Chapter 1

The Phytochemical Constitution of Maltese Medicinal Plants – Propagation, Isolation and Pharmacological Testing

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Additional information is available at the end of the chapter

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1. Introduction

In spite of its small size (31,500 hectares), the Maltese Archipelago hosts a large number of medicinal and aromatic plants that have been utilised medicinally for several centuries. The Maltese Archipelago lies in the middle of the Mediterranean Sea, 35°50′ north of the Equator and 14°35′ east of Greenwich. The climate is characterized by hot dry summers, mild wet winters (an average rainfall of 500 mm and temperatures ranging between 13°C in winter and 35°C in summer) and a high relative humidity all the year round. Most of the wild plants thrive in very shallow soil pockets that, in some cases, contribute to the production of phytochemicals as a means of protection against other plants or other organisms. In general, Maltese soils contain a high amount of calcium carbonate (>53%), which is the parent rock material, a high pH (>8) and a high clay content with a good physical structure but lacking organic matter (<4.5 %).

The Maltese flora comprises around 1284 vascular plants 66% originating from the Mediterranean region while the other 34% originating from the cold European and warm subtropical regions [1]. Out of these, there are about 458 medicinal taxa with approximately 300 originating from the Mediterranean region. The main plant families of medicinal importance are Asteraceae (15%), Lamiaceae (7%), Fabaceae (6%), Umbelliferae (4%) and Rosaceae (4%) amongst others. The biodiversity in medicinal flora is high probably due to several reasons that include:

- favourable Mediterranean climate
- availability of fertile calcareous soils
- considerable area of uncultivated land (wastelands)
- former conquerors of the Maltese Islands
- Maltese interest in herbal medicine

The number of medicinal species is on the decline to the extent that some have already become extinct. This is not mainly attributed to overuse problems but due to various human activities. There were isolated cases where a medicinal plant was under treat due to over-harvesting. One typical example was the seaside squill (*Drimia maritima*) which was over-harvested due to export.

2. Medicinal flora of the Maltese islands

The pharmacological assessment of the Maltese medicinal flora, contributed to a portion of the research conducted on these species. Intensive research has been conducted in other fields, particularly in the ethnobotanical, agronomic, *in vitro* propagation and phytochemical fields. Phytochemistry plays a very important role in medicinal plant research (figure 1). The quality and safety of these plants depends mainly on their phytochemical constitution. These metabolites determine the categorization of plants; whether a medicine, food supplement or cosmetic. The quality and quantity of these metabolites depends mainly on the growing conditions. This instigated researchers to study different aspects of medicinal plants with phytochemistry as the common aspect.

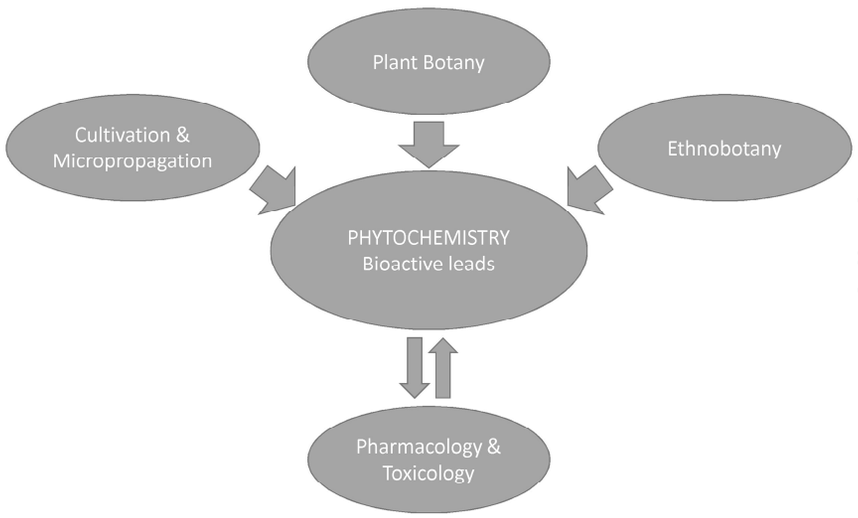

Figure 1. The importance of phytochemistry in medicinal plant research

Medicinal plants have been classified either on their phytochemical constitution or else on their pharmacological activities. These plant contain a myriad of metabolite classes and single

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metabolites. In most cases, more has to be discovered as the information is either unavailable or else still uninvestigated yet. Locally, medicinal plants have been classified on their pharmacological activity. Some would include the following effects: cardiotonic (e.g. squill, oleander), anticancer (e.g. squirting cucumber, borage), immunomodulatory (e.g. squirting cucumber, olive tree), antiflammatory and skin disorders (e.g. marigold, aloe, erica), antihypertensive (e.g. hawthorn), antimicrobial and antifungal (poison ivy, sage, garden basil, sticky fleabane, couch grass, garlic, fig tree, caper plant, pellitory of the Wall), antidiabetic (karela), insect repellents and insecticides (pennyroyal, tree tobacco), antihelmintic (pumpkin), spasmodic and antispasmodic (vervain, henbane), sedative (blue passion flower, orange-flower water, chamomile), kidney stone problems (micromeria), volatile oil (lavander, garden rue, lemon balm, rosemary, laurel, spearmint) and fixed oils (olive tree, castor oil plant). Some of these plants are listed in table 1.

Local ethnobotanical research has contributed towards the discovery of new leads. In such studies, the traditional claims are challenged using scientific methods. Possible conservation strategies were also considered, particularly for endangered species. However, there are limitations since there are no national incentives to conserve these plant species unless cultivated or sold as pot plants. However, there are few plants that are legally bound. A typical example is the carob tree. The grower cannot uproot a carob tree to pursue cultivation needs.

Latin name	Family	Common name	Maltese name
Drimia maritima (L.) Stearn	Asparagaceae	Seaside squill	Basla tal-għansar
Ecballium elaterium (L.) A.Rich.	Cucurbitaceae	Squirting cucumber	Faqqus il-ħmir
Mentha pulegium L.	Lamiaceae	Pennyroyal	Plejju
Salvia officinalis L.	Lamiaceae	Garden sage	Salvja
Verbena officinalis L.	Verbenaceae	Vervain	Buqexrem
Hedera helix L.	Araliaceae	Common ivy	Liedna
Crataegus monogyna Jacq.	Rosaceae	Common hawthorn	Anżalor salvaġġ
Calendula officinalis L.	Asteraceae	Pot marigold	Suffejra
Melissa officinalis L.	Lamiaceae	Lemon balm	Burieħa
Olea europea L.	Oleaceae	Olive tree	Żebbuġa
Urtica dubia Forsk.	Urticaceae	Stinging nettle	Ħurrieqa
Capparis spinosa L.	Capparaceae	Caper plant	Kappara
Ephedra fragilis Desf.	Ephedraceae	Mormon tea	Efedra
Nicotiana glauca RC Graham	Solanaceae	Tree tobacco	Tabakk tas-swar

Table 1. The Maltese medicinal plants in this study.

2.1. *Drimia maritima* (L.) Stearn

Drimia maritima or *Urginea maritima* is one of the local medicinal plants which was harvested and exported. It is a member of the Asparagaceae family, with cardiac glycosides that reside

in the bulb of this plant. It is renowned for its emetic, diuretic, cardiotonic [2], expectorant, rodenticide [3] and anticancer activities. The seaside squill has been extensively studied for its propagation potential. Locally, cultivation studies have been associated with the cardiac glycosidic content while micropropagation has been linked to biomass production.

The main constituents of the seaside squill are the cardiac glycosides and phenolic compounds [4]. It also contains mucilage and calcium oxalate crystals. The squill cardiac glycosides are bufadienolides. In principle, these are similar to triterpenoids having a sugar group and a lactone ring at C17. Scillaren A accounts for about 70% of the total glycosidal content of squill. It contains one unit of rhamnose and one unit of glucose. When scillaren A is hydrolyzed by enzymes, it breaks down to proscillaridin A and D-glucose (Figure 2).

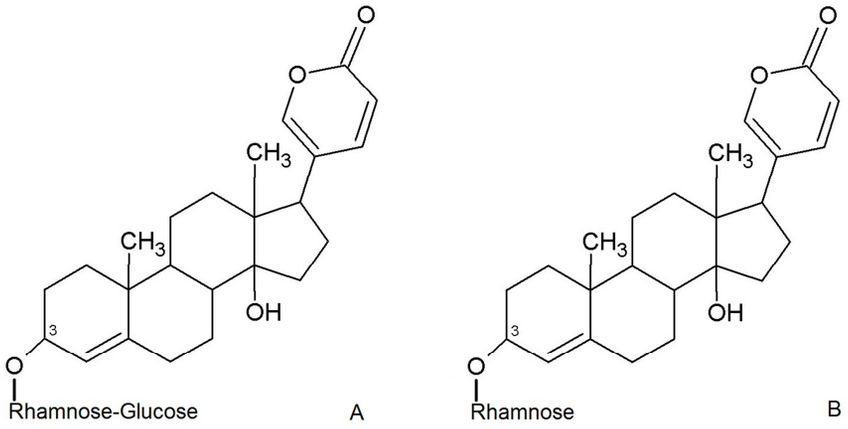

Figure 2. The structure of cardiac glycosides of *Drimia maritima* (L.) Stearn.: (A) Scillaren A and (B) Proscillaridin A

These glycosides act by binding to the Na^+/K^+ ATPase pumps. This occurs due to the presence of the lactone group [5, 6]. These bufadienolides are therefore important cardiotonic, blood pressure stimulating and antitumour agents. The main glycosides with digoxin-like effects are scillaren A and proscillaridin A [7].

Several cultivation parameters were studied for *Drimia maritima* in relation to dry matter yield and the total glycosidal content [8]. These include methods of propagation, planting at different depths, effects of nitrogen (N), phosphorus (P) and potassium (K) fertilizers, cultivation in different soil types, age of harvesting and seasonal timing of harvesting. Propagation by bulb division only takes 10 weeks to produce a seedling as opposed to seed propagation that requires 56 weeks. The type of soil does not contribute to the variation of glycosides in the squill bulb. In fact, Maltese squill grown on four soil types, namely terra soil, xerorendzina soil, carbonate raw and sandy soil exhibited average glycosidal contents of 0.575 % (w/w). Fertiliser studies revealed that the use of different ratios of N, P and K affect the rate of growth but no change in glycosidal content (average of 0.59 % w/w). For the best annual production of dry weight and glycosidal content, it is advisable to harvest squill in the third year after transplanting (table 2) immediately after flowering. The highest glycosidal content is obtained from the roots (Figure 3)

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Year of harvest	Treatment	Mean glycosidal content(%)
First	Control	0.25
	Fertiliser-treated	0.26
Second	Control	0.66
	Fertiliser-treated	0.68
Third	Control	0.57
	Fertiliser-treated	0.58
Fourth	Control	0.58
	Fertiliser-treated	0.58
Fifth	Control	0.38
	Fertiliser-treated	0.40
Sixth	Control	0.31
	Fertiliser-treated	0.34

Table 2. The mean percentage glycosidal content in the squill bulb with year of harvest [8].

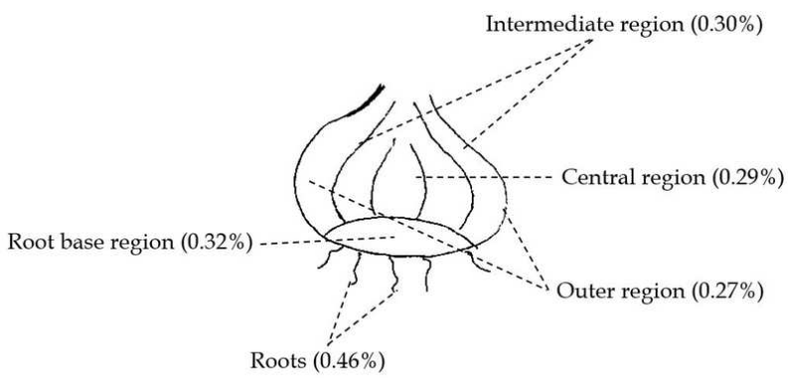

Figure 3. The mean percentage glycosidal content in different parts of the squill bulb [8].

Micropropagation of squill was carried out by direct and indirect organogenesis. Regeneration was successfully achieved using bulb explants by direct organogenesis. Although the process of regeneration was slow, callus cultures maintained in high auxin concentrations (4 mg/l 2,4-D + 2 mg/l NAA) induced root formation, when the plant growth regulators (PGRs) were removed [9].

2.2. Ecballium elaterium (L.) A.Rich.

Ecballium elaterium (squirting cucumber), a member of the Cucurbitaceae family, is a Mediterranean medicinal plant in a monotypic genus. In the past, the squirting cucumber was used as

a purgative, emetic, for the treatment of jaundice and oedema. It was also used for the treatment of otitis, hydrophobia and malarial fever. Locally, it was prepared in various dosage forms such as powders, solutions, semisolid blocks and dried cubes for exportation. It also used to be prepared in the form of lozenges with gum Arabic. The fresh fruit juice was renowned for several pharmacological effects mainly as antibilirubinaemic, antihepatotoxic and lacrimation stimulant. The dried juice, also known as the elaterium, was effective as a laxative, anti-inflammatory, antitumour and as an aflatoxin suppressor [10, 11]. Most of these pharmacological effects have been proven through various scientific investigations.

The main constituents of this plant are the cucurbitacins (Cu), the major ones being CuE and CuB (Figure 4), particularly present in the fruit juice. Other cucurbitacins include cucurbitacins D, G, H, I, R, L, hexanorcucurbitacin I, 16-deoxy-Δ^{16}-hexanorcucurbitacin O, anhydro-22-deoxo-3-epi-isocucurbitacin D, and their glycosides [12-14]. The squirting cucumber also contains sterols, fatty acids, elaterases, tannins [15], complex phenolic compounds and flavonoids [16], amino acids and their derivatives as well as the *Ecballium elaterium* protease inhibitors (EEPIs). These EEPIs are obtained from seed extracts and are effective against at least four different serine proteinases [17]. In fact, these are termed as trypsin inhibitors I, II, and III, also known as the trypsin isoinhibitors (EETIso), chymotrypsin inhibitor (8 kDa), subtilisin inhibitor (9 kDa), and elastase inhibitor, and Astacus protease inhibitor [18].

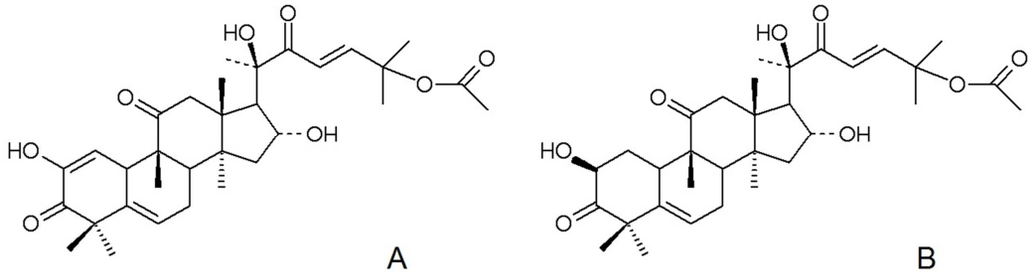

Figure 4. The structures of (A) cucurbitacin E and (B) Cucurbitacin B found in *Ecballium elaterium* (L.) A.Rich.

Although this plant is abundant in wastelands throughout the Maltese Archipelago, micropropagation was attempted for two main reasons. These were as a means to study the responses of explants from the squirting cucumber to different plant growth regulators, and to determine the potential propagation of high-yielding mother plants. In this attempt, seeds were germinated in Murashige-Skoog (MS) medium. Different concentrations and types of PGRs, mainly auxins and cytokinins, were added. Subculturing with the different PGRs was performed every 4 weeks and explants were maintained at about 25 ± 1 °C and 3250 ± 250 lx. Once developed, the plantlets were transferred to Jiffy® pots until rooting and then repotted (compost:peat:perlite, 2:2:1) until flowering [19]. The main four responses of explants were bud multiplication, shoot elongation, callus production and rooting, as illustrated in Figure 5.

A regeneration protocol was devised as follows. Briefly, the seeds were germinated on MS medium (8 - 9 weeks). Bud multiplication of node explants was performed on naphthaleneacetic acid/6-benzylaminopurine (NAA/BAP) medium (for 2 - 3 subcultures every 4 weeks).

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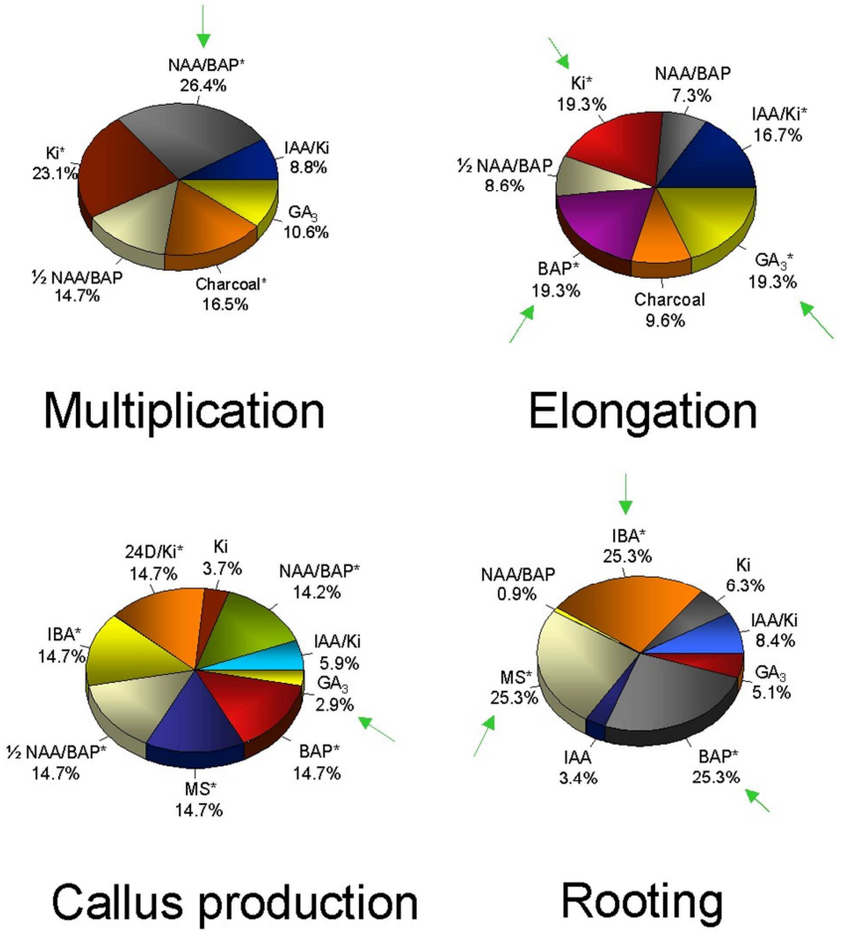

Figure 5. The effects of plant growth regulators on *Ecballium elaterium* explants in tissue culture [20].

shoot elongation was obtained on Gibberellic Acid (GA_3) medium (4 weeks), followed by an auxin shock on Indole-3-acetic acid (IAA) medium (1 week) then, treated with rooting hormone powder and finally transfer to Jiffy® pots (3 - 4 weeks). The plants were then repotting and acclimatised for 4 - 5 weeks [19]. The whole process takes between 24 and 35 weeks.

The *Ecballium elaterium* explants produced a high amount of callus and this led to further studies to determine the production of cucurbitacins in these undifferentiated cells. Callus masses were treated with different PGRs at different concentrations. The best PGR combination for biomass accumulation was 2,4-Dichlorophenoxyacetic acid/kinetin (2,4D/Ki) while for metabolite production, the NAA/BAP combinations showed optimum yields [20]. A growth-linked accumulation of metabolites was observed (figure 6).

The production of cucurbitacins from cultivated sources, is significantly higher in fruit compared to stems and leaves (figure 7). A drop in ambient temperature results in lower production of cucurbitacins [21].

Pharmacological testing has been extensively carried out on this plant. Extracts exhibited a marked effect on prostate cancer cells (IC_{50}= 9.35 nM) and moderate effects on melanoma and breast cancer cells (IC_{50} = 0.87 and 1.95 μM, respectively) *in vitro*. Negligible cytotoxic effects

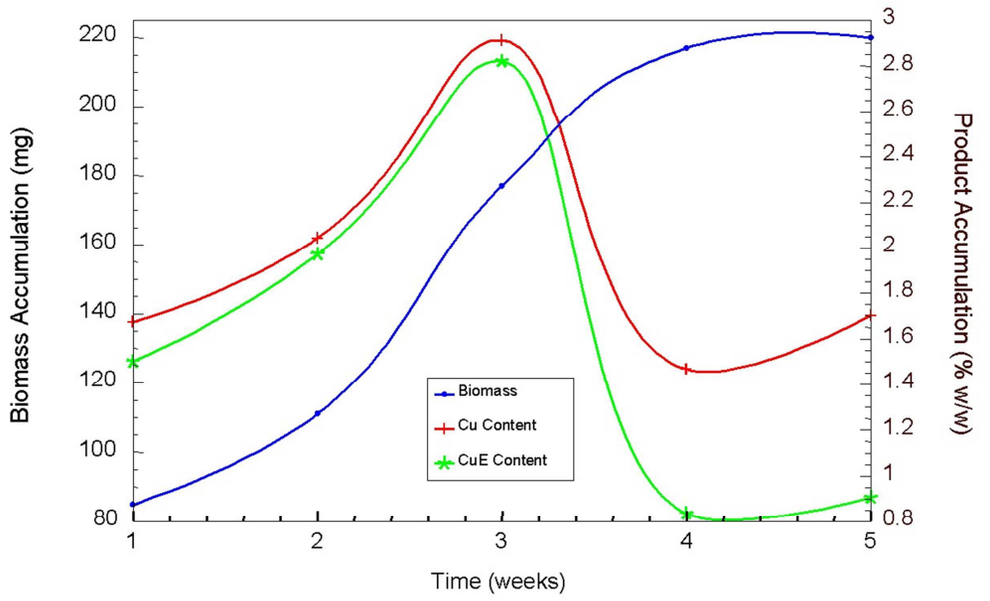

Figure 6. Growth-linked accumulation of metabolites in *Ecballium elaterium* cultures.

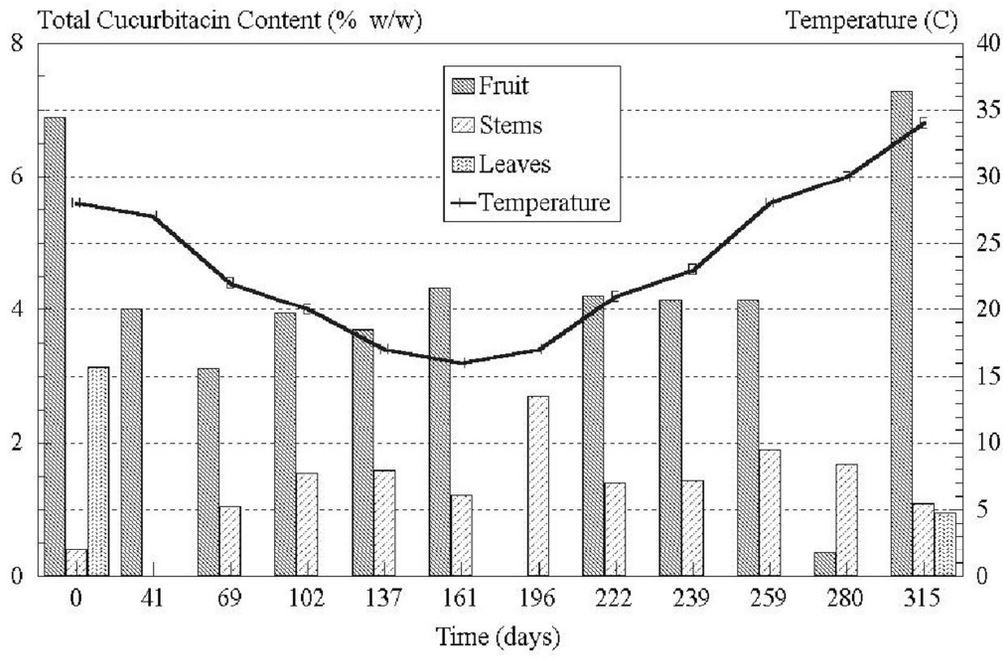

Figure 7. The total cucurbitacin content in elaterium produced from *Ecballium elaterium* fruit, stems and leaves with time and temperature [21].

were observed on normal fibroblasts (IC_{50} = 93.8 μM) [22]. It was demonstrated that CuE provoked apoptosis in cancer cell lines. This was exhibited by the condensation of chromatin and also DNA fragmentation using gel electrophoresis. CuE was also effective as an immune modulator. Human peripheral T-lymphocytes were freshly isolated and challenged with phytohaemagglutinin (PHA) and *Ecballium elaterium* extracts [23]. Cucurbitacins in the juice

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extract of *Ecballium elaterium*, also exhibited potential anti-inflammatory, analgesic and antipyretic activities in rodents [10, 24].

2.3. Mentha pulegium L.

Mentha pulegium L. is a perennial plant, belonging to the Lamiaceae family. During Roman times, the plant was used for several ailments particularly for headaches, flatulence and even as an abortifacient. The name 'pulegium' derived from the Latin word 'pulex' for flea, indicates that in Roman times the plant was used as a flea repellent [25]. Locally, it was well-reputed as a treatment for common cold, as a carminative, emmenagogue but also as an insect repellent [26]. *Mentha pulegium* used to be hung in wardrobes to ward off fleas and placed on windowsills to repel mosquitoes especially during the summer months. The most important extract from this plant is the essential oil, known as the pennyroyal oil.

In a study by [27], pennyroyal oil contained 38.0% piperitone, 33.0% piperitenone, 4.7% α-terpineol and 2.3% pulegone as the major components (Figure 8). The authors concluded that Iranian pennyroyal oil is rich in piperitone/piperitenone. In another study, the pulegone content of Iranian pennyroyal oil ranged between 1.3 – 52.0%, when extracted by supercritical fluid extraction, while hydrodistillation yielded around 37.8% of pulegone. Piperitenone consituted only 6.8% to the extracted essential oil [28]. Similarly, in another study [29], the content of pulegone in Greek pennyroyal oil was in the range of 42.9% and 90.7% attributed to two populations. In other wild populations, the pulegone content did not exceed 35.6%. Such populations were rich in either menthone/isomenthone or in piperitone/piperitenone. In Tunisian pennyroyal oil, 41.8 % of the oil was pulegone [30] while Portuguese pennyroyal oil contained 23.2 % of pulegone [31]. The pennyroyal oil was extracted from wild Maltese populations using hydrodistillation with a yield of 0.73 % [32]. The pulegone content in the oil was 85.8 %, followed by other constituents; (-) limonene (0.984 %), myrcene (0.109 %) and β-pinene (0.191 %). This was determined by GC-FID.

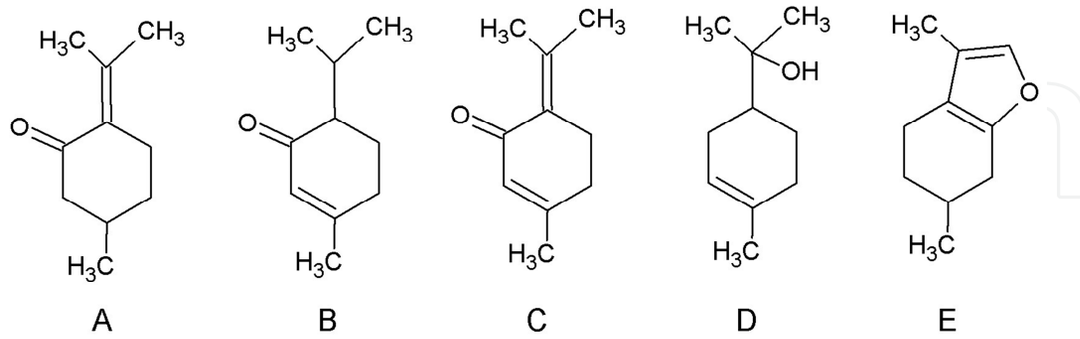

Figure 8. The most abundant monoterpenoids of *Mentha pulegium* L. essential oil: (A) pulegone, (B) piperitone, (C) piperitenone, (D) α-terpineol and (E) menthofuran.

Apart from its abortifacient activity, pennyroyal oil is also hepatotoxic and causes pulmonary necrosis. Hepatotoxicity is mainly attributed to the conversion of pulegone into its epoxide or menthofuran derivatives [33-35].

Insect repellent activity of pennyroyal was determined by using two setups (Figure 9) with citronella oil and distilled water used as positive and negative controls, respectively [32]. Setup 1 consisted of a trough with a diameter of 30 cm and a height of 12 cm. Four zones were designated within the trough (Figure 9A). The mosquitoes were introduced inside the container, and the oil sample was then injected by a syringe. Sixteen mosquitoes were observed every two minutes for a period of 20 minutes and their position within the trough was recorded. After the second minute, 75 % of the mosquitoes were found in the compartment furthest from the injection site. A gradient was achieved at this time interval and the mosquitoes moved away from the source. After the tenth minute, this compartmental difference was no longer observed, most probably due to the fact that the oil must have saturated the trough and hence there was no trend in mosquito distribution. Setup 2 consisted of a glass tube with an internal diameter of 2.5 cm and a length of 150 cm. Seven zones were designated within the tube (Figure 9B). Twenty mosquitoes were observed every two minutes for half an hour and their position recorded, following injection of the pennyroyal oil. As with setup 1, there was a statistical difference between zone 1 and zone 7 of the tube, but this difference became negligible with time. Similar results were observed with citronella. In spite of this similarity, GC-FID determination of the citronella oil revealed the presence of geraniol (60.0 %), citronellal (15.0 %) and camphene (> 15.0 %), but no significant pulegone content. With water a more random distribution of mosquitoes was observed [32].

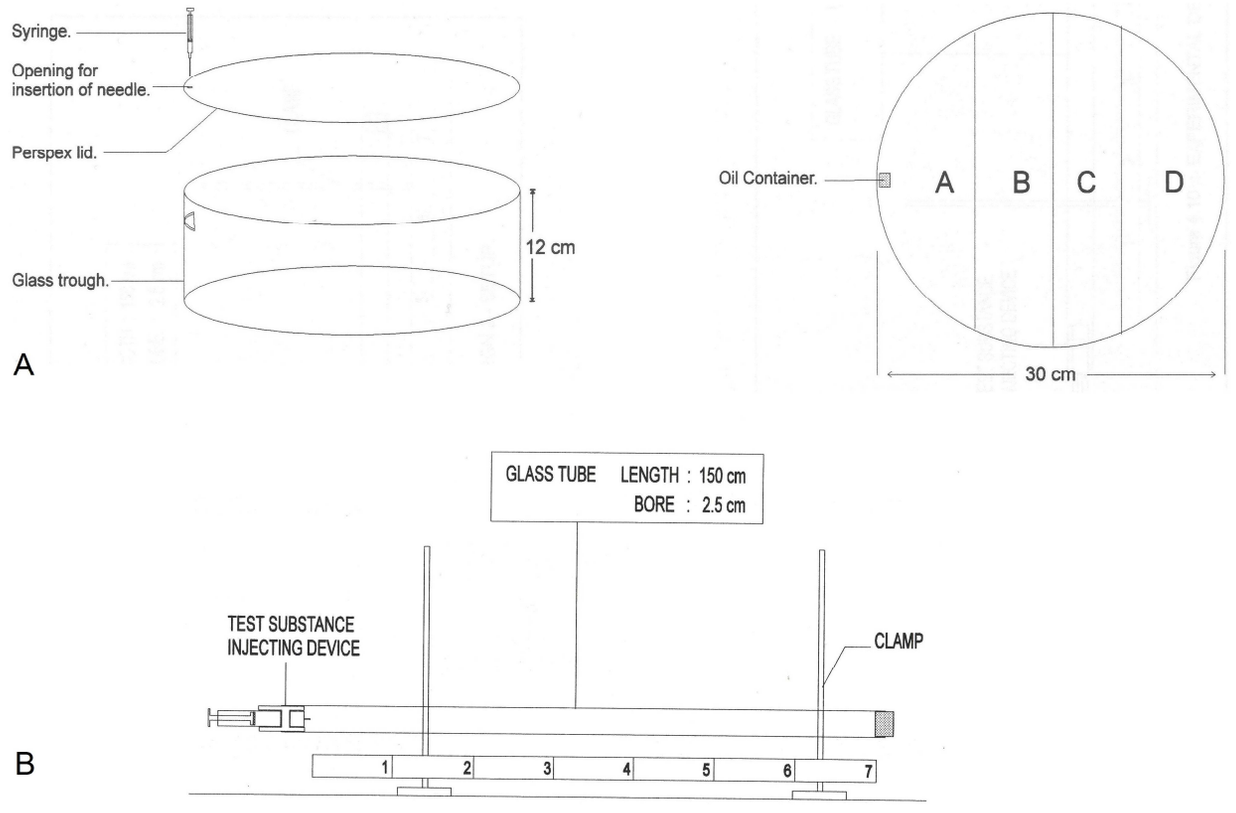

Figure 9. The experimental setups used to determine the insect repellent properties of pennyroyal oil [32].

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Pennyroyal oil exhibited repellent and insecticidal effects. After 90 minutes exposure, none of the mosquitoes were airborne and those that were in contact with the oil were dead. The insect repellent activity was attributed to the high pulegone content [36].

2.4. Salvia officinalis L.

Salvia officinalis, more commonly known as garden sage, is a member of the Lamiaceae family. Sage has been renowned for its healing properties since the Ancient Greeks. The Romans inherited the medicinal knowledge on sage and used it to enhance diuresis, menstruation and to stop bleeding of wounds. It was also used to treat pain associated with colds and rheumatism [37]. Scientifically, sage has several medicinal properties, such as, antioxidant [38, 39], anti-bacterial [40], anti-inflammatory [41] and antiviral effects [42] and is also used to control Alzheimer's disease [43]

Sage contains several metabolites primarily monoterpenoids and sesquiterpenoids, diterpenoids [43], triterpenoids, such as ursolic and oleanolic acid [41, 44], and also flavonoids and phenolic glycosides [45]. The essential oil of Portuguese sage according to [46] contains α-thujone (17.4 %), α-humulene (13.3 %), 1,8-cineole (12.7 %), *E*-caryophyllene (8.5%) and borneol (8.3%) as major constituents. In another study [47], the sage essential oil contained mainly α-thujone (29.1 %), camphor (26.3 %), 1,8-cineole (9.3 %), α-humulene (4.4 %) and terpinen-4-ol (4.0%). Similar results were obtained in a local study [48], where the Maltese sage oil was found to contain mainly α-thujone (29.28 %), camphor (26.61 %) and 1,8-cineole (15.53 %) as the major constituents (Figure 10).

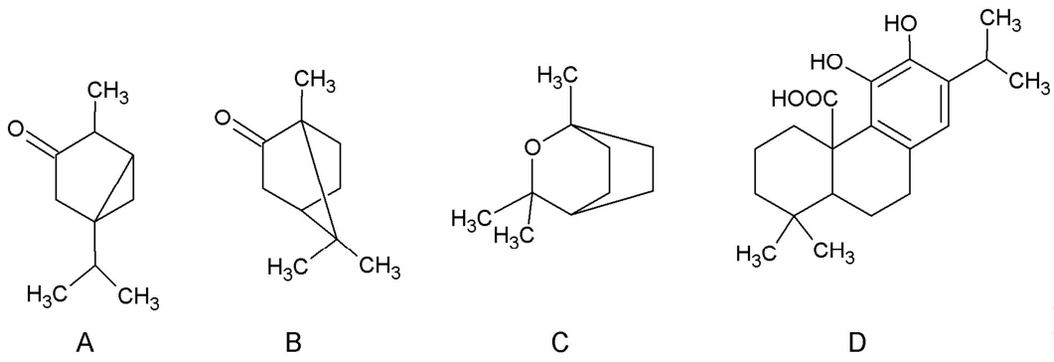

Figure 10. The common constituents of *Salvia officinalis* L. essential oil: (A) thujone, (B) camphor, (C) 1,8-cineole and (D) carnosolic acid.

Another significantly important metabolite in sage is carnosolic acid, a bitter abietane diterpenoid derivative with a carboxylic acid structure. This compound possesses antimicrobial, antioxidant, antiviral and anticancer activities [49]. Carnosolic acid was extracted using Soxhlet extraction and petroleum ether as extractant. The extract was dried and dissolved in pyridine/acetic anhydride. The neutral fraction was then chromatographed using silica gel as support [48].

Cultivation studies revealed that sage is best cultivated under shade conditions with irrigation. Propagation is best performed by cuttings every three weeks during spring after the plants

have ceased to flower. The recommended planting distance is 30 cm in a row with a cultivation density of 10 plants per m^2. Plants should be irrigated immediately after planting of cuttings and twice weekly in summer. The monthly harvesting of leaves produced a variable content of essential oil on fresh weight basis with the peak reached during the month of August (2.24 % v/v) and the least during December (0.52 % v/v) (Figure 11).

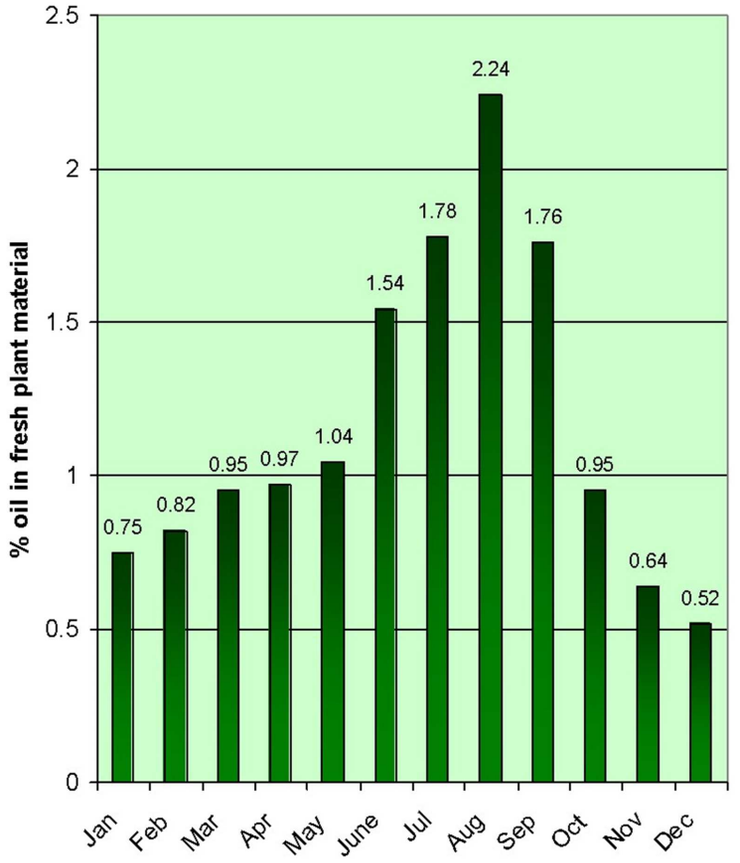

Figure 11. The yield of Maltese sage essential oil throughout the year [48].

2.5. Verbena officinalis L.

Verbena officinalis, a member of the Verbenaceae family, is also known as vervain. This plant is indigenous to Europe, North Africa and Asia but has been introduced to North America and Australia. Some of the common traditional uses of vervain, worldwide, were in the treatment of respiratory problems such as cough, wheezing and shortness of breath [37], as a purgative, in the treatment of haemorrhoids, eye problems [50], wounds, fever and stomach upsets [51]. In Malta, vervain was used in the treatment of many ailments particularly, carbuncles, boils, wounds, eczema, high blood pressure, diarrhoea, dysentery, cough and arthritis [52].

The main constituents of *Verbena officinalis* are iridoid glycosides, namely verbenalin [53], hastatoside [54] and aucubin [55]. It yields an essential oil, with citral, geraniol, limonene and verbenone as main constituents [56]. Other constituents include the flavone derivative artemetin, phenylpropane glycosides verbascoside and eukovoside and the triterpenes ursolic

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acid, β-sitosterol and lupeol [57]. Some of these constituents are highlighted in figure 12. The volume of oil obtained from Maltese sources was negligible [58].

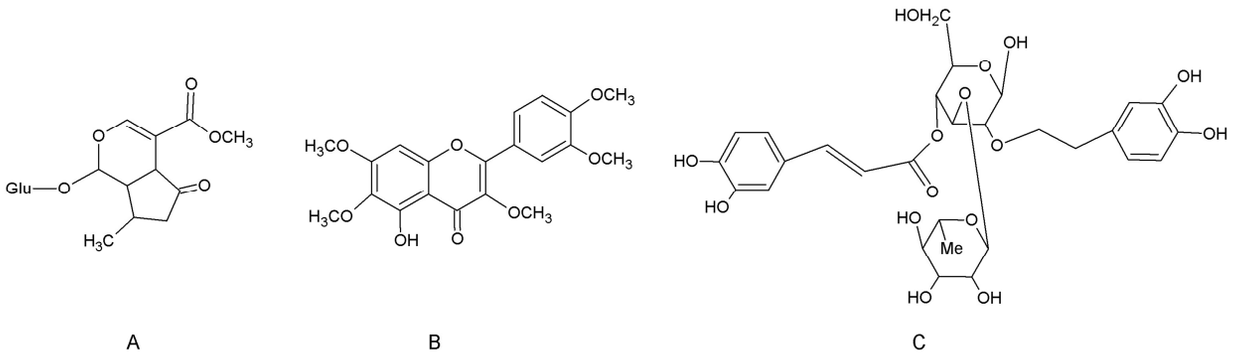


Figure 12. Typical constituents of *Verbena officinalis* L.: (A) verbenalin, (B) artemetin and (C) verbascoside

A hydromethanolic extract of the dried aerial parts of Maltese vervain was obtained by Soxhlet extraction [58]. The constitution of verbenalin was determined by HPLC using Supelcosil LC-18 column, acetonitrile/water-phosphoric acid (pH 2) gradient mobile phase with a flow rate of 1.5 ml/min. The content of verbenalin expressed as dry weight of plant material was 2.09 % (w/w). Previous reports [59] declared that contents of verbenalin were less than 0.1 % when extracted with ether but the content in methanolic extracts varied between 0.12 and 0.50 % [60].

Several pharmacological activities are attributed to vervain, namely, anti-inflammatory [54, 61], neuroprotective [62], antioxidant, antifungal [63], antileukaemic [64] and hepatoprotective [65]. Verbenalin, from Maltese vervain sources, was tested on mammalian intestinal smooth muscle *in vitro* and compared to acetylcholine [58]. Final molar concentrations of acetylcholine (40nM to 10 μM) and verbenalin (21.3 μM to 2.6 mM) were prepared. The smooth muscle was placed in an organ bath with a 30 ml-muscle chamber in freshly prepared Tyrode's solution maintained at 37°C. The muscle was challenged for a period of 30 seconds with the two substances at the stated concentrations (Figure 13). Between additions, the muscle was allowed to achieve baseline activity. The median effective concentration for acetylcholine and verbenalin were 1.54 μM and 0.32mM, respectively, with acetylcholine being approximately 200 times more potent than verbenalin. In spite of its mild effects, the presence of verbenalin in vervain is not recommended in pregnancy [66].

2.6. Hedera helix L.

Hedera helix L. or common ivy, a member of the Araliaceae family, is indigenous to Europe but its presence has been reported in Asia (as far as Japan), Africa and North America. Records of the use of ivy as a medicinal plant, dates back to the times of Hippocrates. The flowers were used to treat dysentery, earache and headache, while the leaves were usedas an emmenagogue [67). Others claimed it to be effective in the treatment of sunburn, ulcers, tuberculosis, bronchitis, whooping-cough, constipation, wounds and various skin diseases [68-70].

The main constituents of *Hedera helix* are the saponins, more commonly known as hederasaponins. This is a group of structurally related triterpenoid glycosides with an oleanane

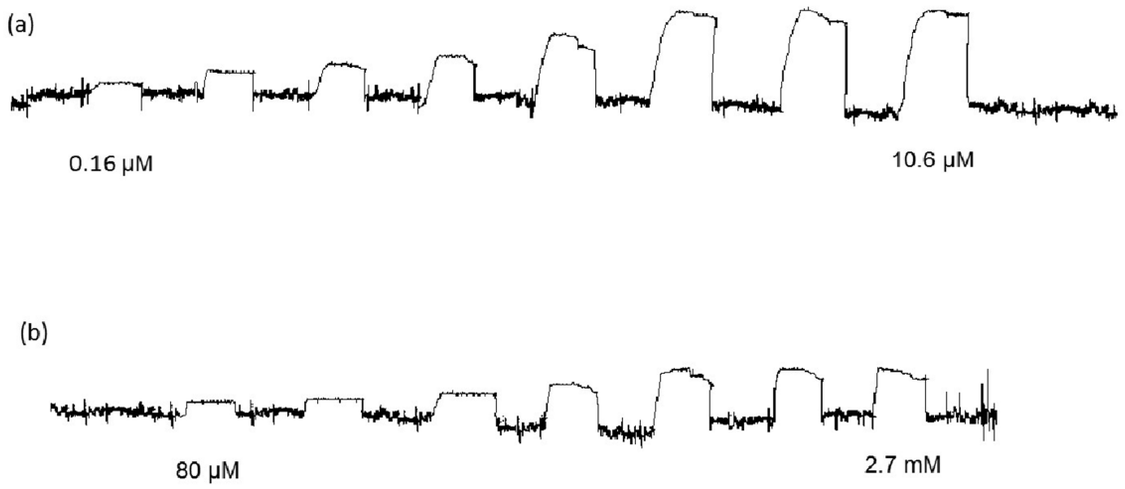
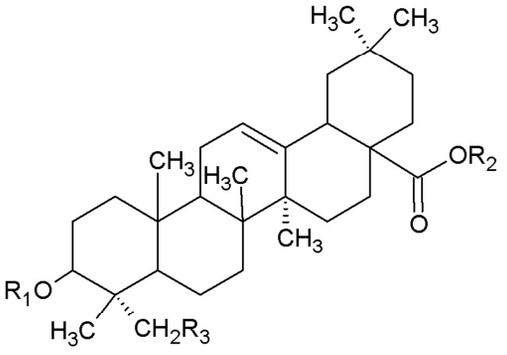


Figure 13. The spasmodic response of the smooth intestinal muscle with (a) acetylcholine and (b) verbenalin [58].

backbone (Figure 14). These are divided into mono- and bidesmosides. Monodesmosides include α-hederin and hederagenin 3-O-β-glucoside, while bidesmosides include hederasaponins C, A, B, D, E, F, G, H and I [71, 72].



	R_1	R_2	R_3
Hederasaponin C	$Rha_{1-2}Ara$	$Rha_{1-4}Glc_{1-6}Glc_1$	OH
Hederasaponin D	Ara_1	-	OH
Hederasaponin B	$Rha_{1-2}Ara$	-	H
α-hederin	$Rha_{1-2}Ara$	H	OH
δ-hederin	Ara_1	H	OH
β-hederin	$Rha_{1-2}Ara$	H	H

Figure 14. The main pentacyclic triterpenoids of *Hedera helix* L.

Another important group is that represented by phenolics (flavonoids, anthocyanins, coumarins and phenolic acids) [71, 73]. The essential oil from ivy stems and leaves contains germacrene D, β-caryophyllene, sabinene, β-pinene, limonene, and α-pinene [74]. Hederasaponins, from ivy grown in Malta, were extracted with 70 % ethanol by Soxhlet extraction [75]. Spring, summer, autumn and winter leaves yielded 12.75 %, 11.82 %, 10.74 % and 10.97 % (w/w) of dried extract. The hederosaponin content was determined by HPLC using Supelcosil LC-18 column, acetonitrile/water-phosphoric acid (0.01 N) gradient mobile phase with a flow rate of 1 ml/min. Hederasaponin C and α-hederin were used as standards. The 70 % ethanolic extract contained 46.7 % hederasaponin C and 6.1 % α-hederin totaling 52.8 %. Purification of the ethanolic extract through an alumina column with methanol as solvent resulted in 62.2 % hederasaponin C and 9.2 % α-hederin. This goes in accordance with other authors [76, 77] who confirmed that hederasaponin C is the main saponin in common ivy.

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Hedera helix was investigated for its pharmacological potential, by many scientists. Typical reported activities include anti-inflammatory [78, 79], antiviral [80], antifungal [81], antibacterial, mucolytic, antispasmodic agent and *in vitro* bronchodilatory [82, 83].

The ivy leaf extracts, obtained from Maltese sources, and the standards were tested for their antimicrobial activity [75]. The tested organisms were *Staphylococcus aureus*, *Escherichia coli*, *Enterobacter aerogenes*, *Klebsiella* sp., *Serrata* sp. and *Candida albicans*. Pure α-hederin was inactive against all organisms presumably due to its poor solubility in water as was reported by [84]. On the other hand, pure hederasaponin C was active against all the tested organisms. It was more active than both ivy extracts against *Staphylococcus aureus*, *Enterobacter aerogenes*, *Klebsiella* sp. and *Serrata* sp. It was just as effective as the purified ivy extract against *Escherichia coli* and *Candida albicans*. The only difference between hederasaponin C and α-hederin is that the former has an extra sugar group. Being a bidesmoside, hederasaponin C is more water soluble. There are no other structural differences that may have contributed to a better antimicrobial activity. In conclusion, the purified ivy extract (62.2 % hederasaponin C) and pure hedersaponin C were more active against *Staph. aureus* and least active against *Candida albicans* (table 3).

Microorganism	Minimum Inhibitory Concentrations (mg/l)			
	hederasaponin C	**α-hederin**	**Ethanolic extract**	**Purified ethanolic extract**
Staph. aureus	0.312	-	1.25 – 2.50	0.625 – 1.25
Escherichia coli	5	-	10	5 - 10
Enterobacter aerogenes	2.5	-	5 – 10	5 – 10
Klebsiella sp.	1.25	-	5 – 10	2.5 – 5
Serrata sp	2.5	-	5 – 10	-
Candida albicans	10	-	-	10

Table 3. Minimum Inhibitory Concentrations (mg/l) for *Hedera* extracts [75].

2.7. Crataegus monogyna Jacq.

Crataegus monogyna (may, quick or common hawthorn) belongs to the Rosaceae family. Records show that it has been used since the Ancient Roman times. Dioscorides and later Paracelsus reported the effects of the shrub in heart conditions [85]. Mediterranean folk medicine utilized the shrub as an astringent, febrifuge, sedative, in the treatment of diarrhoea, whitlow's, heart disease, high blood pressure and to improve circulation [86].

Hawthorn contains several constituents, most of which are either pharmacologically active or have a nutritional value. Triterpenoids, flavonoids, coumarins and amines are the main groups of compounds that possess a significant activity in the treatment of cardiovascular diseases [87].

The two triterpenoids, abundantly found in hawthorns, are ursolic and oleanolic acids (figure 15). These account for 90 % of the total pentacyclic triterpenoids present in the shrub [88]. The triterpenoids oleanolic, ursolic and crataegolic acids were extracted as a crude mixture with 96 % alcohol [89, 90], as an acid-ether extract [91] and as a tincture of *Crataegus monogyna* [92].

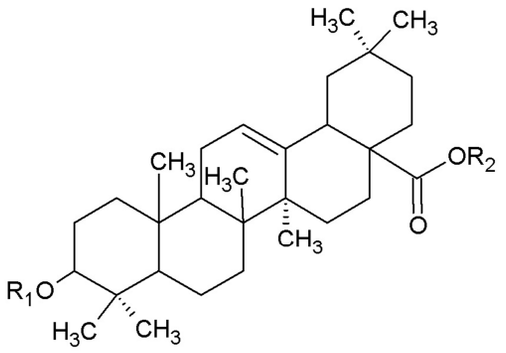

Figure 15. Structure of oleanolic acid and derivatives. For oleanolic acid, R_1 and R_2 are hydrogen atoms. For the triterpenoid glycosides, R_1 and R_2 represent different sugar groups.

Crataegus species are renowned for their flavonoid content [93]. Flavonoids include vitexin, hyperoside [94], rutin, quercetin, luteolin-7-glucoside [95] and apigenin [96]. The most abundant in flowers was the hyperoside [94]. Other flavonoids included catechin, luteolin, epicatechin, quercetrin, quercetrin-3-rhamnogalactoside and luteolin-3',7-diglucoside [87, 97]. Hawthorn contains a large variety of cardiotonic amines in different plants parts especially the leaves and flowers. These include di- and trimethylamine, ethanolamine, ethylamine [87], isoamyl and isobutylamines [92]. Choline and acetylcholine are also present. It contains other minor constituents [98].

Hawthorn extracts have been tested for several pharmacological activities such as antimicrobial, antioxidant [99, 100], peroxysmal tachycardia [101], prevention of cardiac necrosis [102-104], hyperglycaemia [105], atherosclerosis [106] and hypertension [107].

The hydroethanolic extract of *Crataegus monogyna* was studied for its angiotensin-converting enzyme (ACE) inhibitory activity [108]. The direct interaction of extracts and pure compounds with ACE was performed using a microtiter plate method modified for the ACE detection kit (Sigma, MO) at 430 nm (Figure 16). The crude extract contained triterpenic acids, flavonoids and coumarins. The ACE inhibitory activity of the crude extract and pure oleanolic acid (a triterpenoid) were compared to captropril, the latter used as a control drug. The hydroethanolic extract and oleanolic acid showed higher IC_{50} values (335.00 μg/ml and 3.61 μM, respectively) in comparison to captopril (46.9 nM). However, these results suggest that the anti-ACE activity of the hydroethanolic extract from hawthorn is due to oleanolic acid and other triterpenic acids present. This was the first study to suggest that triterpenic acids contribute to the antihypertensive activity of hawthorn. In previous studies, the ACE inhibitory activity of *C. monogyna* extracts was always attributed to flavonoids and proanthocyanidins.

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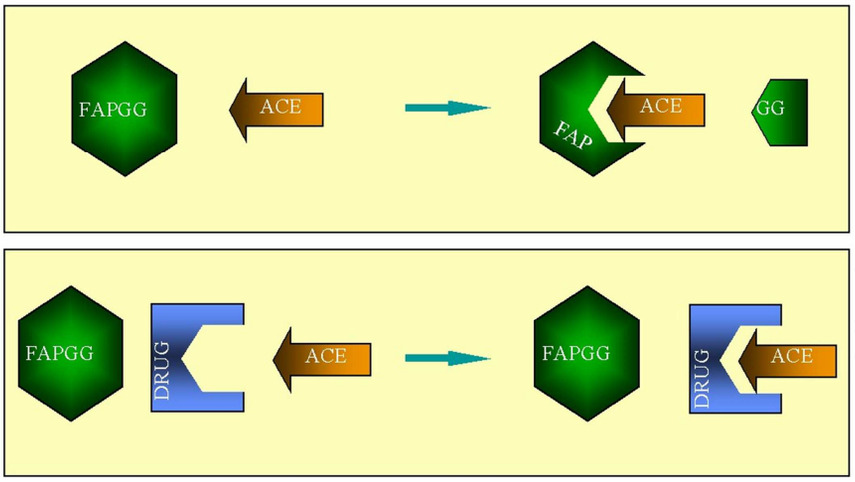

Figure 16. The interaction of angiotensin converting enzyme and compounds (such as captopril and oleanolic acid) with the chromophore N-[3-(2-furyl)acryloyl]-L-phenylalanylglycylglycine (FAPGG).

2.8. Calendula officinalis L.

Calendula officinalis, more commonly known as the pot marigold, belongs to the Asteraceae family. The use of pot marigold for therapeutic purposes has been recognised since the time of St. Hildeguard (1098-1197), who described in her work Causae et Curae and Physica the curative properties of ringula [109]. *Calendula officinalis* was being used internally during the twelfth century for digestive disturbances and also as an antidote against man and animal intoxication. It was also used externally for the treatment of impetigous eczema. Hundred years later (1193-1280), Albert the Great utilized the *Calendula* which he called Sponsa Solis, against animal bites and also to alleviate hepatic pain and pain of the spleen. This plant can also be seen in the herbals of the Renaissance. Leonard Fuchs stated that if the plant was to be boiled and held in the mouth for some time it relieved dental pain. Later during the sixteenth Century, Mattioli (1500-1577) attributed the therapeutic properties of the pot marigold in the constriction of the heart and palpitations as a consequence of menstrual fluid retention. According to this author, the water of the marigold has sudatory properties. He was also the first physician to recommend the herb for its therapeutic use against cancer and accordingly called it Herba Cancri. Mattioli's recommendations of *Calendula officinalis* as a remedy against cancer were fully approved by Osiander and Hufeland [109]. The pharmacist, J. W. Weinmann (1683 - 1741), in his work "Phytantoza iconographica" recommended the aqueous marigold extract for the alleviation of red and inflamed eyes and also used the plant in the treatment of goitre.

Pharmacologically-active classes of compounds, in the marigold, include the terpenoids including the carotenoids, flavonoids, coumarins and polysaccharides [110-112]. The saponosides are particularly abundant in the plant. There are also numerous triterpenoid alcohols which are derived from tarassene, lupene, oleanene and ursene. These are present as free or esterified as monols, diols and triols. The content of monoesters of the triterpenoid diols is between 2 and 45 %, of which 1.85 % is made up of faradiol esters. The most common triter-

penoid is oleanolic acid (Figure 15). The colour of the flowers is determined by the amount of carotenoids which can vary from 1.5 to 3 %. The orange flowers are made up mainly of carotenes particularly lycopene whereas the yellow flowers contain mainly xanthophylls [113]. The heterosides of quercetin and isorhamnetin (flavonoids) are present in the dry *Calendula* drug [114]. Their content varies between 0.25 and 0.88 %. The *Calendula* drug contains 14.75 % of polysaccharides (PS), which are soluble in water. The three main ones are PS I (molecular weight of 15,000), PS II (molecular weight of 25,000) and PS III (molecular weight of 35,000). These are made up of galactose, rhamnose and arabinose subunits. Other constituents include the essential oil, triterpene alcohols, phenolic acids, tannins, sterols, tocopherols, N-paraffins, pyrethrins, sesquiterpenes and coumarins. Monoterpenes and sesquiterpenes make up the essential oil. However, the latter does not contain sesquiterpene lactones. Moreover, 50 - 60 % of the oil present in the seeds is made up of calendulic acid, an unsaturated fatty acid having an unusual chemical structure.

Flowerheads of *Calendula officinalis* were extracted with methanol and following concentration, the extract was hydrolysed with 0.5 M hydrochloric acid. The mixture was centrifuged and the residue was dissolved in chloroform. This was then dried and subjected to column chromatography (silica gel; mobile phase - petroleum ether:dichloroethylene:acetic acid 50:50:0.7). The collected fractions were analysed by melting point determination, Infrared and Ultraviolet spectroscopy. The content of oleanolic acid extracted from the dried flowerheads was 0.13% (w/w) [115].

The marigold has been investigated for its anti-microbial, anti-inflammatory [116, 117], anti-tumour [110] activities, effects on the cardiovascular and nervous systems [118, 119] as well as oestrogenic [120], hypolipidaemic [121], anti-ulcer [122] and spermicidal properties [123].

The antimicrobial activity was conducted for oleanolic acid against a number of organisms [115]. Due to the insoluble nature of oleanolic acid in water, it was incorporated in the nutrient agar for bacterial strains and in Sabouraud's dextrose agar for fungi. In fact, [124] stated that the anti-bacterial agent was soluble in alcohol but not in water. According to the results obtained after 24 hours in the study performed, oleanolic acid was active against Gram-positive organisms (*Strep. faecalis, Strep. viridans* and *Staph. aureus*) except *Staph. albus*. However, for *Staph. albus*, there was slight inhibition which resulted in hazy growth. On the other hand, it was inactive against Gram-negative strains. Although for *Morganella* species and *Pseudomonas aeruginosa*, there was some degree of inhibition, the plate which contained the ethanol instead of oleanolic acid showed the same degree of inhibition. Hence, the anti-bacterial activity in these two cases might be attributable to the presence of ethanol. Oleanolic acid did not show any activity against *Candida albicans*.

The topical in vivo effects of oleanolic acid (2.5 %) on inflamed bites induced by mosquitoes (*Culex pipiens*) was studied [115]. The positive and negative controls included indomethacin (2.5 %), hydrocortisone (1 %) and petroleum jelly. The topical anti-inflammatory activity of oleanolic acid, over a 24-hour period, was comparable to that of hydrocortisone, after being applied at 8 hourly periods. However, when compared to indomethacin, oleanolic acid was found to be less effective ($P<0.01$). In accordance with the study conducted by [125], oleanolic acid was found to have similar effects to those of hydrocortisone. However, other studies relate oleanolic acid to non-steroidal anti-inflammatory agents, like indomethacin [126].

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2.9. Melissa officinalis L.

Melissa officinalis L. is a member of the Lamiaceae family. It is also known as lemon balm or simply as balm. The Latin name ''Melissa'' (balm) refers to the Greek word 'melitos', that is honey. It is believed that the plant attracts honey bees. The plant is found mainly in the Mediterranean region and eastwards to Asia and Siberia. Balm is renowned for its effects on the nervous system and is used to treat nervous agitation, insomnia, hysteria, melancholia, migraine, headache, toothache, earache and nerve pains. It is also useful for gastrointestinal problems such as gastric complaints and lower abdominal pain [127, 128].

Lemon balm contains a volatile oil [129], flavonoids (cynaroside, rhamnocitrin, isoquercitrin, cosmosin), phenolic acids (carnosic acid and rosmarinic acid), and triterpene acids (particularly ursolic and oleanolic acid) [130]. The study by [131] focused mainly on the cultivation parameters that affect the quantity and quality of the lemon balm oil. The oil yield was 0.1 % (v/w) with cis-citral and trans-citral as the major constituents (figure 17).

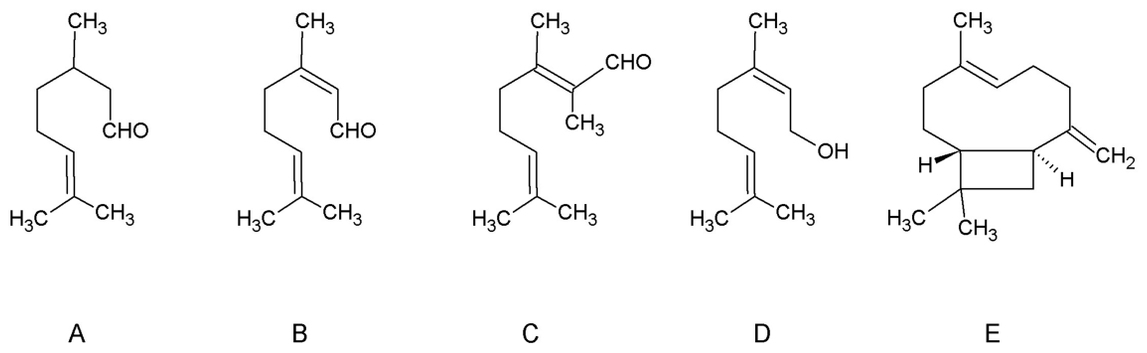

Figure 17. The main constituents of *Melissa officinalis* essential oil: (A) citronellal, (B) geranial (trans-citral), (C) neral (cis-citral), (D) trans-caryophyllene

Seeds were procured from four sources: Maltese (Argotti Gardens), Swiss (Basel Botanic Gardens), German A (Botanischer Garten der Martin-Luther-Universität) and German B (Botanischer Garten der RWTH). The planting distance was of 20 cm in a row with a distance of 50-60 cm between rows. The cultivation density was of 10 – 12 plants per m^2. The plants were irrigated immediately after transplanting and then once every fortnight in winter but twice weekly in summer. Plots were divided into two: half treated with fertiliser (NPK Mg (12+12+17+2) + Trace elements) while the other half left untreated, as a control. The leaves were harvested in May and subjected to steam distillation extraction and GC-MS analysis. Table 4 illustrates the results obtained in this study.

In most cases, the use of fertilizer improved content of the two main terpenoids, geranial and neral. This goes in accordance with [132], stating that nitrogen fertilisers increased the yield of these constituents. In some cases citronellal also showed significant increases with fertilizer application. In another study, the oil yield was found to vary between 0.16 and 0.25% [133]. With farmyard manure, the content of neral (28.23%) and geranial (39.86%) was higher than with other treatments. Oil yield was also significantly affected by planting spacing and nutrient amendments.

Sample		Citronellal	Nerol	Geranial	Neral	Caryophyllene
Maltese	w/o Fertiliser	0.00	0.00	37.11	47.39	1.02
	Fertiliser	0.52	0.00	36.82	47.74	1.26
Swiss	w/o Fertiliser	0.55	0.00	36.08	48.92	2.11
	Fertiliser	1.31	0.55	30.73	45.13	2.67
German A	w/o Fertiliser	1.24	0.57	30.96	45.79	2.62
	Fertiliser	1.25	0.71	32.11	47.23	1.84
German B	w/o Fertiliser	1.65	0.56	31.39	47.63	2.13
	Fertiliser	1.31	0.74	33.42	49.19	1.94

Table 4. The composition of essential oils obtained from lemon balm of different seed origins [131].

2.10. Olea europea L.

Olea europea L. is a typical Mediterranean plant within the Oleaceae family with culinary and medicinal virtues. The typical extract from this plant is the fixed oil obtained from the fruit. In the ancient world, by 2000 BC the olive tree was already in cultivation. Olive and olive oil was used and traded by the Egyptians, Phoenicians, Greeks and Romans. Today, a large number of olive varieties are recognised internationally as table olives and olives for oil production. Extracts from the olive tree were used in the treatment of hypertension, hyperglycaemia, hyperacidity [134], constipation, for treatment of wounds, sunburn and muscle aches [135, 52] amongst others.

The bioactive phenolic compounds present in the olive fruit include phenolic acids, phenolic alcohols, flavonoids and secoiridoids. The main phenolic acids are cinnamic, syringic, p-coumaric, vanillic, caffeic, 3,4-dihydroxyphenylacetic and protocatechuic acid [136]. Phenolic alcohols include 3,4-dihydroxyphenylethanol (hydroxytyrosol) and p-hydroxyphenylethanol (tyrosol) [137-139]. Flavonoids include taxifolin, apigenin, luteolin and lignans represented by pinoresinol and its metabolites [140]. However, an important class of metabolites found in the leaves and fruit of *Olea*, is that of the secoiridoid glycosides. These include oleuropein (figure 18), demethyloleuropein, oleuropein aglycone and elenolic acid [141-144].

Oleuropein was extracted from Maltese olives as follows [145].The leaves were defatted with petroleum ether and then extracted with 50% ethanol for 6-8 hours. The dried extract was then treated with water and sodium chloride was added until saturation was achieved. Chloroform was added and the aqueous extract was collected. Ethylacetate was added to the aqueous extract and following partitioning, the ethylacetate extract was collected. The extract was then subjected to dryness in order to obtain a yellow crystalline substance. Oleuropein in the olive leaf ethanolic extract amounts to 20.6 %, as mentioned by [146], with a content varying from 20 to 25% (w/w) total dry weight.

Olea europea was tested for its antimicrobial [147, 148], antiviral [149], antioxidant [146, 150], antihypertensive, antiatherosclerotic [151, 152] and antidiabetic [153] activities amongst

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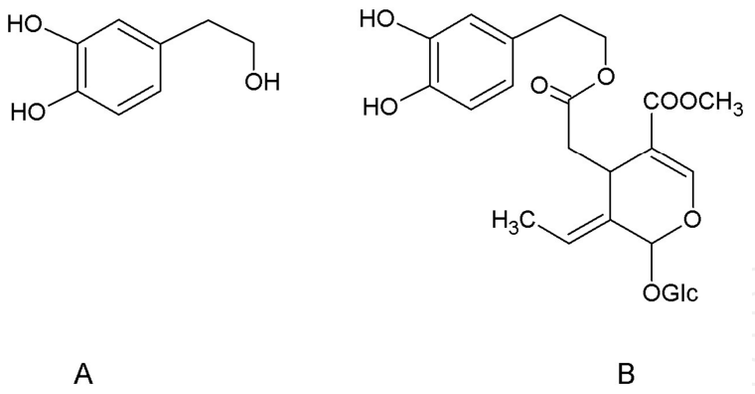

Figure 18. Structures of polyphenolic compounds from olive oil: (A) hydroxytyrosol and (B) oleuropein glucoside.

others. Maltese olive leaf extract was studied for its immunomodulatory activity [145]. Human peripheral blood lymphocytes were isolated and cultured on RPMI medium. Oleuropein (540 – 0.054 μg/ml) was tested alongside phytohaemagglutinin (m-form, Gibco BRL, UK - 1 – 0.0001 % as positive control and *Olea* extracts to final concentrations ranging from 540 – 0.054 μg/ml (oleuropein content). The cells were studied for their survival, death and morphological characteristics using WST-1 assay, LDH (Boehringer-Mannheim, Germany) and the Papanicolau staining procedure, respectively. Oleuropein possesses three α,β -moieties; two α, β -unsaturated keto systems at the 3,4-dihydroxyphenyl part and one α, β -unsaturated aldehyde system on the secoiridoid part, which are important for the non-toxic but stimulatory activity on lymphocytes. From the results obtained, oleuropein was more effective when it formed part of the extract (SC_{50}, < 0.054 μg/ml) than when used in its pure form (SC_{50}, 0.146 μg/ml).

2.11. Urtica dubia Forsk.

Urtica dubia Forsk., stinging nettle, is a member of the Urticaceae family. The *Urtica* species are common weeds found growing wild throughout the temperate zones of both hemispheres worldwide. These species are renowned for their stinging sensation when touched. Since Ancient Greek times, stinging nettle was used as a medical treatment for septic wounds, nosebleeds and as an emmenagogue [154]. They were later used as diuretics and laxatives, in the treatment of asthma, pleurisy, dog bites, tinea and mouth ulcers [155]. In Malta, *Urtica dubia* was used in the treatment of pneumonia, chilblains, as a metabolic stimulant, to improve blood circulation and as a diuretic [156].

Stinging nettle contains bioactive amines such as 5-hydroxytryptamine; flavonoids such as quercetin, kaempferol and their glycosides; coumarins such as scopoletin; organic acids such as caffeic acid and chlorogenic acid; fatty acids such as erucic acid, α-linolenic acid and linoleic acid; an essential oil; carotenoids such as lutein, β-carotene, neoxanthin, violaxanthin and lycopene; agglutinins such as *Urtica dioica* agglutinin (Figure 19); and phytosterols such as β-amyrin, stigmasterol, oleanolic acid and β-sitosterol [157-163]. The isolation of *Urtica dubia* agglutinin (UDuA) was based on a procedure described by [164] with some modification [165]. Briefly, the fresh plant materials (rhizomes, leaves and stems) were homogenised with 0.1N

HCl (200 g/l) and allowed for 24 h shaking. The filtrate was passed through series of extractions with 2N NaOH and $(NH_4)_2SO_4$ solutions. The final agglutinin purified extract was washed with phosphate buffer saline (PBS) which was used as the medium for the bioassays. Phytohaemagglutinin (PHA, Invitrogen) was prepared likewise in PBS. The content of UDuA in the rhizomes, leaves and stems was 0.49 %, 0.65 % and 0.16 %, respectively.

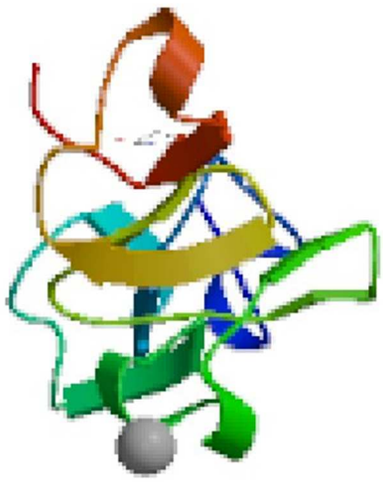

Figure 19. The crystal structure of *Urtica dioica* agglutinin isolectin I [166].

The stinging nettle possesses several pharmacological activities, namely antioxidant, antimicrobial, antiulcer and analgesic activities [167], anti-inflammatory effects [168] and cardiovascular effects [169]. The UDuA extracts from the Maltese *Urtica dubia* were tested for haemagglutination activity on human red blood cells (RBCs) [165]. Briefly, a 1% suspension of RBCs was prepared and 100 μl aliquots were tested with different concentrations of UDuA and PHA. The agglutination was quantified by lysing the precipitated agglutinated cells and read spectrophotometrically at a wavelength of 405 nm at 20, 40, 60 and 80 minutes. Over the 80-min period, the best results were obtained after 60 min, as was observed by [170] for the snowdrop lectin. Extracts from all three plant parts exhibited superior haemagglutination activity (AgA) to the standard PHA lectin (AgA - 3.996 ± 0.259). The highest activity was exhibited by the stems, followed by roots and leaves (AgA - 4.824 ± 0.301, 4.693 ± 0.368 and 4.594 ± 0.417, respectively at 1% concentrations).

2.12. Capparis spinosa L.

Capparis spinosa L. is a member of the Capparaceae family, also known as the caper plant. Today it is renowned for its culinary uses, particularly in the Mediterranean region. When stored in brine, the intensive and slightly pungent taste of the capers is preserved. Capers were used since prehistoric times, although it is believed that other *Capparis* species were actually utilised rather than *Capparis spinosa* [171]. In the past, the root bark and leaves were used as aperient,

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tonic, diuretic and expectorant while the flowers were used as anthelmintic, emmenagogue, analgesic, antimicrobial, antifertility, anti-inflammatory, hepatoprotective, antihyperglycemic, immuno-stimulant and in the treatment of anaemia, diabetes, heart problems, amongst other uses [172]. In Malta, caper extracts were used as diuretics, in the treatment of skin rashes and pain associated with gout [135].

According to [173], the capers contain 79% moisture, 1.6% ash, 5.8% protein, 1.6% fat and 5.4% raw fibre. It contains several minerals such as, Ca (871 ppm), Mg (636 ppm), K (542 mg/100mL), Na (226 ppm), Fe (13 ppm) and P (21 mg/100g). Other valuable constituents include the flavonoids such as rutin, kaempferol and its glycosides; alkaloids (Figure 20) such as cadabicine [174], capparisine A, capparisine B, capparisine C; 2-(5-hydroxymethyl-2-formylpyrrol-1-yl) propionic acid lactone and N-(3′-maleimidy1)-5-hydroxymethyl-2-pyrrole formaldehyde [175]. Other constituents include aldehydes, esters, sesquiterpenes, monoterpenes and sulphur compounds with methyl-isothiocyanate as the main constituent [176], carotenoids with lutein as the main constituent [177], sterols such as β-sitosterol, campesterol, stigmasterol, 5-avenasterol, cholesterol and campestanol [178], and a lectin (*Capparis spinosa* lectin) [179].

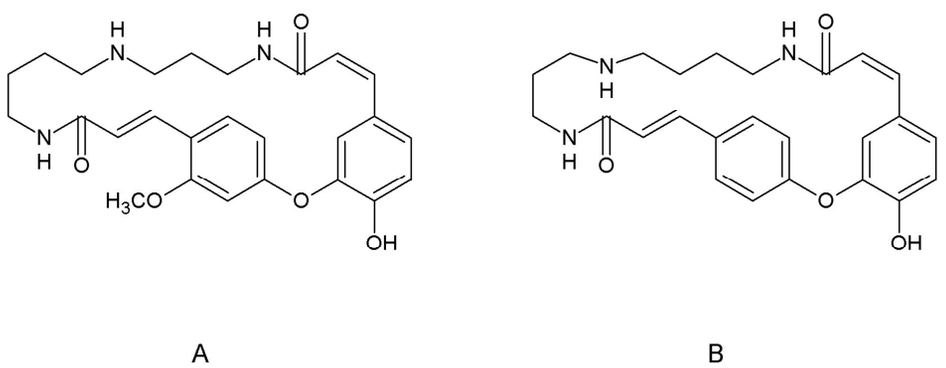

Figure 20. Typical *Capparis spinosa* L. alkaloids: (A) capparisine and (B) cadabicine.

Metabolites from the Maltese caper plant were obtained by extracting the plant material with four different solvents [180]. The xanthoproteic test for proteins [181], Fehling's test for carbohydrates, Sudan IV test for fats and lipids [182], Dragendorff's test for alkaloids [183], triphenyltetrazolium test for terpenoids and the acidified vanillin test for flavonoids [184] were carried out on the extracts. The petroleum ether extract (0.020 % w/w plant material) contained fats and lipids, the aqueous/methanol extract (2.401 % w/w plant material) contained proteins and terpenoids, the methanol extract (1.398 % w/w plant material) contained alkaloids, while the aqueous extract (3.015 % w/w plant material) contained carbohydrates and terpenoids.

The caper plant was tested for several pharmacological activities such as antiviral [185], anti-arthritic [186], anti-oxidant [187], hypolipidaemic [188], antihyperglycaemic [189], chondrocyte protective [190], antiallergic, antihistaminic [191], antifungal [192], anti-Leishmania [193] and antimicrobial [194]. The Brine shrimp test was conducted for the extracts derived from the Maltese caper plants [180]. Briefly, Artemia salina eggs were hatched and challenged with

various concentrations of the extracts ranging between 0.0001 and 1 % as 1 in 10 dilutions. After 24 hour the number of dead larvae (nauplii) was determined. The aqueous extract exhibited the lowest LC_{50} (0.014%) compared to the methanol (0.0475%) and the aqueous/ methanol (0.08%) extracts. The chloroform extract did not reach a 50% lethal effect and therefore the LC_{50} could not be determined. According to [195] the methanol, aqueous and aqueous/methanol extracts were all active as their LC_{50} was below the 0.1% threshold.

2.13. Ephedra fragilis Desf.

Ephedra fragilis Desf., a member of the Ephedraceae family, is also known as Mormon tea. *Ephedra* has been listed amongst the most important herbs used by Ancient Chinese civilisations. It was known as Ma Huang and was used to treat coughs, colds, headache and fever. It was later used by the Chinese to treat asthma [196] and acute nephritis [197]. This plant contains alkaloids [198], amino acids, proteins [199], tannins and fatty acids [200]. The volatile oil of *Ephedra fragilis* contains (E)-phytol (10.1%), pentacosane (5.2%), 6,10,14-trimethyl-2-pentadecanone (5.3%), cis-thujopsene (3.5%), and α-terpineol (3.0%) as the major components [201]. Flavonoids, minerals, and vitamins are also present. The principle alkaloid present in this plant is ephedrine [198] (Figure 21).

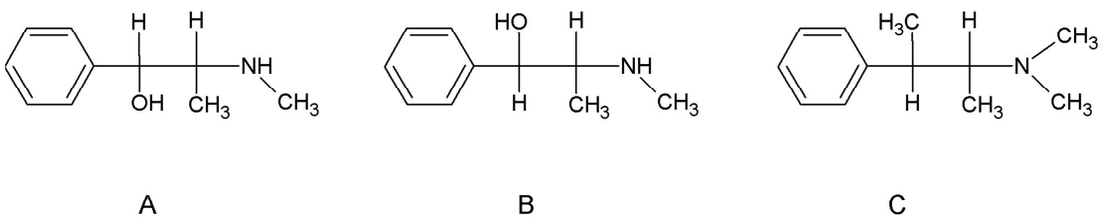

Figure 21. Ephedrine, pseudoephedrine and methylephedrine

Aerial parts of local cultivated *Ephedra fragilis* specimen were dried in an oven at 30ºC for 48 h, pulverized and dispersed in distilled water for 25 min. After thorough mixing for 30 min at 30ºC (twice), the filtrate was treated with sodium carbonate (15 g). An equal volume of benzene was added and then acidifed and treated with acidifed water. After neutralisation to a pH of 7, the precipitate was oven dried. The alkaloidal content in the different plant parts was determined; 1.8675 % (w/w) in flowers, 0.6234 % in seeds, 0.5198 % in pods and seeds, 0.1389 % in dried pods and 0.0547 % in branches [202].

Clinically, ephedra has been tested for its anti-hypertensive [203], bronchodilator [204], decongestant [205], diuretic [206] and immune booster [207]. The immunomodulatory response of ephedrine and the *Ephedra* extract were studied on human peripheral lymphocytes [202]. Cell viability, cytotoxicity and morphological characteristics were recorded for the test substances and phytohaemagglutinin (PHA), a mitogen known to stimulate cell division of T-lymphocytes. Over the 96-hour treatment, ephedrine and *Ephedra* extracts exhibited high cell viability (> 97% viability) and blastogenesis when compared to the untreated control. The control cells measured 6-10 μm, while treated cells measured 20-40 μm in diameter. The ephedrine present in *Ephedra* extracts exhibited a direct effect on lymphocytes *in vitro*.

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2.14. Nicotiana glauca RC Graham

Nicotiana glauca RC Graham belongs to the *Solanaceae* family and is known as tree tobacco. This was native to South America but is now naturalized in North America, the Mediterranean, and Africa. Since, this plant was considered as poisonous [208], it has been rarely used in tradition. The more toxic counterpart, *Nicotiana tabacum* was used for several conditions particularly to expel leeches [209], against snakebite [210] and scabies [211].

Tree tobacco contains pyridine alkaloids [212], as for other *Nicotiana* species. The major pyridine alkaloids are nicotine and anabasine (figure 22). Nicotine predominated in *Nicotiana tabacum* [213] and *Nicotiana* rustica [214] whereas anabasine predominates in *Nicotiana glauca* [214, 215].

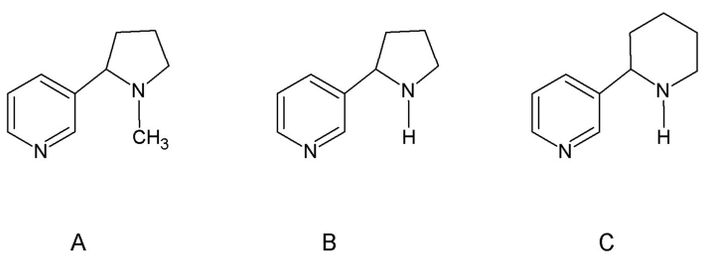

Figure 22. (A) Nicotine, (B) Nornicotine and (C) Anabasine, the main pyridine alkaloids of *Nicotiana* species.

The leaves of Maltese *Nicotiana glauca* were dried and extracted with 200ml of 0.5% sodium hydroxide [216]. After volume reduction, chloroform was added to extract the alkaloids in this organic phase. This phase was then treated with acidified water (0.05M hydrochloric acid) and then neutralised with ammonia solution to a pH of 7. The presence of alkaloids was tested at each step using the Dragendorff's reagent [217] and the anabasine content was determined by HPLC. A Shimadzu LC-10A HPLC (Shimadzu, Kyoto, Japan) using a C18 MicroBondapak column, 250 x 4.6mm, 10mm was used. The mobile phase consisted of 40 % methanol containing 0.2 % phosphoric acid buffered to pH 7.25 with triethylamine [218]. The anabasine standard was used for calibration and for the determination of anabasine in Nicotiana extracts. In the Maltese study, the anabasine content (0.258 ± 0.0042 %) concords very closely with the results obtained in a study in Arizona (0.233 ± 0.0061 % anabasine) [219]. In another HPLC determination, the anabasine content of *Nicotiana glauca* plants in California, was 0.143 % [220].

The nicotine and anabasine have been widely used as pesticides. Nicotine is a powerful insecticide towards aphids [221] and larvae of lepidopterous pests [222]. Anabasine and nicotine exert their insecticidal effect by interacting with nicotinic acetylcholine receptors [222, 223]. Anabasine and *Nicotiana glauca* extracts were tested for their effects against *Pieris rapae* larvae [216]. The paralysis of the larvae was an indicator of activity. Standard anabasine produced an effect on *Pieris rapae* larvae (EC_{50} - 0.572 mg/larva or 0.286 %) which was higher to that provoked by the extract (EC_{50} - 1.202 mg/larva or 0.601 %). It is possible that alongside anabasine there may be other metabolites that interfered with anabasine hence reducing the response of the caterpillars to anabasine.

3. Conclusion and further directions

The studies on the fourteen Maltese medicinal plants, presented herein, demonstrate a wide array of experimental work that is all associated with phytochemical research. This is a very small fraction of the Maltese medicinal flora, but in terms of research, this represents a diversity of research protocols that may be adopted for medicinal plant research. In some cases, phytochemical analysis is the end-point of the research whereas in others, phytochemical analysis leads on to further studies, including pharmacological testing.

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