphate. The second stage involves the stepwise

polymerisation of the isoprene units to form the acyclic polyprenyl precursors of the terpenoids such as geranyl, farnesyl and geranylgeranyl pyrophosphate. The third stage involves the cyclisation of these to form the underlying carbon skeleta of the various families of terpenes. The final stage involves establishing the sequence and stereochemistry of the various hydroxylations and oxidations which lead to the individual families of terpenoid natural products. The last step is the formation of individual terpenoids.

1.4.10 Mevalonate Pathway

All terpenoids are derived from the basic five-carbon building units, isopentenyl diphosphate (IPP) and its allylic isomer dimethylallyl diphosphate (DMAPP) [73]. The MVA pathway in plants consists of six steps and starts with the Claisen-type condensation of two molecules of acetyl-CoA to acetoacetyl-CoA (AcAc-CoA) catalyzed by acetoacetyl-CoA thiolase (AACT). After an aldol condensation reaction catalyzed by HMG-CoA synthase (HMGS), AcAc-CoA is combined with a third molecule of acetyl-CoA to form the C6-compound *S*-3-hydroxy-3-methylglutaryl-CoA (HMG-CoA). The prenyl diphosphate intermediates built by condensation of these five-carbon units are used as precursors for the biosynthesis of terpenoids with fundamental functions in growth and development and for the formation of a large number of terpenoid compounds with more specialised roles in the interaction of plants with their environment [74].

The mevalonate pathway is responsible for the formation of IPP/DMAPP, the basic five-carbon unit of terpenoid biosynthesis. Synthesis of each IPP/DMAPP unit requires three molecules of acetyl-CoA. Both the MVA and the DXP elucidation Pathways graphes are existing at the appendix part of this thesis.

1.4.11 Deoxyxylulose 5-Phosphate (DXP) Pathway

In this pathway, pyruvate reacts with thiamine pyrophosphate (TPP) to yield a two-carbon fragment, hydroxyethyl-TPP, which condenses with glyceraldehyde 3-phosphate. TPP is released to form a five-carbon intermediate, 1-deoxy-*D*-xylulose 5-phosphate, which is rearranged and reduced to form 2-*C*-methyl-*D*-erythritol 4-phosphate and is subsequently transformed to yield IPP.

1.4.12 General Approach of Terpene Biosynthesis

The major subclasses of terpenoids are biosynthesised from the basic five-carbon unit, IPP, and from the initial prenyl (allylic) diphosphate, dimethylallyl diphosphate, which is formed by isomerisation of IPP. In reactions catalysed by prenyltransferases, monoterpenes (C10), sesquiterpenes (C15), and diterpenes (C20) are derived from the corresponding intermediates by sequential head to-tail addition of C5 units. Triterpenes (C30) are formed from two C15 (farnesyl) units joined head-to-head, and tetraterpenes (C40) are formed from two C20 (geranylgeranyl) units joined head-to-head. The figure emphasizing this approach is available at the appendix part.

1.4.13 Saponins

Saponins are naturally occurring structurally and functionally diverse phytochemicals that are widely distributed in plants. They form a complex and chemically varied group of compounds consisting of triterpenoid (C30) or steroidal aglycones (C27) linked to oligosaccharide moieties. Numerous studies have been conducted indicating that triterpenoid saponins show different bioactivities, including anti-inflammatory [75], anti-cancer [5], anti-microbial [76], insecticidal and anti-herbivore [77, 78] activities. Saponins are compounds whose active portions form colloidal solutions in water, which produce lather on shaking and precipitate cholesterol. These properties arc due to the amphiphilic character of the molecule (as it contains lipophilic and hydrophilic moieties). Because of their notable pharmacological activities, plants rich in triterpenoid saponins are often utilised as sources of drugs. Yet, the availability of triterpenoid saponins is restricted due to their low yield in crude drug extraction and difficulties in purification.

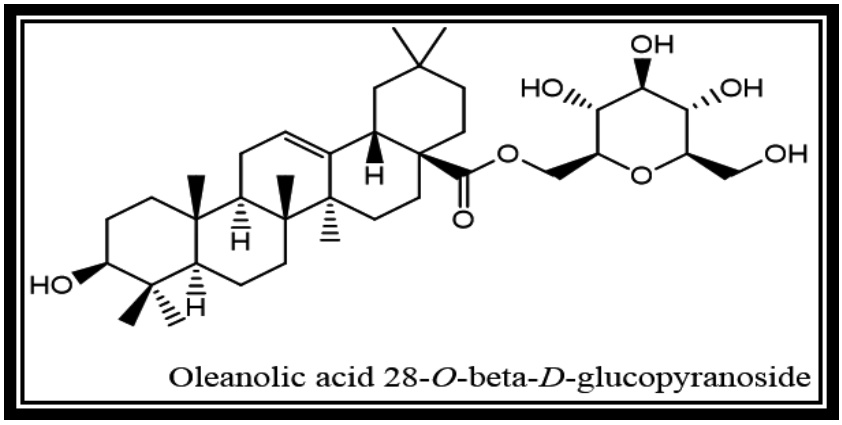
1.4.14 Biosynthesis of Triterpenoid Saponins

The first step in the synthesis of triterpenoid saponins is the cyclisation of 2,3-oxidosqualene to provide one of a number of different potential products [79]. Since the majority of plant triterpenoid saponins are obtained from the oleanane or dammarane skeletons, lupanes are also form a common source [79]. This cyclisation provides an offshoot in the sterol biosynthetic pathway, in which 2,3-oxidosqualene is cyclised, for instance to lanosterol, (in animals and fungi) or to cycloartenol (in plants). Sterols are essential membrane components and also assist as progenitor for hormone biosynthesis. The deprotonation, rearrangement and cyclisation reactions leading to the many products displayed in the figure (the idea which has been initially proposed

as the “biogenetic” isoprene rule) have been studied extensively [80-82]. The enzymatic cyclisation of 2,3-oxidosqualene into sterols moves in the “chair-boat-chair” form to produce the C-20 protosteryl cation, which is later turned to cycloartenol or lanosterol. The 2,3-oxidosqualene cyclases (OSCs), cycloartenol synthase (CS) and lanosterol synthase (LS), respectively are responsible for these cyclisation steps. In contrast, triterpenoid synthesis includes cyclisation to the “chair-chair-chair” formation of the substrate to yield the tetracyclic dammarenyl cation. This cation may thereon be turned to dammarene-like triterpenoids by the OSC dammarenediol synthase (DS), or may be subjected to other rearrangements driving the formation of pentacyclic triterpenoids, such as lupeol, β-amyrin and α-amyrin. Triterpenoid synthesis Pathway graph is existing in the appendix part of this thesis.

1.4.14.1 Oleanolic acid 28-*O*-beta-*D*-glucopyranoside

Oleanolic acid 28-*O*-beta-*D*-glucopyranoside, is a triterpenoid saponin found in *Panax japonicus* var. *major*. Plants are famous for their valuable properties, and the roots of *Panax* species (Araliaceae) are extensively used in Chinese herbal medicine or as a food stuff in Asian countries. The natural ingredients in *Panax* species include a new natural product triterpenoid saponins that can be categorized into two groups depending on their sapogenin skeleton, namely the dammarane- and oleanane-types. Although it has been established that Oleanolic acid-type ginsenosides seem to be typical constituents of ginseng species and are particularly characteristic for *P. ginseng*, *P. pseudoginseng* subsp. *himalaicus* (Himamayan ginseng), *P. vietnamensis* (Vietnamese ginseng), *P. zingiberensis* (ginger ginseng), and *P. japonicus* (Japanese ginseng or Zhujie-Shen) [83, 84].



Oleanolic acid 28-*O*-beta-*D*-glucopyranoside

1.5 Objectives

- Extraction of some unique plants of Yemen and neighbouring areas.
- Biological evaluation of extracts against nematodes, bacteria and fungi.
- Identification of the most active plant extracts and further purification and isolation of the bioactive metabolites.
- Production and subsequent evaluation of nanosized particles of the most active plant parts for activities against selected nematodes, bacteria and fungi.

CHAPTER II

Nematicidal and antimicrobial activities of methanol extracts of 17 plants, of importance in ethnopharmacology, obtained from the Arabian Peninsula

2.1 Introduction

Though significant progress has been achieved during the last 50 year in fighting infectious diseases, they still remain an important cause of morbidity and mortality globally [85]. Infections cause an estimated 50% of all deaths in tropical countries where as much as three million preschool children die each year solely due to infections of the gastrointestinal tract [86]. Besides bacteria and fungi, nematodes transmitted from the soil cause diseases which affect 25% of the world's population, again mostly in the tropics. They are known to lead to anemia and to cause retarded physical and mental growth [87, 88]. The negative effects of nematodes on agricultural livestock are also well documented [89, 90]. As for bacteria Enteropathogenic strains of Gram-negative *E. coli* are known to cause acute & chronic diarrhea, vomiting and fever in infants [91]. The Gram-positive bacterium *S. aureus* can multiply and spread widely in tissues resulting in an enteric infections, boils, skin sepsis, endocarditis, and pneumonia. Their heat-stable endotoxins cause diarrhea, fever, abdominal cramps, and vomiting with an attendant electrolyte imbalance [92]. Owing to their ability to thrive better in warm humid environments, fungal infections are equally problematic and more rampant in the tropics and sub-tropics than any other place in the world. They cause diseases ranging from superficial mycoses, cutaneous mycoses, sub-cutaneous mycoses and systemic mycoses; and are usually very difficult to treat [93]. These organisms also cause diseases in domestic and farm animals resulting in massive economic losses. Unfortunately, many drugs currently available for the treatment of infections are expensive and often not readily available or are easily counterfeited. Furthermore, the development of resistances to these drugs is a major setback to their continued use in humans and livestock [94-96].

Interestingly, the tropics where most of these infections are rampant, at the same time are also amazingly rich in a diversity of plants and fungi. Given the WHO report that medicines derived from plants serve the health needs of approximately 80% of people globally [97], it is important to screen plants that are used in ethnopharmacology and ethnomedicine for activities against nematodes, bacteria and fungi. Such plants may provide new and, above all, inexpensive and locally available drugs and improve the health of people in economically under-developed or developing countries.

In the light of the above, this study has investigated the nematicidal and antimicrobial properties of methanolic extracts of seventeen plants used in ethnopharmacology and ethnomedicine around the tropics and sub-tropics, and particularly in Saudi Arabia and Yemen. The primary aim of this investigation has been to uncover phytochemical products that can be produced locally and in better sufficient commercial quantities and used to improve Medicine and Agriculture, especially in some of the developing economies of the world. Details of the plants, the parts harvested and their uses in Folk Medicine have been obtained from published literature and traditional users of the plants [10, 98-100], and are summarized in Table 2.1.

2.2 Materials and Methods

This chapter describes the methods and results of a study which we have published recently by the Jacob group (Al-marby A. *et al*, 2016). The description of experimental part and results will therefore be concise, as further details may be found in our literature.

The plant materials used as part of this study were collected between the months of March and April 2014 at different locations in Al Baha town, and its outskirts, Saudi Arabia. *Dendrosicyos and Dracaeana* plants were collected from the island of Socotra between November and December 2014. Those plants were identified taxonomically at the Department of Botany, Faculty of Science, Aden University, Republic of Yemen. Voucher specimens of the plant materials were deposited at the Pharmacognosy Department, Faculty of Clinical Pharmacy, Al Baha University, Saudi Arabia for the Saudi plants and at the Department of Botany, Faculty of Science, Aden University, Yemen for *Dracaena* and *Dendrosicyos* plants. These institutes hold permission to harvest, process and also donate small amounts of plant specimens for research purposes.

Figure 2.1. This is the first figure of the second chapter showing the map of Arabian Peninsula and the areas where the plants have been collected.

2.2.1 Nanosizing of Dried Fruit of *S. incanum*

This part of study was performed in cooperation with Dipl. Pharm. Sharoon Griffin, PhD student at the Institute of Bioorganic chemistry, University of Saarland.

In brief, nanosizing of the dry and locally pre-processed powders obtained from Yemen (see figure 3.1, chapter 3) was performed by a combination of rotor-stator high speed stirring (HSS) and subsequent high pressure homogenization (HPH) in the presence of the natural surfactant Plantacare. The latter is a plant derived, food-grade uncharged tenside commonly used to stabilize particles destined for medical or agricultural applications. Particle size analysis was performed using Photon Correlation Spectroscopy (PCS), Laser Diffraction (LD) and light microscopy (MP Biomedicals, Solon, OH, USA).

In the first step, the powdered, crude plant material was subjected to dry milling using a Fast Prep 24 Instrument (MP Biomedicals, Solon, OH, USA). Precellys Kits (Bertin Technologies, Montigny-le-Bretonneux, France) were used as a source of ceramic beads for dry milling (metallic beads were avoided as they may contaminate the sample with biologically active metal ions). After initial dry milling, and for the purpose of stabilization (*i.e.*, avoidance of aggregation), the material was suspended in 1% Plantacare®2000 UP (alkyl-polyglycoside, BASF, Ludwigshafen, Germany) in distilled water to yield 1% macro-suspensions of finely milled plant materials.

Subsequent pre-homogenization of these macro-suspensions was performed using a MICCRA D-9 Homogenizer–Disperser (MICCRA GmbH, Müllheim, Germany). This homogenization procedure was followed by further homogenization employing an APV Gaulin LAB 40 (APV GmbH, Mainz, Germany) High Pressure Homogenizer. The initial homogenization included three cycles at 200, 500 and 1000 bar pressure, respectively, whereas final homogenization was achieved through ten consecutive cycles at 1500 bar pressure.

In order to assess the general quality and properties of the homogenized samples, three different analytic techniques were used during the various stages of milling and homogenization, namely; LD, PCS and light microscopy. LD measurements were performed on a Mastersizer 2000, PCS measurements on a Zetasizer Nano ZS (both from Malvern Instruments, England, UK). The shape and size of the particles was assessed further by light microscopy, employinga Leica DM 1000 LED microscope (Leica icrosystems, Wetzlar, Germany). Microscopy also provided basic information regarding the homogeneity of the samples.

2.2.2 Preparation of Plant Extracts

The preparation of plant extract has been reported by us in the literature (Al-Marby A,*et al*, 2016). The plant parts harvested were air-dried under the shade at ambient temperature and powdered with a blender. The powdered plant material (10 g) was extracted with absolute methanol (4 × 100 mL). The extractions were carried out at room temperature with the constant shaking of the extraction set-up. Thereafter, the mixtures were filtered, and the filtrate evaporated to dryness in vacuo at 40°C to yield the methanol extracts subsequently used as a part of our studies. The yields of each dried extract were calculated in %. The resulting dried crude extracts were stored at 4°C until they were analyzed for nematicidal and antimicrobial properties. These plants samples were harvested by Prof. Nasser A. Awadh-Ali group, Department of Pharmacognosy, Faculty of Clinical Pharmacy, Al Baha University, Saudi Arabia.

2.2.3 Nematicidal Activity

Steinernema feltiae was purchased from Sautter & Stepper GmbH (Ammerbuch, Germany), as a powder cake product and stored in the dark at 4 ºC. Fresh samples were ordered before each experiment and each opened batch was discarded after six days. Prior to each experiment, a homogeneous mixture of nematodes was prepared by suspending 200 mg of powder cake in 50 mL of distilled water at 27ºC in order to revive the nematodes. The suspension was allowed to stand at room temperature with occasional rocking and in moderate light for 30 min. Thereafter, the viability of the nematodes in suspension was determined with a microscope at four-fold magnification (TR 200, VWR International, Belgium). A viability of more than 80 % was considered optimal and seen as a prerequisite for each experiment.

Each plant extract (100 mg) was dissolved in 5 mL of 2% DMSO in water to yield a 20 mg/mL stock solution. From this stock solution, a series of dilutions in water was prepared with 0.5, 1, 3, 5, 10 and 15 mg/mL solutions which were then used for the experiments. To each well in the 96- well plate, 10 µl of the nematode suspension was added (which usually contains 30-40 nematodes per well). Thereafter, 100 µl of each concentration of the plant extracts was added to each well. The control experiment was performed with the DMSO/water vehicle in place of the extracts. The well plates were then assessed immediately for viability under the microscope before incubation in the dark at room temperature for 24 h. After 24 h, 50 µl of distilled water at 50 °C was added to each well to stimulate the movement of the nematodes. Thereafter live and dead nematodes were counted under the microscope (four-fold magnification). Each

concentration was tested in three different wells per experiment, and each experiment was repeated three times to yield a total of nine repeats per individual experiment.

The viability of the nematodes was expressed as percentages. The viability values were calculated using the equation:

$$\text{Viability (\%)} = \left[\frac{V_{24h}}{V_{0h}}\right] \times 100$$

Where V_{24h} is number of live nematodes after 24 h and V_{0h} is number of live nematodes at 0 h.

2.2.4 Antimicrobial Activity

Two bacterial strains, *Staphylococcus carnosus* TM 300 and *Escherichia coli K2* representing Gram-positive and Gram-negative bacteria species respectively, as well as the fungus *Saccharomyces cerevisiae* were chosen as representative model organisms for the antimicrobial investigations.

The disc diffusion assay [101] was used to determine the antimicrobial activities of the extracts investigated. Nutrient Luria-Bertani and Yeast Extract-Peptone-Dextrose (YPD) (Sigma-Aldrich, Steinheim, Germany) were used as media. Sterile plates were inoculated evenly using sterile swab sticks. Sterile qualitative filter paper discs of 6 mm diameter (VWR International GmbH-Darmstadt, Deutschland, ref. No. 601110, lot.06513) were impregnated with 20 µl of each extract solution (equivalent to 4 mg/disc). The paper discs were allowed to dry before being gently placed on the surface of the inoculated agar plates, at positions that were equidistant from each disc. The plates were kept for 3 h in a refrigerator to enable pre-diffusion of the substances into the media. A mixture of penicillin-streptomycin-smphotericin-B was used as positive control, whilst the solvent (methanol) was used as negative control. Plates inoculated with bacteria and yeasts were incubated for 18-24 h at 37ºC. Inhibition zone diameters around each disc (diameter of inhibition zone plus the diameter of the disc) were measured and recorded at the end of the incubation time. An average zone of inhibition was calculated for the three replicates. An inhibition zone of 8 mm or more was considered as indicative of high antimicrobial activity [102].

The Minimum Inhibitory Concentration (MIC) was determined using the broth microdilution method of Bolivar with slight modifications [103]. Fresh cultures of bacteria on LB agar and

yeast on YPD agar were prepared and incubated for 18-24 h. From these cultures, inocula were prepared by suspending colonies of the respective organisms in sterile 0.85% NaCl solution and then adjusted to 0.5 of the McFarland standard (1.5×10^8 CFU/mL for bacteria and 1.5×10^6 CFU/mL for yeast). Different concentrations (0.5, 1.0, 2.5, 5.0 and 10 mg/mL) of the plant extracts were added to the LB or YPD broth in 96-well plates and the inocula were subsequently added to each well. Thereafter the plates were incubated at 37°C for 18-24 h. The assay was conducted in triplicate and three independent experiments were performed on different occasions implying that each specific experiment was repeated a total of nine times. Antibacterial activity was detected by adding 20 µL of 0.01% sodium resazurin (Sigma) and incubating the plates for 1 h. A change from blue to pink indicates a reduction of resazurin and therefore bacterial growth. The MIC is defined as the lowest drug concentration that prevents the colour change. In the case of *Saccharomyces cerevisiae*, 50 µL of 0.5% triphenyltetrazolium chloride (TTC, Sigma) were added and the plates were incubating for 3 h. The MIC was generally defined as the lowest concentration that prevents this colour change by TTC staining agent. A mixture of penicillin, streptomycin and amphotericin was used as a reference antibiotic control at a concentration 10 µg penicillin, 10 µg streptomycin and 25 µg amphotericin B (Sigma-Aldrich, Steinheim, Germany).

Statistics

The data generated was subjected to descriptive statistical analysis and the results are presented as mean ± SEM. Differences between the means (test *versus* control) were assessed for statistical significance using a one-way ANOVA test with the significance threshold fixed at $P < 0.05$. The GraphPad Prism software (GraphPad Inc., USA) was used for all statistical analyses. The results are presented in Tables and Figures and statistical significances in the Figures are marked as *, **, or *** for the $P < 0.05$, < 0.01 or < 0.001, respectively.

2.3 Results

For all plant materials under investigation, suitable extracts could be obtained in good quality and yield. Table 2.1, briefly summarises the individual yields of extraction for the different extracts. It should be emphasised that the fruits of *W. somnifera* yielded the most extract (8.5%) while the leaves of *A. biebersteinii* yielded the least (2.6%) (Table 2.1). The average yield was approximately 5%. The extracts obtained were subsequently investigated for biological activity, first against the nematode *S. feltiae,* and subsequently against different bacteria and the fungus *S. cerevisiae.*

Table 2.1. Selected medicinal and aromatic plants of Yemen

Species	Plant Family	Part tested (Yield in %)	Local name	Traditional uses
Achillea biebersteinii Afan.	Asteraceae	Fl, L (1.5,2.6)	Thafra	antispasmodic and for[1] kidney inflammation.
Calotropis procera (Aiton) W.T.Aiton	Asclepiadaceae	L (4.2)	Alashur	leprosy and filariasis
Chenopodium murale L.	Amaranthaceae	F (7.3)	Jakheara	leishmaniasis[1]
Dendrosicyos socotranus Balf.f.	Cucurbitaceae	L (4.3)	Al-khiayar	severe constipation[2]
Dodonaea viscosa Jacq.	Sapindaceae	L (3.5)	Shath	chronic ulcers, burns, leishmaniasis[2]
Dracaena cinnabari Balf.f.	Asparagaceae	Re (3.5)	Dam Al-akhawaen	antispasmodic, wound healing[2]
*Euphorbia helioscopia*L.	Euphorbiaceae	AP (4.2)	Al-dehin	antiseptic
Lavandula dentata L.	Lamiaceae	AP (2.9)	Al-shiah	As antispasmodic, antiseptic when the leaves chewed[1]
Pulicaria crispa SCH.BIP	Asteraceae	AP (3.1)	Arararabi	antimalarial, stomache disorders[2]
Punica granatum L.	Punicaceae	Fl (2.5)	Al-roman	anthelmintic, antiseptic[2]
Ruta chalepensis L.	Rutaceae	L (5.2)	Al-shathab	antimicrobial[2]
Solanum incanum L.	Solanaceae	F, L (7.6,3.9)	Al-hadak	antiseptic[2] leaves as dressing for healing wounds, paste of fruits for treating leishmaniasis[2]
Verbesina encelioides (Cav.) Benth & Hook. F. ex a. Gray	Asteraceae	L (3.7)	Aafeara	wounds, skin diseases[2]
Withania somnifera (L.) Dunal	Solanaceae	F, L (4.6,8.5)	Alobeb	chronic dermatitis[2]

[a] : AP, aerial parts; F: fruits; L: leaves; Re: resins; Fl: flowers[b]: [1]most information obtained from a reference [8-10] and [2]interviewing the local population.

2.3.1 Nematicidal Activity

In the case of nematodes, a distinctively different activity could be observed for the methanolic extracts of aerial parts of *C. murale, P. crispa, E. helioscopia, L. dentata*, leaves of *D. viscosa, V. enceloides, A. biebersteinii*; leaves of *W. somnifera, C. procera, D.socotranus, R. nervosus, R. chalepensis, S. incanum; resins of C. myrrha, D. cinnabari;* fruits of *S. incanum, W. somnifera*; flowers of *P. granatum, A. biebersteiniii* and *A. nobilis*. These activities are summarised below in Table 2.2 and figures (2.2 - 2.10). Whilst some extracts exhibited considerable activity against *S. feltiae*, others were hardly active. The extract from the leaves of *S. incanum* was the most potent as it resulted in statistically significant mortality of the nematodes at the lowest concentration tested (0.5 mg/mL) (fig. 2.2). Purely for comparison: this concentration corresponds to 2 mM of a chemically pure compound with a molecular weight of 250 g/mol..

The next most active extracts in order of activity were those from *S. incanum* and *W. somnifera* fruits, *R. nervosus* leaves, *P. crispa* aerial parts, and resins of *C. myrrha*, each showing statistically significant nematicidal activity at a concentration of 1 mg/mL. This was followed by extracts from *E. helioscopia, D. viscosa, A. biebersteinii, P. granatum, D.socotrana* and *D. cinnabari*, each with statistically significant nematicidal activity at a concentration of 2.5 mg/mL. In contrast, extracts from *C. murale* (10 mg/mL), *L. dentata* (10 mg/mL) and *C. procera* (20 mg/mL) were hardly effective as nematicides.

Table 2.2 Nematicidal activity of plant extracts against *S. feltiae*

Species	Concentration of Extract (mg/mL)						
	0.5	1.0	2.5	5.0	10.0	15.0	20.0
Achillea biebersteinii	–	–	**	**	***	nt	nt
Anthemis nobilis	–	–	–	*	***	nt	nt
Calotropis procera	–	–	–	–	–	–	***
Chenopodium murale	–	–	–	–	**	nt	nt
Commiphora myrrh	–	*	**	***	***	nt	nt
Dendrosicyos socotranus	–	–	–	*	**	nt	nt
Dodonaea viscosa	–	–	**	***	***	nt	nt
Dracaena cinnabari	–	–	*	**	***	nt	nt
Euphorbia helioscopia	–	–	***	***	***	***	***
Lavandula dentata	–	–	–	–	***	***	***
Pulicaria crispa	–	*	**	***	***	nt	nt
Punica granatum	–	–	*	**	***	nt	nt
Rumex nervosus	–	**	***	***	***	nt	nt
Ruta chalepensis	–	*	***	***	***	nt	nt
Solanum incanum leaf	***	***	***	***	***	nt	nt
***Solanum incanum* fruit**	–	**	***	***	***	nt	nt
Verbesina encelioides	–	–	–	**	**	nt	nt
Withania somnifera leaf	nt	–	–	*	***	nt	***
***Withania somnifera* fruit**	–	**	***	***	***	nt	nt

nt stands for "not tested"; *, **, and *** show significant differences at $P < 0.05$, < 0.01 and < 0.001 respectively. The green color represents the highly active extracts, the yellow color represents the secondly active extracts and the red color represents the less active extracts.

Figure 2.2 shows the nematicidal activity of three plant extracts at different concentrations (0.5-10 mg/mL). The nematicidal activity of leaf extracts of *Solanum incanum* exhibited the highest nematicidal activity against *S. feltiae* at 0.5 mg/mL ($P < 0.001$) than *Rumex nervosus* and *Ruta chalepensis* which also have been considered also considerably active at 1.0 mg/mL ($P < 0.001$). The plants responsible for activity have been displayed below the viability-concntration diagram.

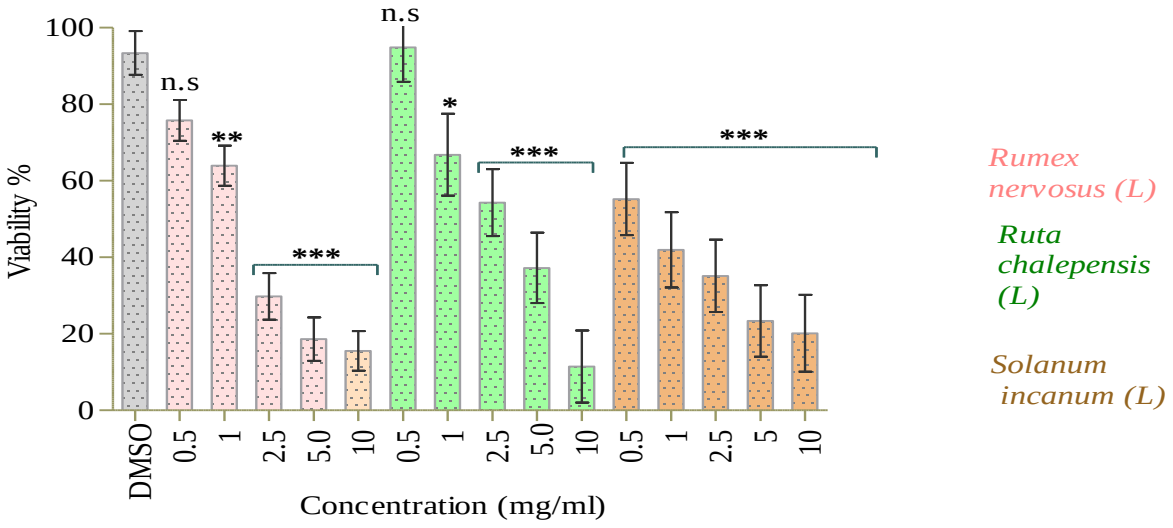


Figure 2.*2* Nematicidal activity of leaf extracts of *R. nervosus, R. chalepensis* and *S. incanum*

These images reflect three plants which showed high activity against *S. feltiae*

Figure 2.3. demonstrats the nematicidal activity of two plant extracts at different concentrations (0.5-10 mg/mL). The nematicidal activity of resin extracts of *Commiphora myrrha* had more toxicity against *S. feltiae* at 1.0 mg/mL than *D. Cinnabari* at 2.5 mg/mL ($P < 0.05$) in which the activity has been considered moderate in comparison to *C. myrrha, S. incanum and rumex nervosus*.

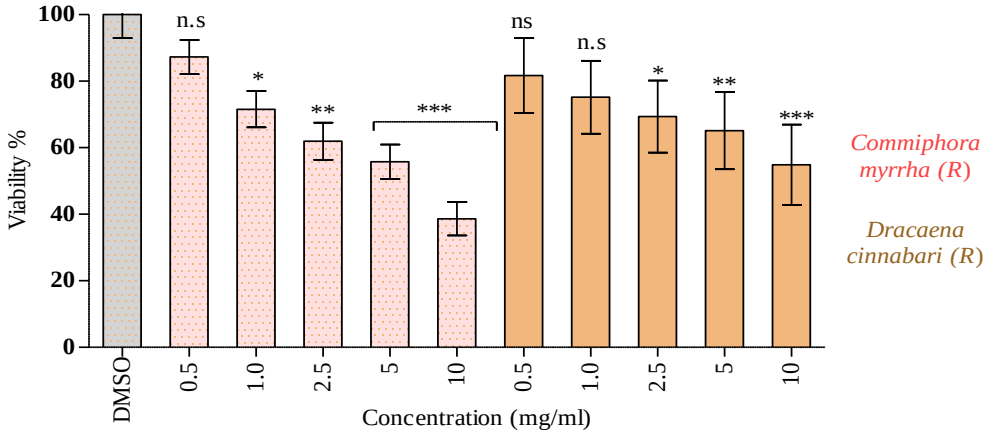
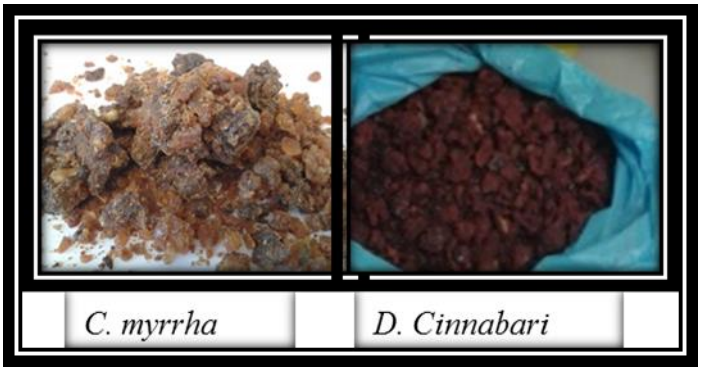


Figure 2.3. Nematicidal activity of resin extracts of *C. myrrha* and *D. Cinnabari*



This picture shows the resins of the two plants which showed high to moderate activity against *S. feltiae.*

Figure 2.4**.** highlights the nematicidal activity of leaf extracts of *Solanum incanum* and *Withania somnifera* against *S. feltiae***.** The nematicidal activity of the fruit extracts of *S. incanum* and *W. somnifera* revealed the same nematicidal activity against *S. feltiae* at 1.0 mg/mL. Considering this activity at such a low concentration, the two plant extracts have been considered considerably active.

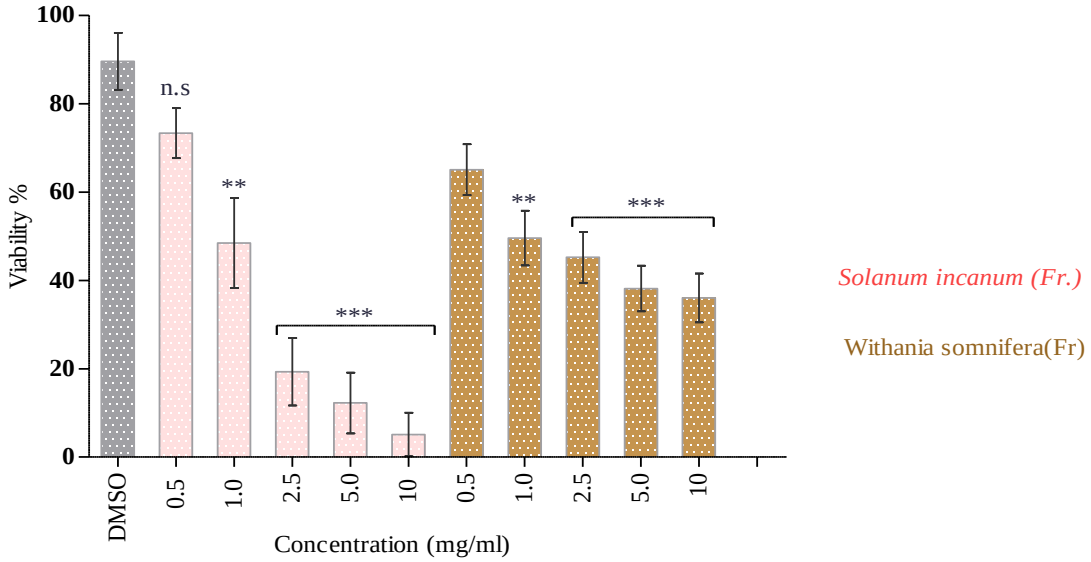


Figure 2.4. Nematicidal activity of the fruit extracts of *S. incanum* and *W. somnifera*

This picture represents the fruits of the two plants which exhibited high activity against *S. feltiae*

Figure 2.5**.** highlights the nematicidal activity of flower extracts of *Punica grantum*, *Achillea biebersteinii* and *Anthemis nobilis* against *S. feltiae.* In this Figure, the extracts revealed moderate (2.5 mg/mL) to mild activity (5.0 mg/mL) in comparison to the extracts of *R.* nervosus (1.0 mg/mL), *R. chalepensis* (1.0 mg/mL), and *S. incanum* (0.5 mg/mL) as the nematicidal activity here started at 2.5 mg/mL for *P. grantum* and *A. biebersteinii* and at 5.0 mg/mL for *A. nobilis.*

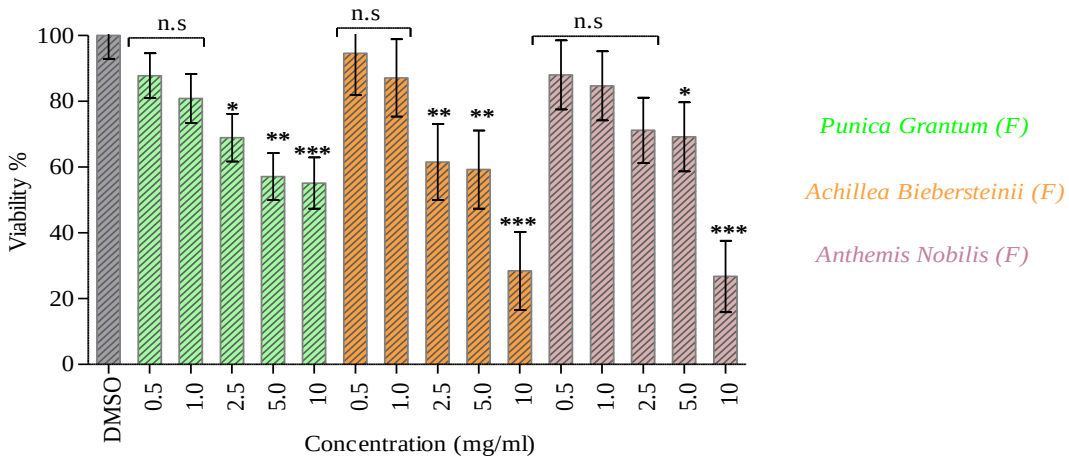


Figure 2.5. Nematicidal activity of flower extracts of *P. grantum*, *A. biebersteinii* and *A.Nobilis*

This picture depicts the flowers of the three plants which exhibited moderate to mild activity against *S. feltiae*

Figure 2.6**.** highlights the nematicidal activity of leaf extracts of *Dodonaea angustifolis*, *Verbesina enceloides* and *Achillea biebersteinii* against *S. feltiae*. In this Figure, the leaf extracts displayed moderate (2.5 mg/mL) to weak (10 mg/mL) activity in comparison to the ones before as the nematicidal activity here started at 2.5, 5.0 and 10 mg/mL for *D. angustifolis*, *V. enceloides* and *A. biebersteinii* respectively, whereas the ones before exhibited toxicity at very low concentration (0.5 - 1.0 mg/mL)

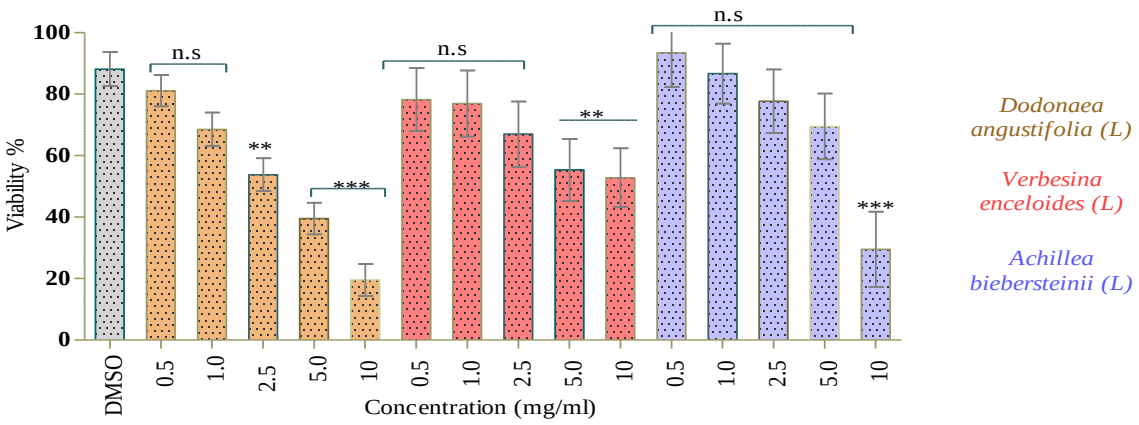
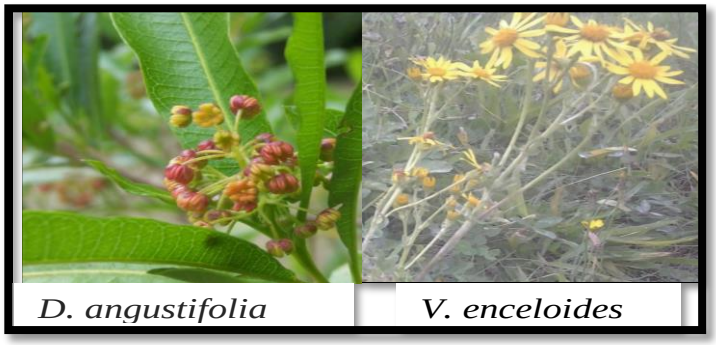


Figure 2.6. Nematicidal activity of extracts of *D. angustifolis, V. enceloides* and *A.biebersteinii*



This image portrays two plants which showed moderate to mild activity against *S. feltiae*. *A. biebersteinii* picture has been shown in the figure before, in which the plant showed weak nematicidal activity against *S. feltiae*.

Figure 2.7. highlights the nematicidal activity of the extracts of *Euphorbia helioscopia* and *Lavandula dentata* against *S. feltiae*. In this Figure, the *E. helioscopia* extracts revealed higher activity in comparison to the *L. dentate* extract as the last was showing weak nematicidal activity at 10 mg/mL, whereas *E. helioscopia* revealed moderate activity at 2.5 mg/mL.

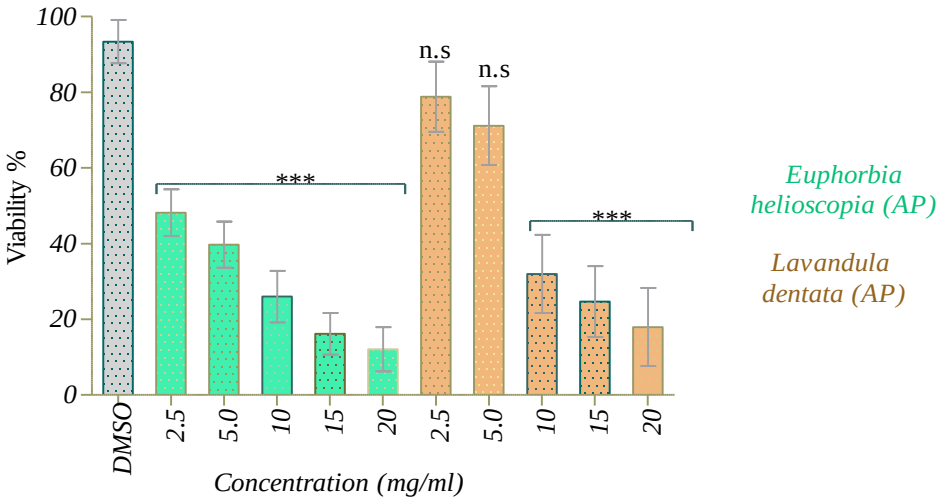


Figure 2.7. Nematicidal activity of extracts of *E. helioscopia* and *L. dentate*

This image portrays two plants which showed moderate to weak activity against *S. feltiae*

Figure 2.8**.** highlights the nematicidal activity of the extracts of *Chenopodium murale* and *Pulicaria crispa* against *S. feltiae*. In this Figure, *P. crispa* extracts revealed significant nematicidal activity at lower concentration (1.0 mg/mL) than the *C. murale* extracts (10 mg/mL).

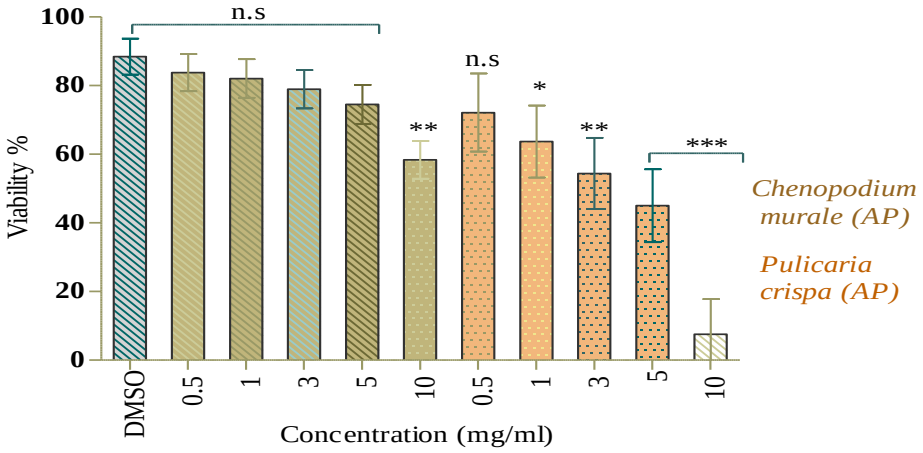
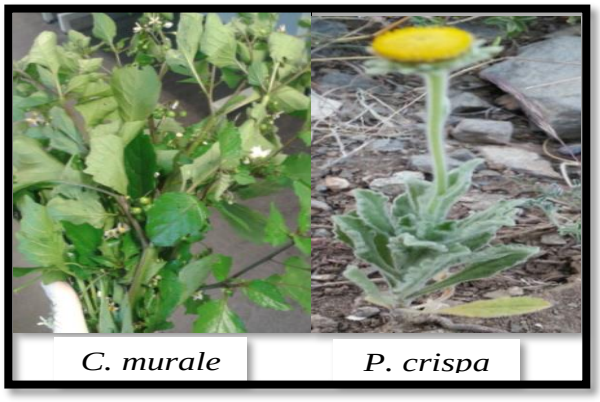


Figure 2.8. Nematicidal activity of extracts of *C. murale* and *P. crispa*



This image portrays two plants which exhibited weak to high activity against *S. feltiae*

Figure 2.9**.** highlights the nematicidal activity of the leaf extracts of *W. somnifera* and *C. procera* against *S. feltiae***.** In this Figure, the *W. somnifera* extracts revealed mild nematicidal activity at concentration (5.0 mg/mL) while *C. procera* showed very low activity at (20 mg/mL).

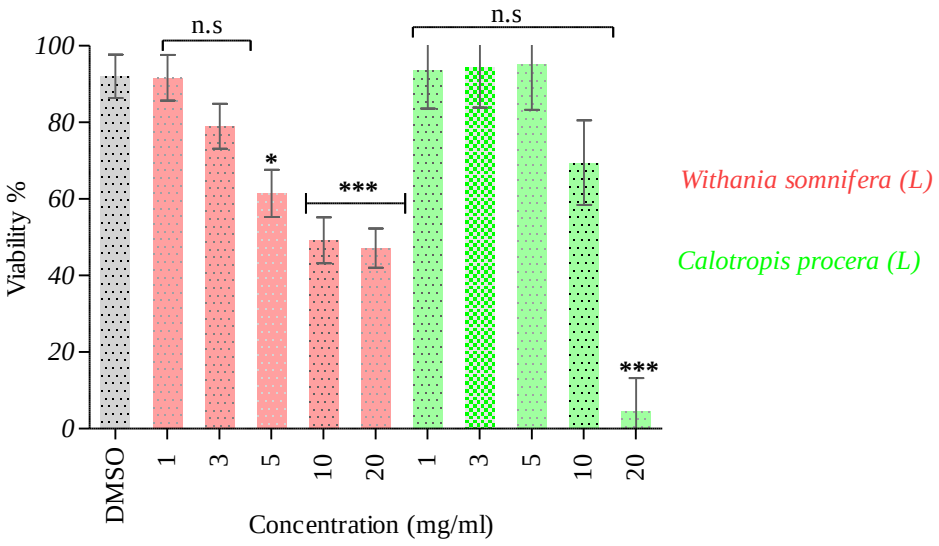


Figure 2.9. Nematicidal activity of extracts of *W. somnifera* and *C. procera*

This image portrays two plants which showed mild nematicidal activity (*W. somnifera*) to nonactive (*C. procera*) against *S. feltiae*

Figure 2.10**.** highlights the nematicidal activity of the leaf extracts of *D. socotranus***.** In this Figure, the water extract showed nematicidal activity (2.5 mg/mL) more than the methanol extract (5.0 mg/mL).

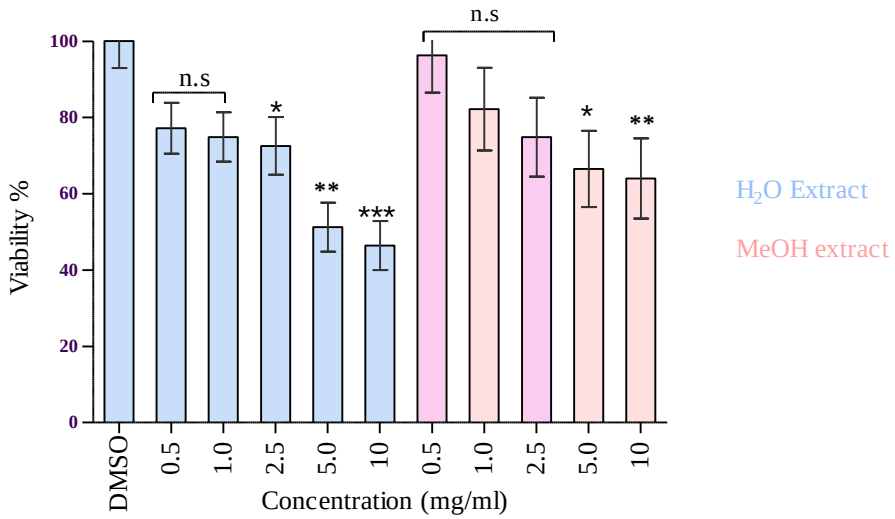
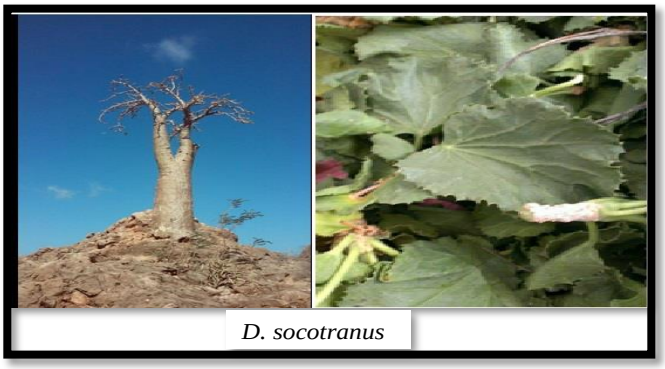


Figure 2.10. Nematicidal activity of H_2O and MeOH extracts of *D. socotranus*



Picture of *D. socotranus* plant which exhibited moderate nematicidal activity against *S. feltiae*.

2.3.2 Antimicrobial Activity

The extracts were then tested for their activity against two bacteria, *S. carnosus* and *E.coli*, which are representative of Gram-positive and Gram-negative bacteria, respectively. An activity profile similar to the one in nematodes could be observed. In the case of *S. carnosus*, the extract from the fruits of *S. incanum*, but also extracts of the resins of *D. cinnabari* and *C. myrrha* and the aerial parts of *E. helioscopia* resulted in high zones of inhibition and low MIC values against this strain of *Staphylococcus*. *E. coli* appeared to be even more sensitive to a wider range of extracts, such as extracts obtained from the leaves of *C. murale*, *S. incanum*, *D.socotranus*, *A. biebersteinii*, and *V. enceloides*, from the flowers of *P. granatum* and *A. nobilis*, the resins of *C. myrrha*, and the aerial parts of *P. crispa*. All of these extracts resulted in high zones of inhibition and low MIC values against *E. coli*.

Ultimately, extracts from the resins *C. myrrha*, the leaves of *D .socotranus* and *S. incanum*, and from the flowers of *A. nobilis*, provided the best antibacterial activity for both Gram-positive and negative bacteria (Table 2.3).

Table 2.3 Antimicrobial activity of plant extracts against,*S. carnosus, E. coli* and *S. cerevisiae.*

Botanical name	Inhibition zones (mm) Mean ± SD			MIC (*mg* /mL)		
	S.c	*E.c*	*S.ce*	*S.c*	*E.c*	*S.ce*
Achillea biebersteinii	8 ± 2	13 ±4	–	4.0	**2.5**	**1.5**
Anthemis nobilis	8 ± 3	15 ± 4	–	4.0	**2.5**	**2.5**
Calotropis procera	–	–	–	–	–	–
Chenopodium murale	15 ± 5	13 ± 4	–	2.5	2.5	2.5
Commiphora myrrha	12 ± 3	13 ± 6	–	**2.5**	**2.5**	–
Dendrosicyos socotranus	8 ± 2	12 ± 2	–	4	2.5	5.0
Dodonaea viscosa	–	–	–	–	–	–
Dracaena cinnabari	13 ± 4	15 ± 2	–	2.5	4	–
Euphorbia helioscopia	12 ± 2	15 ± 3	–	2.5	2.5	–
Lavandula dentata	–	–	–	–	–	10.0
Pulicaria crispa	–	11 ± 4	–		**2.5**	**2.5**
Punica granatum	8 ± 2	6 ± 1	–	4	2.5	5.0
Rumex nervosus	–	5 ± 1	–	–	4	5.0
Ruta chalepensis	–	15 ± 3	–	-	2.5	2.5
***Solanum incanum* (leaf)**	8 ± 2	13 ± 4	–	**4.0**	**2.5**	10.0
***Solanum incanum* (fruit)**	12 ± 2	15 ± 4	–	**2.5**	**2.5**	**2.5**
Verbesina encelioides	–	11 ± 2	–	–	2.5	–
Withania somnifera	–	10 ± 2	–	–	4	5.0
Withania somnifera(fruits)	–	10 ± 3	–	–	4	–
0.01 mg penicillin, 0.01 mg streptomycin and 0.025 mg amphotericin B	30 ± 1	30 ± 2	–	0.01	0.01	0.025

S.c, E.c and S.ce represent *Staphylococcus carnosus* TM 300; *Escherichia coli* K2; and *Saccharomyces cerevisiae, respectively*; no activity observed for negative control. The green color here represents the highly active extracts, while the yellow represents the second more active extracts and the red represents the less active extracts against most of the microorganisms tested.

A very similar picture also emerged with the single-cell fungus *S. cerevisiae* (Table 2.3). Since the broth microdilution method is known to be more reliable and useful in testing plant extracts for activity [104], we have collected data via both, the disc diffusion and the microdilution method. Indeed, in our hands, the latter was also more reliable with regard to toxicity against *S. cerevisiae*. Extracts of the, leaves of *A. biebersteinii, C. murale* and *R. chalepensis*, aerial parts of *P. crispa,* flowers of *A. nobilis* and fruits of *S. incanum* inhibited the growth of *S. cerevisiae* at low concentrations. Other extracts, such as those of the leaves of *R. nervosus* and *W. somnifera* and the flowers of *P. granatum* also inhibited the growth of *S. cerevisiae*, though to a lesser extent. The extracts from the other plants showed little or no effect with respect to inhibiting the growth of yeast.

Overall, extracts from 14 of the 17 plants under investigation exhibited high activity against at least one organism tested while the other three plants, *D. viscosa*, *C. procera* and *L. dentata* showed only limited activities against all of the organisms tested. Among the active plants, *S. incanum* clearly attracted most attention. Intriguingly, extracts of the fruits of *S. incanum* (fig. 2.11), appeared to possess high activity against all four organisms tested, while the leaves of this plant had limited activity against *S. cerevisiae* and *S. carnosus*.

Figure 2.11. The most active plants against three of the four organisms tested.

Preparations from other plants were also rather active, such as extracts of the aerial parts of *P. crispa* and extracts of the resins of *C. myrrha* (fig. 2.11), which gave high activities against three of the four organisms tested.

Less active, but still of interest, due to possible harvesting and processing, were the flowers of *A. nobilis* and leaves of *A. biebersteinii* (fig. 2.12), which showed high activities against two of the four organisms tested, namely against *Steinernema feltiae*, *Staphylococcus carnosus*, *Escherichia coli* and *Saccharomyces cerevisiae*, respectively.

Figure 2.12. Two of most active plants against two of the four organisms tested

2.4 Discussion

Though synthetic nematicides and antimicrobials used in Medicine and Agriculture are effective and rapid-acting, the challenges of resistance to these agents by microorganisms and the concerns to human health and the environment raised by their use in agriculture have spurred research efforts at developing "green" plant-based or plant derived alternatives. In fact, various phytochemicals are known to be safe to both humans and the environment. When used in agriculture, as in the case of nematicides, they are "biodegradable" and usually do not persist in the fields for longer periods of time than is really necessary [105]. Naturally, therefore, research efforts have been geared towards plants that are used in Folk Medicine and Agriculture from different cultures, with a view to identifying those that can be used to develop "green" phyto-protectants, antimicrobials and pesticides. Many of these plants contain a cocktail of phytochemicals, true treasure chests for bio-activity against the myriad of microorganisms that pose challenges to Medicine and Agriculture, especially in developing countries of the tropics.

Our study has therefore investigated the nematicidal and antimicrobial properties of methanolic extracts of leaves, aerial parts and resins from seventeen plants used in traditional ethnopharmacology and ethnomedicine in Saudi Arabia, Yemen and neighbouring countries of the Arabian Peninsula. Deliberately, methanol was used as the solvent of extraction based on our experience over the years in working with plant products and reports from other researchers working on similar subjects [106]. Indeed, several studies have shown that methanol is the solvent of choice for the extraction of antimicrobial constituents of plants [107-110]. Compared to ethanol, methanol is also less controversial culturally.

Interestingly, extracts from five of the plants studied -namely *S. incaum*, *P. crispa, C. myrrha, A. biebersteinii* and *A. nobilis* exhibited high activities at low concentrations against two or more of the organisms tested. *Solanum incanum*, in particular, which is also known as Jericho tomato, attracted our particular interest, as several parts of this plant seem to be extraordinarily toxic. This plant is particularly promising for several reasons. Firstly, it grows readily, widely and requires little care. Secondly, it can be harvested and processed easily. Thirdly, it is not used for any other purposes, hence has little "value". And finally, its extract is amenable to further purification, which may improve activity significantly. Indeed, the literature available to date on the most active plants show that *S. incanum* is rich in phytochemicals (Fig. 2.13) such as incanumine, solasodine, carpesterol, *β*-sitosterol, stigmasterol and khasianine [108].

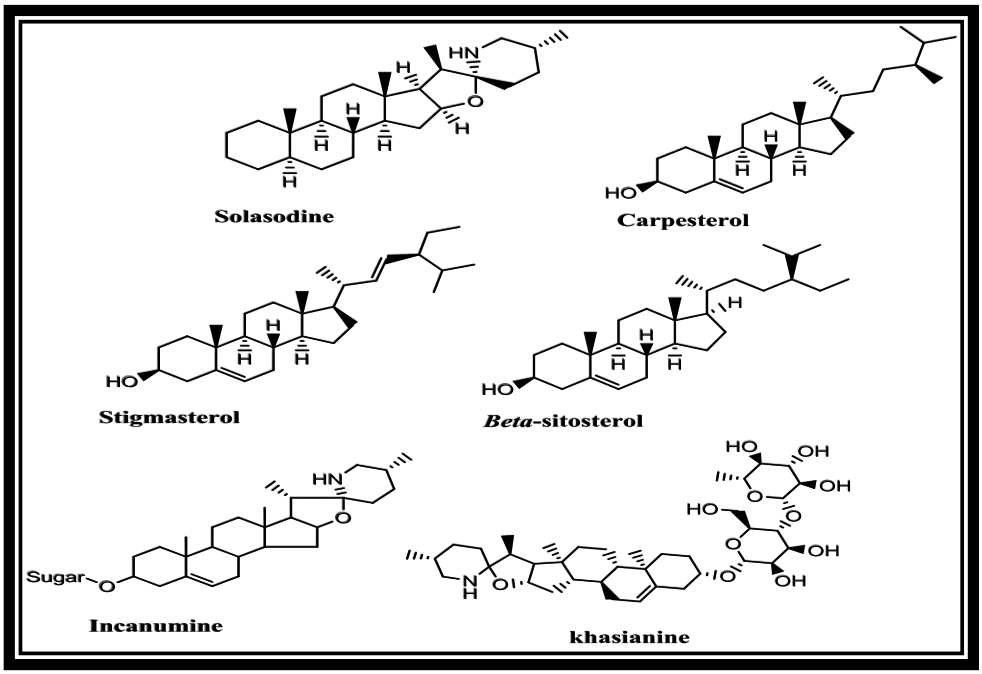


Figure 2.13. Bioactive steroidal alkaloids and phytosterols found in *S. incanum*

Lin *et al*. [111] also reported the presence of quercetin, kaempferol, and astragalin (Fig. 2.14) in parts of the plant. Besides, members of the *Solanaceae* family have been known for a long time to possess antibiotic activity, which is likely due to the presence of glycosides and alkaloids [112].

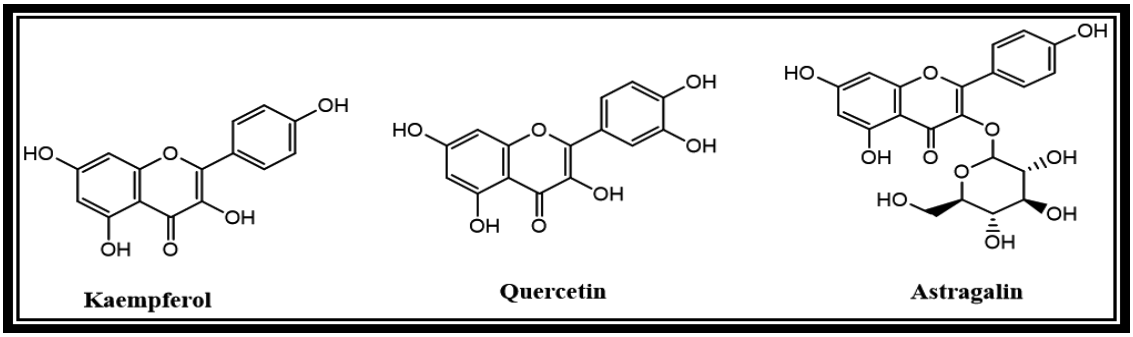


Figure 2.14. Some *S. incanum* flavonoids phytochemicals

Ultimately, a partially purified, stabilised and appropriately conserved extract of the fruits and/or leaves of the Jericho tomato may well be suitable for applications in the field of agriculture and possibly also medicine. Within this context, possible toxic effects on humans and higher animals need to be considered and addressed in earnest, and the possibility of a synergism in the action of the individual chemical components contained within the different extracts may have to be accounted for.

Indeed, the activity observed for extracts of *S. incanum* and the various other extracts may arise from a variety of chemical components and biochemical mechanisms. Though we do not currently possess data on the mechanisms of action, it is known, for instance, that plant extracts often exert their lethal effects through the disruption of cell membrane permeability in organisms that come in contact with them [113]. Within this context, variations observed in the effectiveness of the extracts in killing the nematodes, bacteria or fungi may indeed be explained by the

biological differences which exist between the organisms, for instance, differences in cell wall structure and composition. In fact, it has been reported in other studies, and corroborated by this study, that plant extracts often show a higher activity against bacteria when compared to fungi and this may, in part, be due to differences in the cell wall synthesis and structure [110, 114, 115]. Specific phytochemicals, such as tannins, furthermore, have the capacity to bind to and subsequently denature or disrupt proteins, and if such proteins are vital structural or catabolic proteins such substituents may well result in the death of the organism [116-118]. There are obviously many other possible mechanisms and mode(s) of action associated with the plethora of phytochemicals found in those plants.

Whilst we cannot list all of the ingredients contained within our most active extracts and their suspected mode(s) of action, it is worth mentioning a few. Many potent phytochemicals have been found in *P. crispa,* especially sesquiterpene lactones and guauanolide Sesquiterpenes (Fig. 2.15) [119, 120]. Possibly, the *in vitro* antimicrobial activity and known antileishmanial activities of the methanolic extract of this particular plant are due to the presence of these phytochemicals [121, 122].

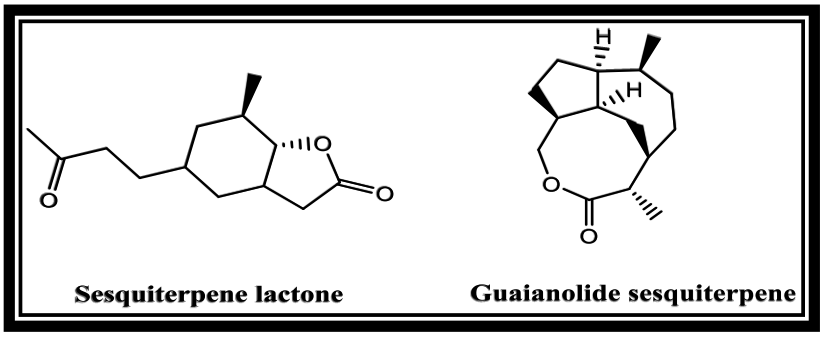


Figure 2.15. Some sesquiterpense potent phytochemicals of *P. crispa*

Similarly, the genus *Commiphora* in general and *C. myrrha*, in particular, is a true hotspot of biologically active secondary metabolites, with more than three hundred of them identified and many of them associated with a pronounced activity against a variety of different microorganisms [123]. Those include flavonoids, alkaloids, tannins, glycosides, steroids, saponins and terpenoids, and among them biologically highly active molecules such as myrracadinol A, B and C, and myrracalamene A, B and C, and triacont-1-ene [124].

Similarly, flowers of *A. nobilis*, a plant referred to in German language as "Alles zutraut", meaning "capable of anything" [125], have been used for a long time and are documented in more than 27 national pharmacopoeias. Indeed, this Chuck Norris of medicinal plants has been studied for centuries, and over a century ago, in 1914, Power and Jun reported that the flowers contain essential oils, anthemene, anthemol and anthesterol [126].

More recently, the terpenoids, bisabolol, chamazulene and sesquiterpenes, the flavonoids apigenin, luteolin and quercetin and the coumarins umbelliferone and scopoletin-7-glucoside have been described as biologically active constituents of *A. nobilis* (Fig. 2.16). Other active substances contained within that particular plant include angelic and tiglic acid esters, anthemic acid, choline, tannin, polysaccharides, phenolic and fatty acids [127].

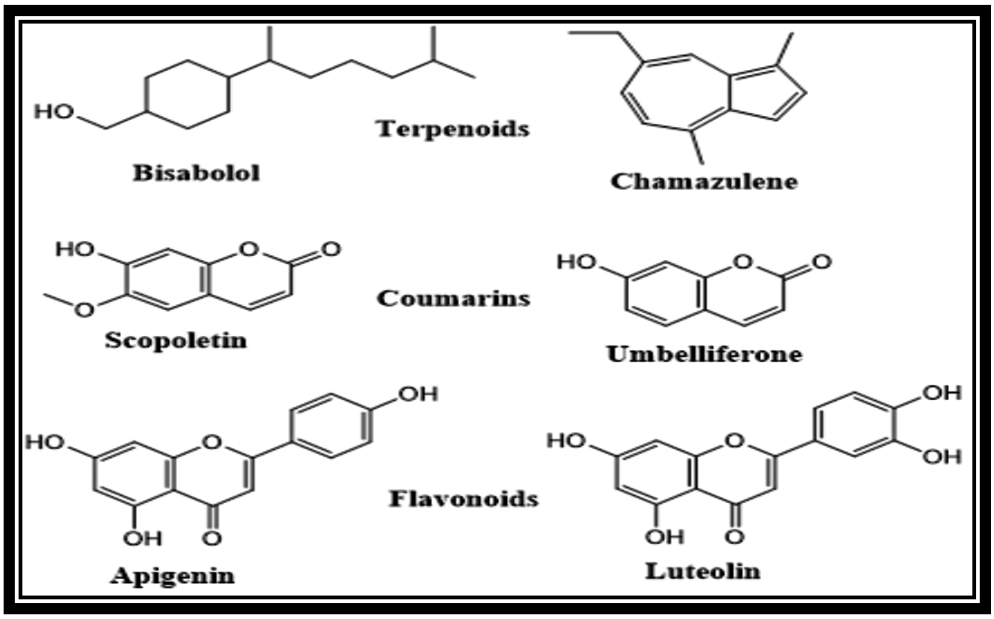


Figure 2.16. Some biologically active constituents of *A. nobilis*.

Finally, the extracts of *A. biebersteinii* contain large quantities of *β*-sitosterol, stigmasterol, sesquiterpene lactones, guaianolide, germacranolide and flavonoids [128]. This list of phytochemicals for the five most active plants is clearly not exhaustive and is without prejudice to the apparent rich phytochemical constitution of the other ten plants that did show at least some activity against at least one of the organisms studied. Ultimately, it is, therefore, permissible to speculate that the nematicidal and antimicrobial activities observed in this study derive from the

rich milieu of phytochemicals found in these very active plant extracts. These aspects require further attention as part of future studies in this field.

2.5 Conclusion and Outlook

A future search for potent phytochemicals as an integral part of future "green" medicine and agriculture should consider the five plants identified by us as most active in more detail, with a particular focus on *Solanum incanum*. Isolating and characterising the active compounds contained therein and elucidating the mode(s) of action will indeed be very important yet also challenging. It may also be useful to develop agents based on combinations of different extracts, such as combined extracts of the fruits and leaves of *S. incanum*. Eventually, it may also be feasible to blend active phytochemicals from different plants, such as *S. incanum* with *A. biebersteinii* and *R. nervosus* with *C. myrrha*. Moving on from natural products to synthetic chemistry, one may also envisage the design and development of synthetic analogues of the natural compounds, yet this task will be more challenging scientifically and also does not address the matter of local availability at a low cost. After all, the plants selected by us are readily available, easy to cultivate and harvest which render their utilisation cost effective in the Arabian Peninsula and parts of Africa.

CHAPTER III

Turning Waste into Value: Nanosized Natural Plant Materials of *Solanum incanum* Promising Nematicidal Activity

3.1 Introduction

As extraction methods are often cumbersome, difficult to perform, and in any case require subsequent purification and formulation steps, we have turned our attension to a possible alternative. Nanosizing is a fast one- or two-step method, which can be used to move from a (dried) part of the plant to a readily applicable nanosuspension. This approval seems to provide an interesting and elegant alternative, specially in the field of agriculture and where more sophisticated extraction methods are not available or feasible.

Many plants are known to harbour biologically active ingredients, for instance against pathogenic bacteria, fungi and other microbes [129]. In order to unlock this "treasure chest" of biological activity for nutritional, pharmaceutical or agricultural uses, it is often necessary to employ a vast and expensive barrage of techniques, from extractions with organic solvents to the fractionation and isolation of the active substances. Once obtained, those compounds of interest subsequently have to be processed further to suitable forms of delivery (e.g., pills, crèmes, sprays, granules). Not surprisingly, most regions, especially in the developing world, lack the kind of industrial manufacturing basis to embark on such a sophisticated production process. This is rather tragic, as those regions, at the same time, are also rich in many plant species, which, at least in theory, may be useful in the fields of medicine and agriculture. Alternative application forms, *i.e.*, crude extracts or milled materials that exhibit adequate potential for treatment, would open up a new perspective for their use in developing countries.

Furthermore, *S. incanum* grows quickly and it has been known for over 30 years that some of its alkaloids possess an amazing antimicrobial activity against bacteria, yeasts, dermatophytes, and even some pathogens affecting agricultural produce [29]. Whilst currently of no practical use, the Jericho tomato therefore contains a vast variety of substances which may be used for a range of practical applications if only turned into a "deliverable" form or format.

Faced with the problem of ready availability of considerable amounts of plant material on the one hand and the lack of adequate means to render these materials into a useful form on the other, we have therefore turned our attention to nanosizing little-processed, crude plant materials (please note that the expression "nanosizing" is employed here to describe the arsenal of milling and homogenization methods employed and does not imply that the materials obtained by these methods necessarily also consist of particles with diameters in the nanometer range). Here, we have posed the question: could simple milling of locally readily available and otherwise useless material (due to lack of solubility and hence low bioavailability) unlock the biological activity in a "useful" manner?

In order to achieve this goal, we have focussed on adequate, yet also readily available and economical (*i.e.*, cheaper) methods. After the initial step, *i.e.*, simple milling, a technique with a sufficiently high diminution efficacy has been selected, which is capable of destroying hard plant material and elastic plant fibres at the same time. Here, wet milling is the method of choice if very fine particle sizes are required [130]. Among the different processes for wet milling available today, *i.e.*, milling with colloid mills, bead milling and high pressure homogenization (HPH), the latter is the method of choice for the samples discussed here: the high energy input during the

homogenization process enables an efficient diminution of the material within a short period of time [131]. HPH is also a well-known and hence well-established, straightforward and safe technique which is frequently applied in practice, not only in the pharmaceutical industry, but also in the field of cosmetics and food production. In addition, large scale production is possible [132]. It should also be noted that the basic HPH equipment is accepted by regulatory authorities, is of relatively low cost (around €10k) and available worldwide.

3.2 Materials and Methods

This chapter describes the methods and results of a study which we have published recently by the Jacob group (Griffin S. *et al*, 2016). The description of experimental part and results will therefore be concise, as further details may be found in our literature.

In order to investigate the possibility of turning waste into value, we have chosen the above-mentioned plant, *i.e. S. incanum*, as it represents one common plant native to one region of the developing world, and a region with a particular need of effective medicines, such as antibiotics, and antimicrobial agents for various agricultural uses, for instance as phyto-protectants.

The weed *S. incanum* was harvested on the 25 September 2015 in the Wasab District, Yemen Republic (GPS coordinates: 14°20′4″ North, 43°48′41″ East). The plant was then been identified taxonomically at the Department of Botany, Faculty of Science, Aden University, Yemen, with a voucher number CP-131. Various parts of the plant, including its fruits, were subsequently dried and milled with a coffee grinder to yield the raw material shown in Figure 3.1, which was used for further investigations (see below).

Figure 3.1. Dried fruits of *S. incanum* can be ground easily to form a green, powder-like material which in itself cannot be applied in practice. It can be processed further, for instance by extraction with organic solvents or via nanosizing.

3.2.1 Nanosizing of Dried Fruit of *S. incanum*

This part of study was performed in cooperation with Dipl. Pharm. Sharoon Griffin, PhD student at the Institute of Bioorganic chemistry, University of Saarland.

As described in chapter II, nanosizing of the dry and locally pre-processed powders obtained from Yemen (Figure 3.1) was performed by a combination of rotor-stator high speed stirring (HSS) and subsequent high pressure homogenization (HPH) in the presence of the natural surfactant Plantacare. The latter is a plant derived, food-grade uncharged tenside commonly used to stabilize particles destined for medical or agricultural applications. Particle size analysis was performed using Photon Correlation Spectroscopy (PCS), Laser Diffraction (LD) and light microscopy (MP Biomedicals, Solon, OH, USA). More details are mentioned in chapter II.

3.2.2 Extraction Methods

As described in chapter II, nanosizing was paralleled by more traditional extraction methods. For this purpose, 10 g of the powdered fruits of *S. incanum* (Fig. 3.1) were extracted with methanol (4 × 100 mL) at room temperature and under constant shaking. Thereafter, the extracts were combined and filtered, the filtrate was collected and the solvent evaporated *in vacuo* at 40°C to yield the crude dry (methanolic) extract, which was stored at 4°C until further use. The yield was calculated in percentage.

3.2.3 Biological Activity Assays

As in the previous chapter, these biological activity assays involve *Steinernema feltiae, Escherichia coli* and *Saccharomyces cerevisiae*. The nanosized and stabilized particles were suspended in distilled water and then tested against threse model organisms [133]. Nematicidal assays were performed in the morning and antimicrobial assays in the early afternoon. Relevant experimental conditions and procedures for individual assays, e.g., data collection and evaluation, and statistical analysis have been reported in chapter II of this thesis, materials and methods and informations on some other similar assays can be found in the relevant literature [134-137].

3.3 Results

In essence, the results obtained support the notion that it is possible to employ nanosizing as a two-step method to render crude plant materials otherwise of little or limited use into preparations with substantial biological activities. The quality of the particles obtained was acceptable, yet could probably be enhanced further and the nanosized material of *S. incanum* was particularly effective against the model agricultural nematode *S. feltiae*, although it showed less activity against *E. coli*. These findings will now be presented in some more detail.

3.3.1 Homogenized Particles of *S. incanum*

This part of the study was carried out in close cooperation with Dipl. Pharm. Sharoon Griffin and was performed at the Department of Pharmacy, Institute of Pharmaceutical Technology and Biopharmaceutics, Philipps University, Marburg, Germany.

The crudely milled *S. incanum* (Fig. 3.1) contained large particles of varying shapes and sizes. It is not soluble in water and cannot be used for any biological studies or applications. HSS reduced the size of the particles to about 10% of the original size and subsequent HPH led to a further break-up of the particles (Fig. 3.2). Interestingly, an attempt to further reduce the size of the particles and/or plant cells employing a homogenization above 1,500 bar pressure did not result in a better quality of sample as this approach caused a slight agglomeration of the particles. For this reason, the samples obtained after the initial HPH cycles (*i.e.*, at "just" 1000 bar pressure), were selected for subsequent investigations in biological test systems as they encompassed the optimal, *i.e.*, smallest achievable particle sizes. These particles were more or less round in shape, and their size varied between 2 and 3 µm (Fig. 3.2c). Eventually, the micro-particles of *S.*

incanum seemed to release fibres, proteins and sugars, and hence, their physical stability was somewhat impaired and some aggregation could be observed [138]. Still, the particles, stabilized by the natural surfactant Plantacare (Figure 3.2a, b), formed a clear suspension in water which in stark contrast to the original sample shown in Figure 20 could be employed subsequently for biological tests.

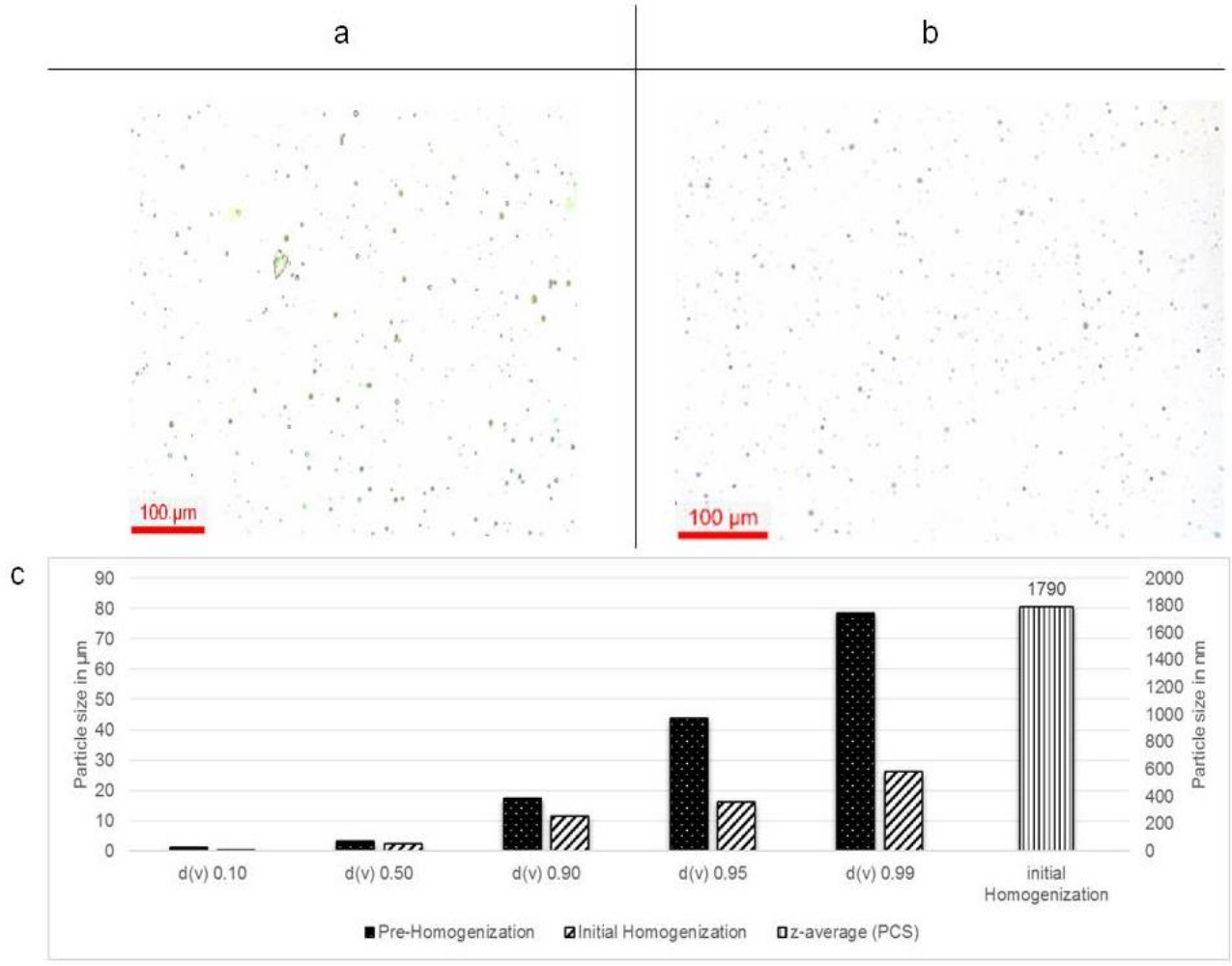


Figure 3.2. Microscopic examination of *S. incanum*.(**a**) Microscopic examination of the pre-homogenized sample of the fruit of *S. incanum* (200-fold magnification). (**b**) The same sample after initial HPH up to 1,000 bar pressure. (**c**) Characterization of the samples of *S. incanum* at different stages of homogenization. Here, homogenization was sufficient to generate particles with diameters below 2 µm (see (**b**)), whereas further HPH at 1,500 bar resulted in samples prone to aggregation (diameters above 2 µm, not shown).

As for *S. incanum*, the results obtained by PCS analysis also argued against further HPH (*e.g.*, more than ten cycles at 1,500 bar), as this may lead to subsequent agglomeration. It seems that HPH can only be performed up to a certain extent depending on the properties of the individual plant material under investigation. In any case, the particle suspensions obtained by HPH were clear in appearance and quite stable at room temperature. Still, they were stored in a cool environment to reduce the possibility of long(er)-term agglomeration (see Materials and Methods).

3.3.2 Biological Activity of Processed Samples and Respective Extracts

When tested for activity against any of the three representative target organisms, the particle suspension of *S. incanum* fruits, (up to a concentration of 1%) was inactive against bacteria and yeast, yet exhibited a concentration-dependent and ultimately statistically significant activity against the nematode *S. feltiae* (Figure 3.3). Here, a 1% particle suspension of *S. incanum* reduced the viability of the nematode to around 75%. At the same time, the negative controls, which included distilled water and a 1% Plantacare solution, showed no significant activity, whilst the processed methanolic extract of *S. incanum*, which was employed as a "conventional" benchmark control, was also active, reducing viability to less than 40% when used at a concentration of 1 mg/mL. Whilst it is, *a priori*, difficult to compare the particle suspension with the extract solution due to major differences in composition, consistency and concentration, it seems that both preparations under the experimental conditions used show an acceptable activity against *S. feltiae*.

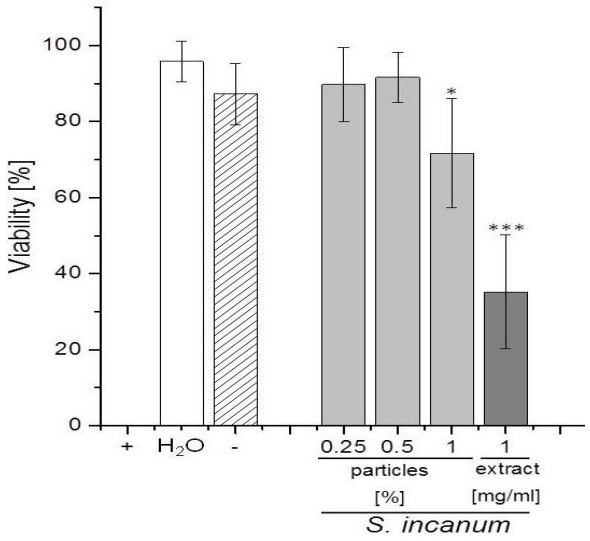


Figure 3.3 Nematicidal activity of particle and extracts of *S. incanum*. Activity of particle suspensions and methanolic extracts of *S. incanum* against the nematode *S. feltiae*. Experimental details are provided in the text. Negative controls (H_2O) and Plantacare 1% (−) and a positive control (+) of 70% ethanol were used. All experiments were performed in triplicate and on at least two different occasions. Statistical significances were calculated using one-way ANOVA (OriginPlus). * $P < 0.05$, *** $P < 0.005$.

3.4 Discussion

The results obtained with the processed, homogenized particles of *S. incanum* support the use of such crude materials against pathogenic organisms, such as agriculturally relevant nematodes related to *S. feltiae* (*S. feltiae* itself is a model but not a target). Still the results also point towards possible limitations of the method and scope for further improvement, particularly in the context of particle quality and stability, aspects of release and level of activity.

The nanosizing process, in particular, deserves further attention. Whilst a combination of HSS and subsequent HPH seems to provide a simple, straight-forward and comparably rapid method to produce samples for initial biological testing, it is also apparent that there is still considerable room for further improvement. Whilst the shapes and sizes of the particles obtained by these procedures *i.e.*, for the fruits of *S. incanum*, no doubt are adequate for biological tests, it would be desirable to achieve particles of more uniform shapes and smaller sizes, and particularly of higher stability for practical applications in the future. Here, more refined methods, such as more appropriate stabilizers or more effective nanonization methods, e.g., ART Crystal Technology, may be considered, bearing in mind that one of the prime aims of this study has been the investigation of *simple* methods which explicitly may be applied locally in developing countries [139]. Hence future studies may have to consider a balance between economical, straight-forward methods on the one side and good quality and stable particles on the other.

Similarly, our study should also be seen as preliminary when considering issues of why such particles are active. Such considerations almost certainly will raise questions related to nanosafety and/or the release of active substances from those particles. Here, we still lack information

regarding the release of biologically active compounds from them. Still, one may speculate that the particles employed serve as natural delivery systems for toxic agents such as incanumine, solasodine, carpesterol, *β*-sitosterol, stigmasterol and khasianine, whilst other effects on cells and organisms, such as interactions with membranes or the entire blockage of pores (in nematodes) would also have to be considered.

In any case, to unlock the biological activity of active ingredients contained within plants, it should be possible to nanosize a wide variety of such different plants and parts thereof. As long as they can be dried and do not contain excessive amount of fats or oils, such natural plant materials should be suitable for simple milling and homogenization procedures. In fact, the activity of the particle preparations derived from the fruits of *S. incanum* compares rather well with the ones observed for the respective extracts. This, in turn, may point to wider practical applications not only of the nanosizing method, but also of the plant involved in this study. Whilst *S. incanum* seems to be primarily promising in a more agricultural context as a possible nematicide, it may possess some antibacterial properties which could be useful in the context of simple infections, for instance affecting the gastrointestinal tract or the skin. In both cases, toxic effects on human cells obviously need to be investigated in earnest. Yet as far as we can judge, neither agricultural nor topical applications seem to bear any excessive risks.

Besides the Jericho tomato, which has been used here simply to showcase the potential of the homogenization method, one may also envisage a wide range of additional plants commonly grown and highly abundant often even as weed or waste in developing countries with a rich flora, in particular in Africa, Asia and South America. Promising examples include, for instance, *Nauclea latifolia Sm.* or *Ocimum gratissimum* L., which already have some reputation as being

effective against malaria and intestinal parasites [9]. Other sources of particular interest may well include various parasitic plants, which often also behave as weeds, and on one side are rich in biologically active ingredients and on the other tend to lack chlorophyll and other readily degrading substances. Indeed, there is plenty of choice as far as suitable plants are concerned.

Eventually, comprehensive studies on the nanosizing techniques, the particles obtained and their respective biological activities, physico–chemical and release properties will decide if such methods indeed provide a viable alternative to the extraction, isolation, formulation and delivery methods traditionally employed to move from a crude plant materials to a practical applications.

As nanosizing “in one go” covers all these conventional methods from extraction to formulation, and since our initial results are certainly not negative, it is definitely worthwhile to give such methods and the resulting particles further consideration.

3.5 Conclusion and Outlook

In essence, our study lends support to the idea that nanosizing of plant materials may enable us to move from a crude, dried plant material to an applicable, "complete particle" based delivery and release system in just one or a few simple steps. Whilst there are plenty of questions which remain to be addressed and answered in the future, none of these issues is insurmountable. Depending on the funds available, the methods for nanosizing can be varied and refined, ultimately leading to more defined and stable particles. Release properties can also be controlled by such processes, and so can be the (physical) properties of the particle itself and the biological activity caused by the substances released from it.

Future studies will therefore not only focus on the preparation of a wide range of particles from an equally wide range of local plants, and of different, application-specific quality. They will also consider a much wider spectrum of possible applications in the fields of nutrition and cosmetics, in preventing diseases and therapies. Here, cardiovascular, anti-inflammatory, anti-cancer and anti-infective agents may be at the forefront of such investigations.

Ultimately, a prime focus will also be on agricultural applications, as the amounts required in agriculture are considerably higher than in medicine, whilst the potential risk for humans is lower. *S. incanum* is one example for such possible agricultural applications, and for turning waste into value, but there are many more. "As long as it can be nanosized, it should be all right."

CHAPTER IV

Bioassay-guided Isolation and Characterisation of Nematicidal and Antimicrobial Compounds of Active Substances

4.1 Introduction

Chapter II nad III have considered the biological activity of crude extracts and nanoparticles designed for readily delivery. These chapters have not addressed the question which compounds or class of compounds are responsible for this activity. This crucial question will now be approached in form of bio-assay guided isolation and characterization of nematicidal and antimicrobial compounds from *D.socotranus* Balf. leaves.

Nematodes pose significant problems in medicine and agriculture, causing diseases in a quarter of the global population [140], and accounting for an estimated USD 80 billion in crop losses globally [141]. Traditionally, the menace of nematodes is managed using synthetic chemicals (nematicides) and soil fumigants. Currently, the management of nematodes has become difficult due to the restricted use and sometimes outright ban of nematicides (such as methyl bromide) given their human and environmental safety concerns [142]. It has also been reported that plant-parasitic nematodes are able to repopulate lands sprayed with some synthetic nematicides [143]. Not surprisingly, there is a high demand for environmentally friendly "green" nematicides.

Pathogenic microorganisms, especially bacteria and fungi, represent significant health challenges particularly in humid tropical countries where the organisms thrive better [144]. Despite the advancements in the development of pharmaceuticals for the treatment of diseases caused by these pathogens, the development of resistance by several microbial species has ensured that pathogenic organisms remain a major cause of morbidity and mortality globally [145]. Here, bacterial resistance to antimicrobials (even chemically unrelated ones) is on top of the list [146], leading to the emergence of multidrug resistant bacteria which make current drugs ineffective

[147]. The challenges of drug resistance, safety, affordability and compliance have led to a renewed search within the scientific community for phyto-antimicrobials that can serve as "green" alternatives to currently available drugs.

In response to these challenges, we have recently begun a screening of plants from the Arabian Peninsula [148] for compounds that can be used as antimicrobials and nematicides in themselves, or as novel lead compounds for the development of better drugs. As mentioned already in the previous chapters, the island of Socotra, Republic of Yemen, has witnessed a long geological and geographical isolation. This, coupled with its adverse climatic conditions (typified by droughts and high temperatures) has created a unique flora on the island, including *Dendrosicyos socotranus* Balf. of (Cucurbitaceae), the cucumber plant, (Fig. 4.1) which is one of the most widespread species in Socotra and is used traditionally throughout Yemen (where it is known as Al-khiar) for the treatment of urinary retention, liver diseases, constipation and diabetes [149, 150]. This chapter reports studies on the phyto-compounds responsible for the nematicidal and antimicrobial properties of extracts and fractions of *Dendrosicyos socotranus* leaves harvested from the island of Socotra. This plant has been selected from all the other plants because of its uniqueness and its broad activity against bacteria, nematodes and yeast.

Figure 4.1. *Dendrosicyos socotranus* Balf. f leaves

4.2 Materials and Methods

Fresh leaves of *Dendrosicyos socotranus* were collected on the 20th of November 2014 from the island of Socotra (GPS coordinates: 12°30′N 53°55′E). The leaves were identified taxonomically at the Department of Botany, Faculty of Science, Aden University, Yemen, and a voucher specimen (voucher number CP-14-1) was deposited at the herbarium of the Department.

4.2.1 Extraction and Fractionation of *Dendrosicyos Socotranus* Leaves

The leaves were washed thoroughly with tap water, rinsed with distilled water and air-dried at room temperature, in the shade. They were subequently powdered to obtain a fine powder. The powdered leaves (350g) was macerated in 1,500 mL of absolute methanol, with constant shaking, for 48 h. Thereafter, the mixture was filtered to obtain the methanol extract of the leaves which was subsequently evaporated to dryness *in vacuo* at 40°C to obtain the extract. The yield was 7.7%. It was stored at 4 °C until it was tested for biological activity or fractionated.

The different steps of the fractionation procedure are shown in figure 4.2. The dry residue from the methanol extraction (*i.e* material which was insoluble in methanol) procedure was extracted further with water to yield an aqueous extract which was freeze-dried and stored at 4 °C until tested for biological activity.

Fractionation of the methanol extract was carried out by successive extraction with solvents with increasing polarity *viz*: petroleum ether, dichloromethane, ethylacetate and methanol (Fig.4.2). The yields of the fractions were 31.5%, 31.5%, 11.0% and 25.9%, respectively. The fractions were dried *in vacuo* at 40 °C and stored at 4 °C until they were needed for biological tests or elucidation of the constituent phytochemicals.

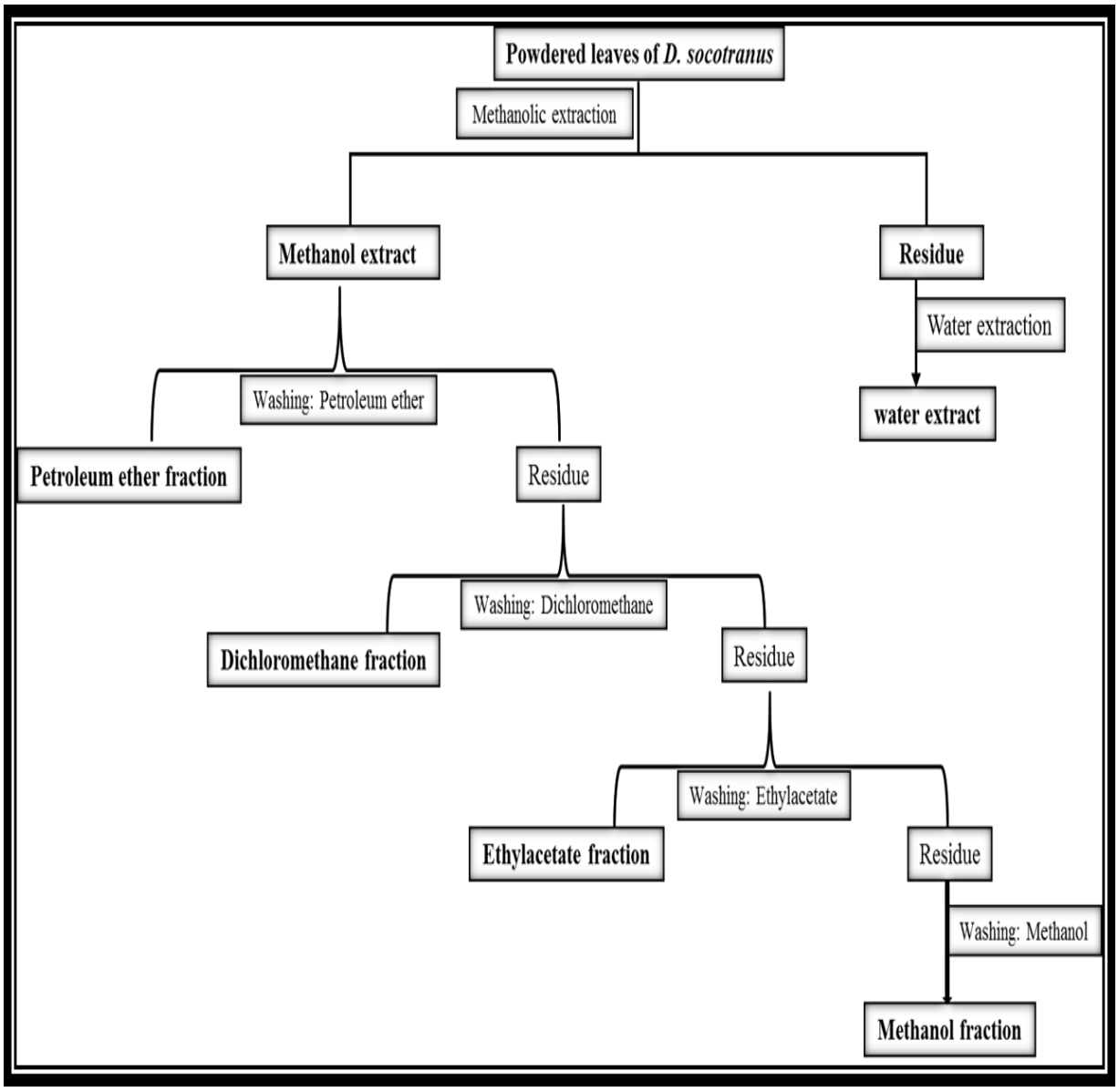


Figure 4.2. Scheme for the fractionation of the methanol extract of *D. socotranus* leaves. See text for deatails.

4.2.2 Phytochemical Screening of Extracts and Fractions

The methods used for the screening of the plant extracts and fractions have been recently published by the Jacob group (Al-Marby A. *et al.*) [148] and they will only be mentioned briefly here.

4.2.3 Nematicidal Activity

This nematicidal activity used for the preparation of stock solution and the other different concentrations of the plant extracts and fractions have been described carefully in chapter II of this thesis and will only be mentioned briefly here.

Briefly, 10 µl of the nematode suspension (approximately 30–40 nematodes) was incubated with 100 µl of each concentration of extract or fraction in the dark at room temperature for 24 h. Thereafter the nematodes were stimulated with 50 µl of distilled water at 50 °C and live and dead nematodes were immediately counted under the microscope (four-fold magnification).

4.2.4 Antimicrobial Activity

The disc diffusion assay (Bauer *etal*., 1966) was used to determine antimicrobial activities of the extracts investigated. The Minimum Inhibitory Concentration (MIC) of the extracts and fractions were determined using the broth microdilution method of Mann and Markham (1998) [149], with minor modifications. Both ways were described in detail in chapter II of this thesis.

4.2.5 Qualitative Phytochemical Screening of the Extracts/Fractions

Qualitative phytochemical analyses of the fractions/extracts of the leaves were carried out to determine the presence of phytochemicals such as polyphenols, flavonoids, alkaloids, tannins, terpenoids, triterpenoid saponins, cardiac glycosides, and coumarins following the methods reported by Harborne [151] and Trease and Evans [152].

4.2.6 Identification of Active Ingredients

Preparative TLC was performed on the fractions using high-purity grade silica gel powder (P/UV 254 with fluorescent indicator; Merck, Darmstadt, Germany). TLC plates (20 cm x 20 cm) were evenly coated with a slurry of the gel to a thickness of 1 mm. The solvent was evaporated and the adsorbents activated by heating the plates in an oven at 110 °C for a 90 min. Following separation using $(CH_3)_2CHOH$: NH_4OH: H_2O, (3:1:1) as the solvent system, spots on the plates were detected with UV light. The spots were scrapped-off and dissolved in methanol to enable the separation of the compound of interest from the gel.

Structural elucidation of the compounds isolated was initiated electrospray ionization mass spectrometry (ESI-MS). ESI-MS spectra were recorded with an API 2000, International Equipment Trading Ltd, Mundelein, Illinois 60060 USA, under appropriate conditions. These conditions included: flow rate 30 µl/mL, curtain gas (CUR) 50 psi, ion spray voltage (IS) 5500 V, temperature 500°C, declustering potential (DP) 76 V, entrance potential (EP) 11 V, ion source gas (GS1) 45 psi and ion spray gas (GS2) 50 psi and mass spectrum 0.5 seconds.

4.2.7 Statistics

The data generated were subjected to descriptive statistical analysis and the results are presented as mean ± SD. Differences between the means (test *versus* control) were assessed statistically for significance using a one-way ANOVA. A significance threshold of $P < 0.05$ was adopted. The GraphPad Prism software (GraphPad Inc., USA) was used for all statistical analyses. The results are presented in Tables and Figures.

4.3 Results

Virtually all relevant phytochemicals screened for were present in the methanol extract, whilst only triterpenes and triterpenoid saponins were found in the aqueous extract. None of the phytochemicals were found in the ethyl acetate fraction, and only triterpenoids were present in the dichloromethane fraction. Phenolics, tannins, flavonoids and triterpenoid saponins were present in the methanol fraction while alkaloids, triterpenoids, coumarins and cardiac glycosides were present in the petroleum ether fraction (Table 4.1).

Table 4.1 Phytochemicals present in the extracts and fractions of *D. socotranus* leaves

Phytochemical	H_2O extract	MeOH extract	Methanol Fractions			
			PE fraction	DCM fraction	EtOAc fraction	MeOH fraction
Phenolics	–	+	–	–	–	+
Flavonoids	–	+	–	–	–	+
Alkaloids	–	+	+	–	–	–
Triterpenoids	+	+	+	+	–	–
Tannins	–	+	–	–	–	+
Triterpen Saponins	+	+	–	–	–	+
Coumarins	–	+	+	–	–	–
Cardiac Glycosides	–	+	+	–	–	–

Key: + and − stand for present and absent, respectively; DCM, PE, EtOAc and MeOH epresent Dichloromethane, Petroleum ether, Ethyl acetate, and Methanol, respectively. Green and yellow colors depict the relevant phytochemicals available in aqueous extract because our interest just in these two phytochemicals as the aqueous extract showed the nematicidal activity more the the methanol extract and also its fractions.

Compared to the control, the aqueous extract of *D. socotranus* leaves showed significant ($P < 0.05$) concentration-dependent nematicidal activity against *S. feltiae*, beginning at a concentration of the 2.5 mg/mL. The methanol extract exhibited significant ($P < 0.05$) nematicidal effect against the test organism at slightly higher (*i.e* 5.0 and 10.0 mg/mL) concentration of test doses, relative to the control (Fig. 4.3). Just the methanol fraction of the extract showed significant nematicidal

activity ($P < 0.05$) at 5.0 mg/mL and 10.0 mg/mL compared to the control. Its activity was concentration-dependent. The other fractions had nematicidal activities that were statistically significant when compared to the control ($P > 0.05$) to the control (Fig. 4.3).

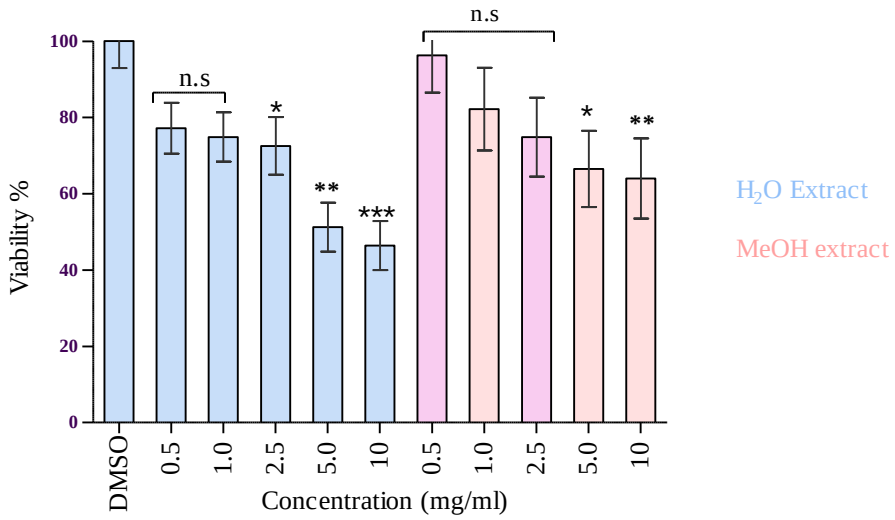


Figure 4.3 Nematicidal activity of aqueous and methanol extracts of *D. socotranus* leaves. This figure highlights the significant nematicidal activity of aqueous extract happening at (2.5 mg/mL) activity in comparison to methanol extract (5.0 mg/mL).

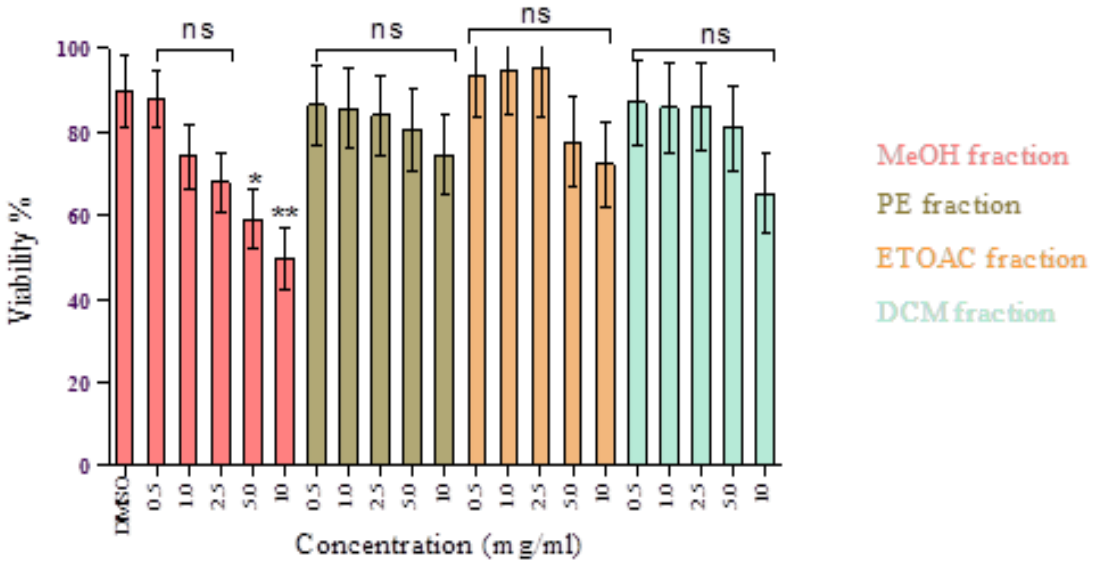


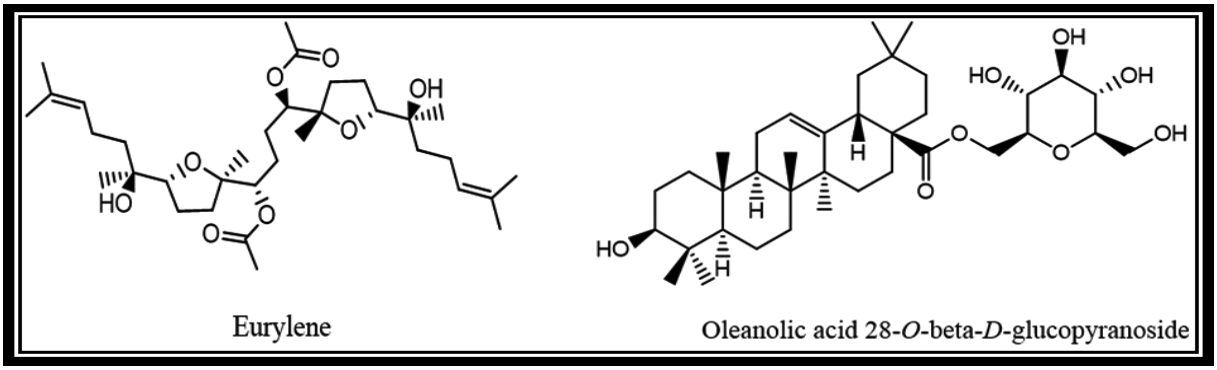
Figure 4.4 Nematicidal activity of fractions of the methanolic extract of *D. socotranus* leaves. Only the methanolic fraction showed the nematicidal activity beginning at 0.5 mg/mL.

The aqueous extract of *D. socotranus* leaves exhibited the highest activity against all the bacteria and fungi investigated followed by the methanol extract and fraction. The aqueous extract was also the most potent against *E. coli* (MIC = 1.3 ± 1.0 mg/mL). The inhibition zone diameters obtained with all the organic fractions (except the methanol fraction) were 7.0 ± 3.0 mm or less while their MIC values were greater than 5.0 mg/mL. The positive control (70% ethanol) nonetheless was a lot more potent than all the extracts and fractions studied (Table 4.2).

From the fractions and classes of natural compounds contained therein, the subsequent focus has shifted to individual compounds.

The ESI-MS spectra show that the aqueous extract of *D.* socotranus leaves contains eurylene (a squalene-type triterpene; molecular formula, $C_{34}H_{58}O_{13}$; molecular weight, 594.8 g/mol) and

oleanolic acid 28-*O*-beta-*D*-glucopyranoside (a pentacyclic triterpenoid saponin; mol. formula, $C_{36}H_{58}O_8$; mol. wt., 618.84 g/mol) (Fig. 4.5). These compounds may be responsible for the activities observed. They are not new compounds *per se*, but this is the first time they have been identified in the leaves *D. socotranus*.



The structural formulas of eurylene (a squalene-type triterpene) and oleanolic acid 28-*O*-beta-*D*-glucopyranoside of the *D. socotranus* leaves. These two compounds may be accountable for the activities observed.

Table 4.2 Antimicrobial activity of extracts and fractions of *D. socotranus* leaves

	Inhibition zones (mm)		MIC (*mg* /mL)		
	S. carnosus	*E. coli*	*S. carnosus*	*E. coli*	*S. cerevisae*
D. socotranus H_2O extract	13.0 ± 3.0	12.0 ± 3.0	2.5 ± 1.0	1.3 ± 1.0	2.5 ± 1.0
D. socotranus MeOH extract	8.0 ± 2.0	12.0 ± 2.0	5.0 ± 3.0	2.5 ± 1.0	5 ± 1.0
Petroleum ether fraction	5.0 ± 1.0	7.0 ± 3.0	**> 5.0**	**> 5.0**	**> 5.0**
Dichloromethane fraction	6.0 ± 1.0	6.0 ± 2.0	**> 5.0**	**> 5.0**	**> 5.0**
Ethylacetate fraction	6.0 ± 2.0	7.0 ± 3.0	**> 5.0**	**> 5.0**	**> 5.0**
MeOH fraction	7.0 ± 2.0	11.0 ± 2.0	2.5 ± 1.0	2.5 ± 1.0	2.5 ± 1.0
Control	30.0 ± 1.0	30.0 ± 2.0	0.01 ± 0.00	0.01 ± 0.00	0.03 ± 0.00

n.t= not tested; S. carnosus, S. cerevisiae and *E. coli* represent *Staphylococcus carnosus* TM 300; *Escherichia coli* K2; and *Saccharomyces cerevisiae*; Negative control did not show any activity. Amixture of 6 mg penicillin, 6 mg streptomycin and 25 µg amphotericin B was applied as a positive control.. As before, the green color indicates the most active (aqueous extract) fraction/extract in terms of antimicrobial activity in comparison to the methanol extract or fraction, yellow color indicates the second active as antimicrobial and the red color indicates the fractions which are not active.

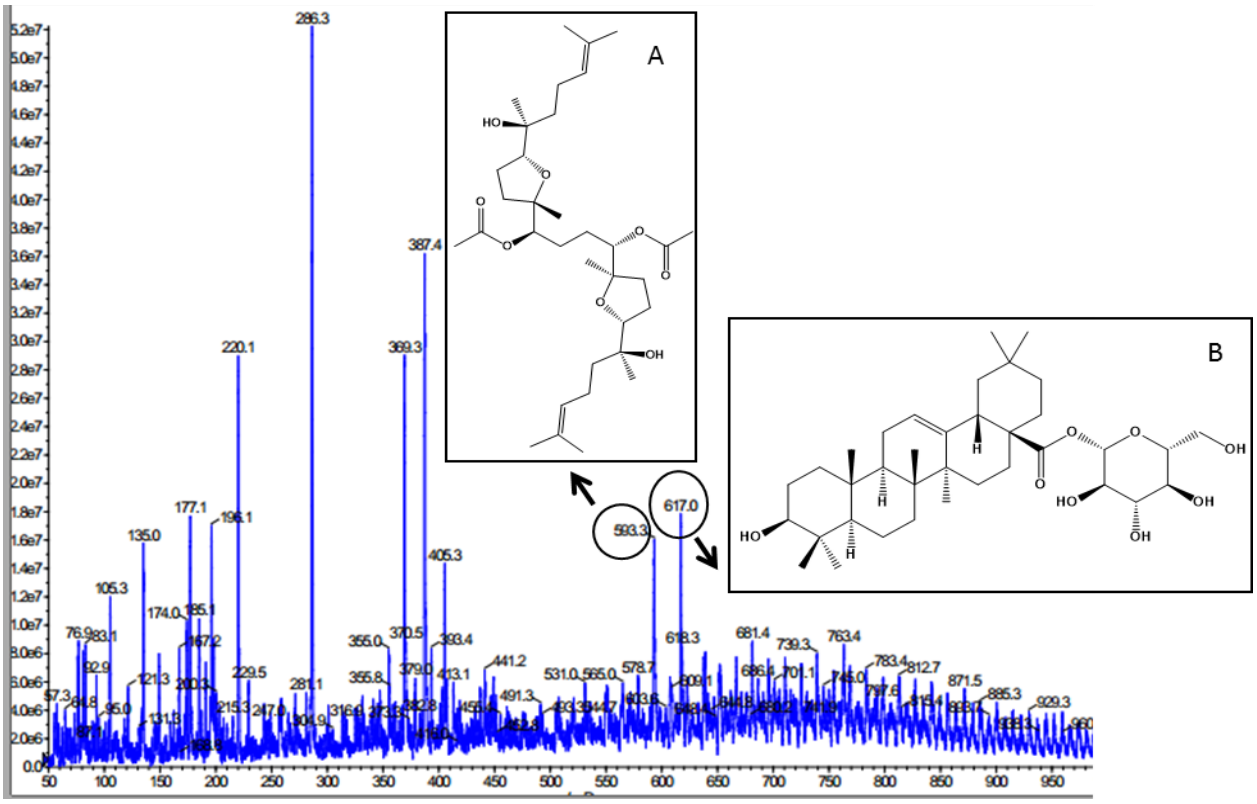


Figure 4.5. ESI-MS spectra of eurylene (A) and oleanolic acid 28-*O*-beta-*D*-glucopyranoside(B) ESI-MS spectra of eurylene (A) and oleanolic acid 28-*O*-beta-*D*-glucopyranoside(B) after positive electrospray ionization and collision-induced dissociation of the aqueous extract of leaves *of D. socotranus*.

4.4 Discussion

The identification of bioactive phytochemicals (including nematicides and antimicrobials) is currently *in vogue*, especially given the public distrust for "synthetic" compounds that are known to have deleterious effects on humans and the environment [142]. Furthermore, given the support for traditional medicines by the World Health Organization [153], a considerable effort has been underway in order to unravell the "mysteries" of plants used by traditional operators in medicine and agriculture. Based on the above premise we have begun a screening of plants from the Republic of Yemen, and as part of these samples, have zeroed in on the leaves of *D. socotranus* for this report.

In agreement with the results reported in chapter II with our earlier report [148] the leaves of *D. socotranus* were found to exhibit activity against *Steinernema feltiae* (our model nematode), *Staphylococcus carnosus* TM 300, *Escherichia coli K2* (our model bacteria species), and *Saccharomyces cerevisiae* (our model fungi). Interestingly, the aqueous extract showed more activity than the methanol extract. The crude methanol extract yielded a higher activity when it is compared to all the fractions (its bioactivity was, however, close to that of the methanol fraction). The nematicidal and antimicrobial properties observed are obviously due to the phytochemicals present in the extracts/fractions. In this regard, triterpenoid saponins appear to be the most active phytochemicals in the extracts/fractions of the leaves of *D. socotranus*. The absence of significant activity in the fractions that did not contain this particular class of phytochemicals lends credence to this observation. The ESI-MS data corroborates the above assertion as it shows that two triterpenes, eurylene and oleanolic acid 28-*O*-beta-*D*-glucopyranoside were the most likely phytochemicals present in the aqueous extract. Some

synergism between triterpenoids and their triterpenoid saponin sub-class may, however, be responsible for the slightly higher activity found in the aqueous extract. This is supported by the slightly lower activity of the methanol fraction which lacked the triterpenoids although it contained triterpenoid saponins. Synergism has in the activity of phyto-compounds have been reported [28], and it offers important prospects for future drug development. The finding that two different triterpenes are most likely to be responsible for the nematicidal and antimicrobial properties of the extracts/fraction of *D. socotranus* is in line with reports that triterpenes are responsible for the cytotoxicity of many members of the Curcubitaceae [154].

It is interesting to note that the presence of phenolic substances, alkaloids and coumarins – which are known to show antioxidant properties [155] – in the methanol extract may be responsible for the attenuated effect of the said extract relative to the aqueous extract. This "antagonism" is plausible as it is observed that the presence of the antioxidant phytochemicals appear to blunt completely the potency of the triterpenoids found in the petroleum ether fraction (as against the observation that triterpenoids in the aqueous extract apparently contribute to heightened activity). It is therefore conceivable that the active principles in the extracts/fractions caused lethality to the test organisms by means of oxidative insults/assaults. The above may explain the reduction of the effects of the extracts/fractions in the presence of antioxidant species. This apparently suggests a vital clue towards improving the potencies of the compounds identified. This notion is not far fetched as there are reports of antagonism in the activity of phytochemicals, especially with the co-occurrence of alkaloids and saponins [156]. Modulation of the redox status of organisms is known to be a potent way of controlling cellular processes. If properly harnessed, these phytochemicals may be used in the development of natural and synthetic compounds that

may active against infections and infestations [157]. Besides this particular interest in biological activity, the study also reveals some exciting phytochemicals.

Though Tang *et al.* [158] first reported the presence of oleanolic acid 28-*O*-beta-*D*-glucopyranoside in the bark of *Aralia taibaiensis* and other researchers have found the compound in a couple of other plants; and whereas Itokawa *et al*. [61] first reported the presence of eurylene from *Eurycoma longifolia* and again other researchers have found the compound in other plants, this is the first report of both compounds in *Dendrosicyos socotranus* and indeed all the *Dendrosicyos species.* Similarly, this is the first report of the nematicidal, antibacterial and antifungal activity of the two compounds. Even though there are no reports on the nematicidal and antimicrobial activities for the compounds identified, existing reports on related phytochemicals (from both *A. taibaiensis* and *E. longifolia*) show that they have been investigated largely for their activity against a variety of chronic conditions [159, 160]. A few studies have, however, investigated other properties of *D. socotranus*, reporting antiplasmodial activity against the malaria parasite [42], and toxicity of its aqueous and methanol extracts against the protoscoleces of the tapeworm *Echinococcus granulosus* [161]. The methanol extract of *Dracaena cinnabari*, which is related to *D. socotranus* also, shows a high antiplasmodial activity (IC_{50} of 2.1 μg/mL) [162]. The activities are thought to be due to the presence of curcubitacins – highly oxygenated tetracyclic triterpenes – found in the Cucurbitaceae [163]. The potency of these compounds provides new vistas of research opportunities into phyto-molecules that can be useful in managing chronic diseases as well as infections/infestations.

4.5 Conclusion and Outlook

In conclusion, the nematicidal and antimicrobial properties of extracts and fractions of the leaves of *Dendrosicyos socotranus* have been investigated and the active phytochemicals have been identified preliminary by MS. The aqueous and methanol extracts show significant nematicidal activity against *Steinernema feltia,* moderate antibacterial activity against *Staphylococcus carnosus* TM 300 and *Escherichia coli* K2, and modest antifungal activity against *Saccharomyces cerevisiae*. Upon fractions, just the methanol fraction of the methanolic extract showed significant activity against all four model organisms. Two triterpenes – eurylene and oleanolic acid 28-*O*-beta-*D*-glucopyranoside – were found to be responsible for the observed activities.

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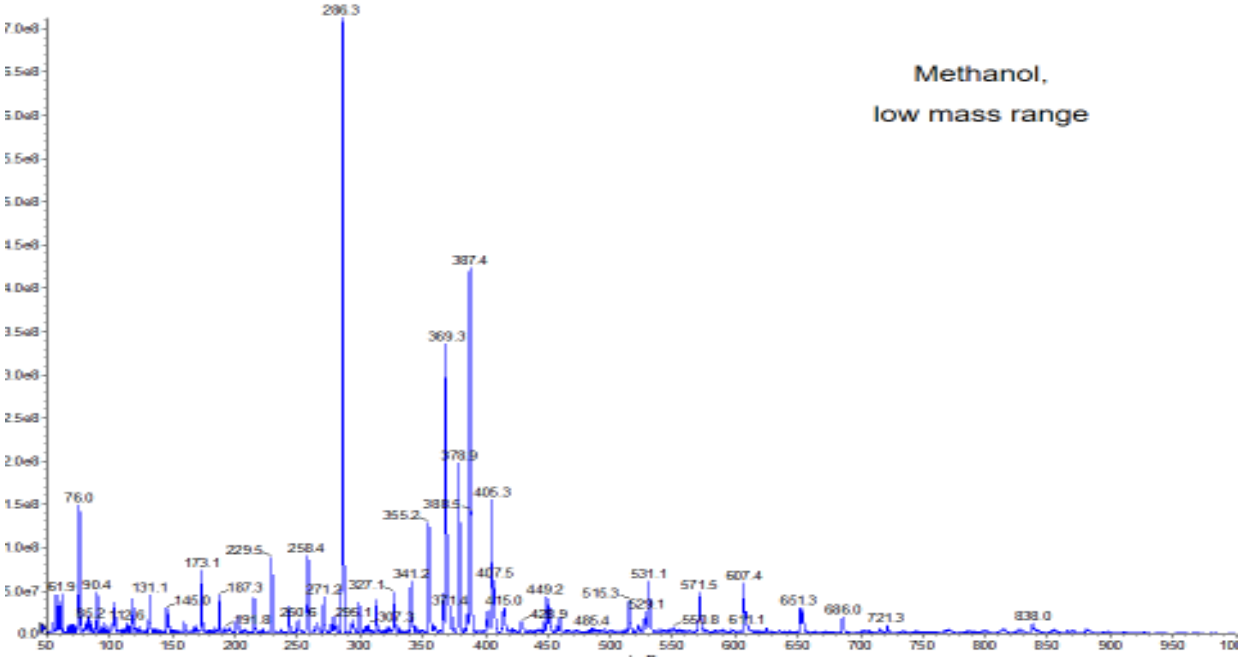
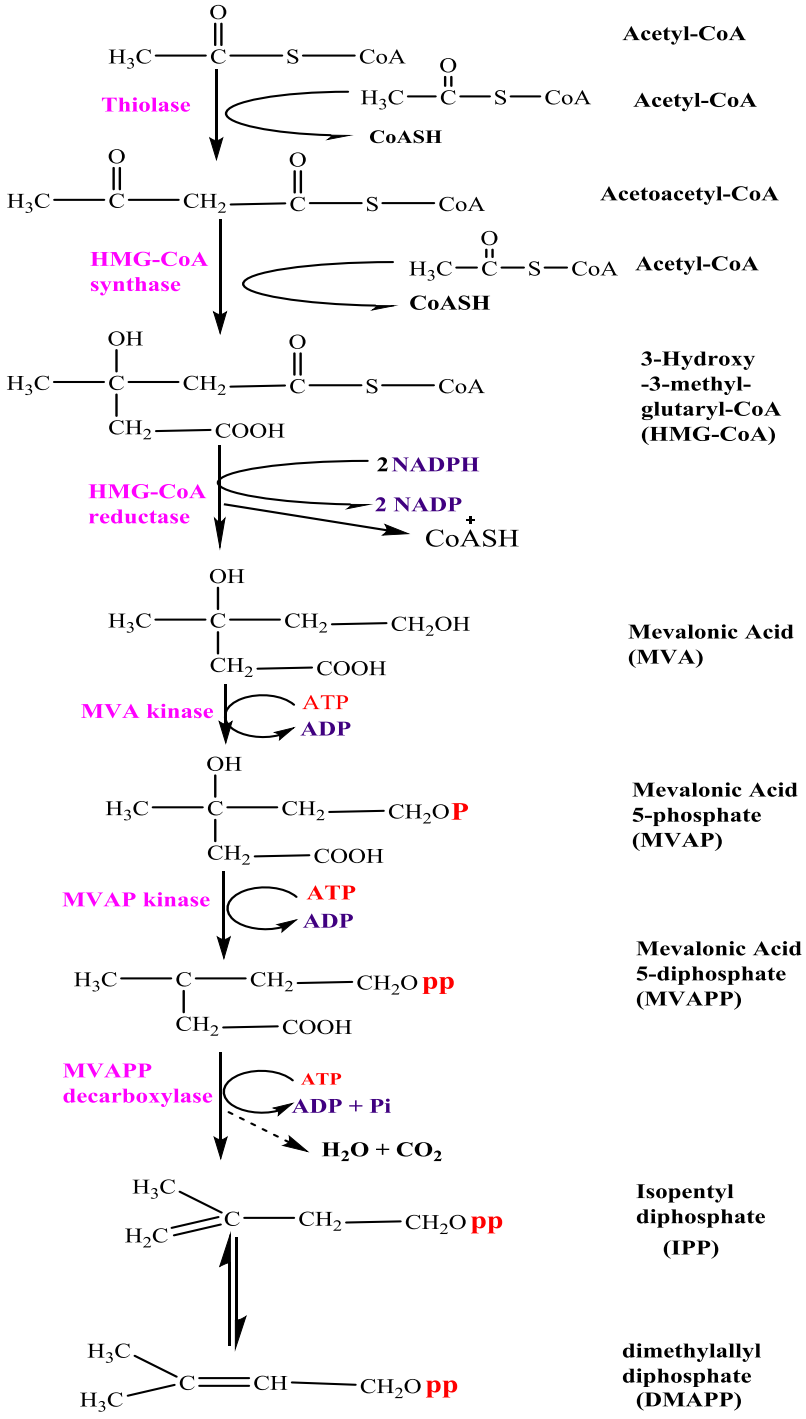
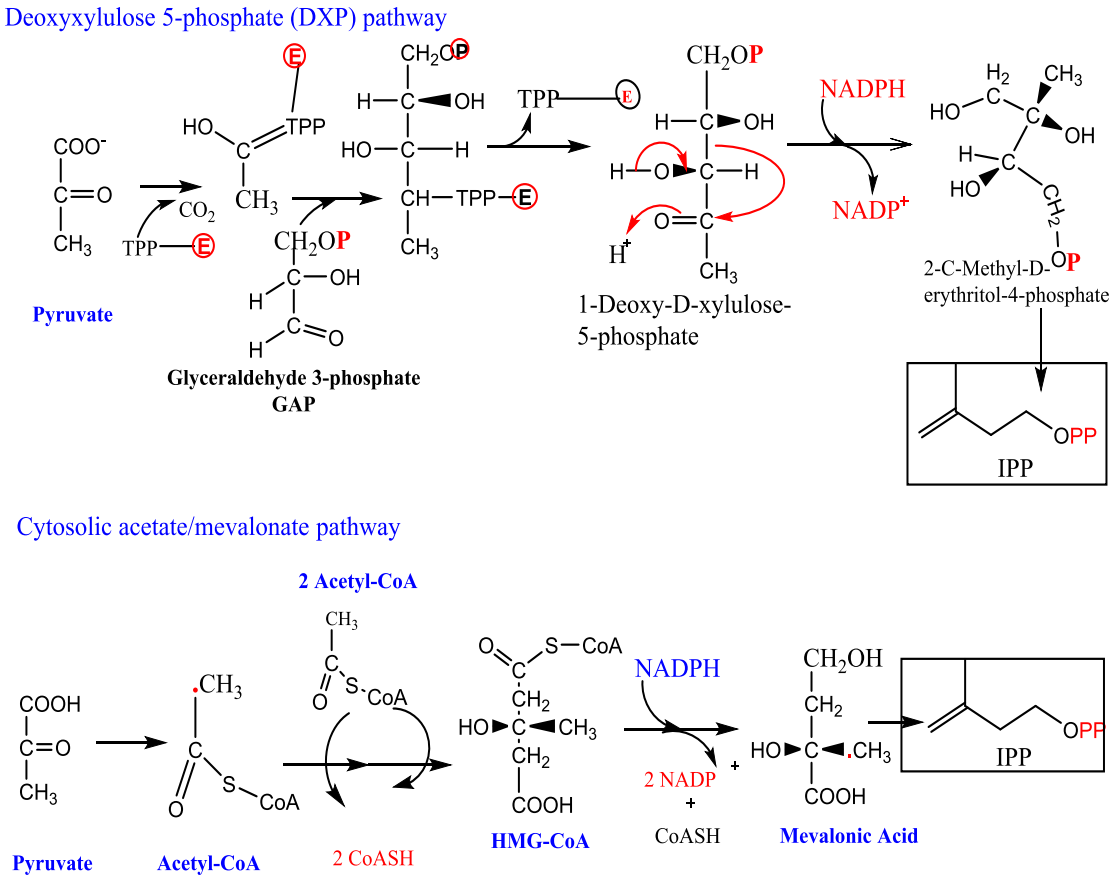
Appendix



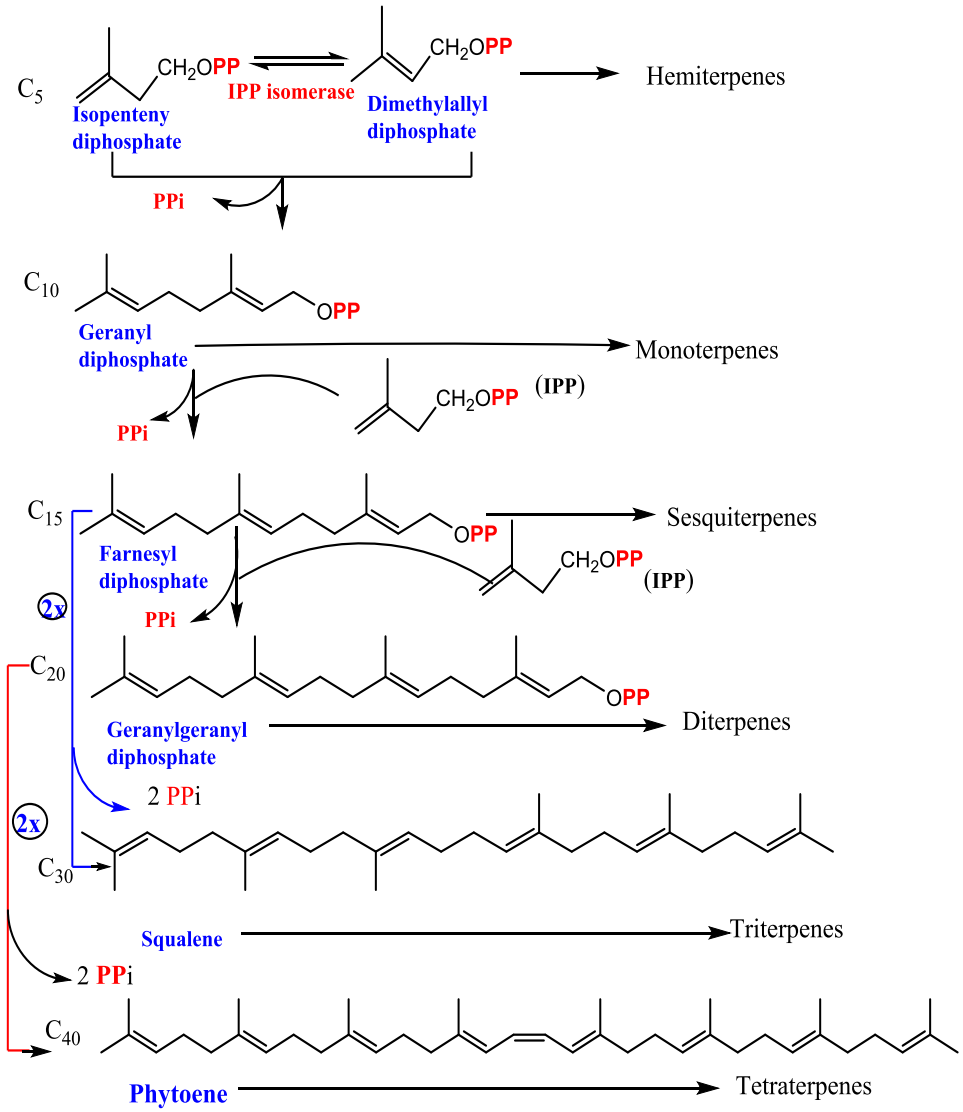
ESI-MS spectra of the blank 1



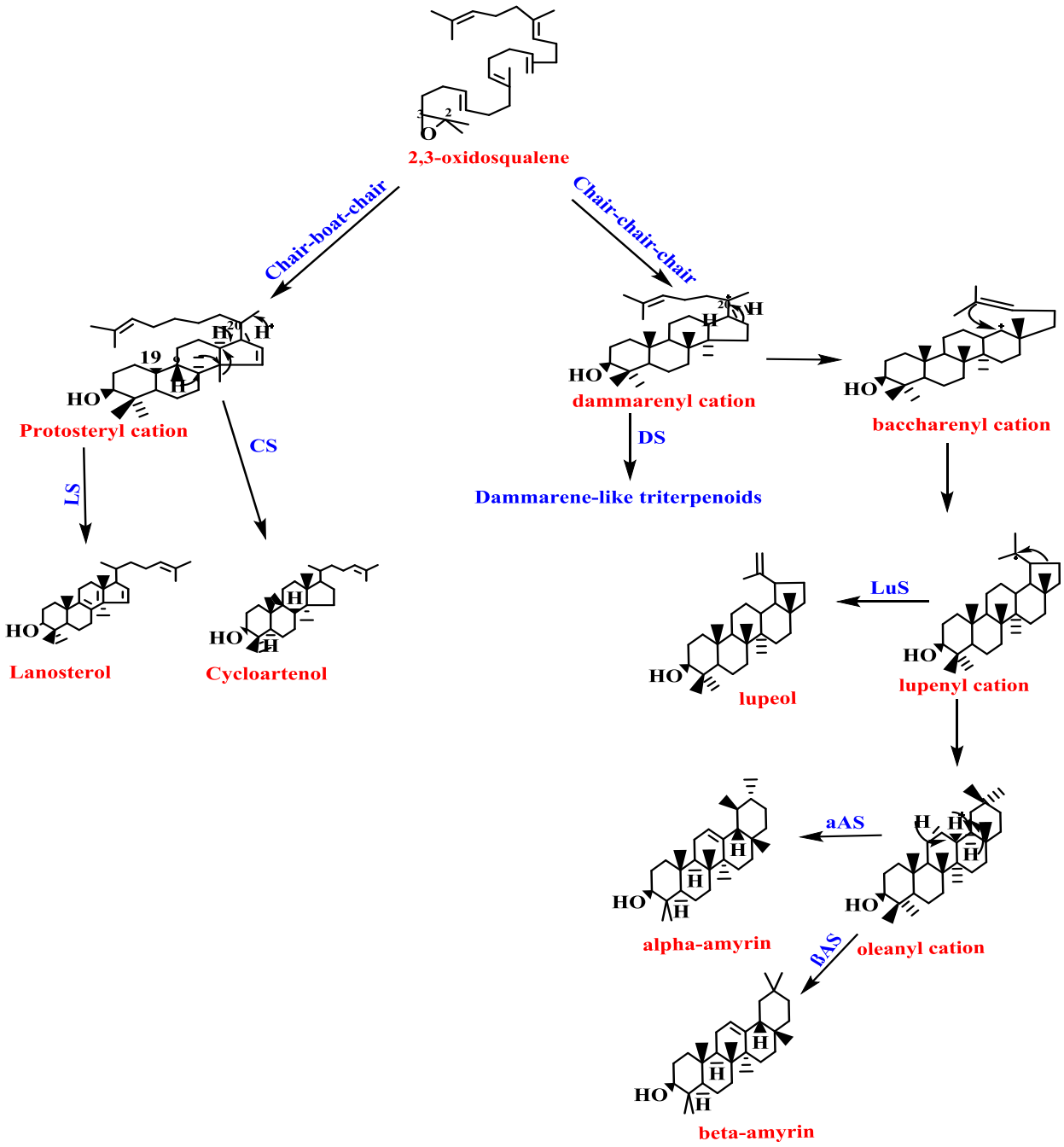
The mevalonate pathway for the formation of IPP/DMAPP



The two pathways of isoprenoid biosynthesis.



The major subclasses of terpenoids biosynthesis



Cyclization of 2, 3-oxidosqualene to sterols and triterpenoids.

The 2,3-oxidosqualene cyclase enzymes that catalyse the formation of the different products are indicated: LS, lanosterol synthase; CS, cycloartenol synthase; DS, dammarenediol synthase; LuS, lupeol synthase; bAS, b-amyrin synthase; aAS, a-amyrin synthase