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The Phytochemical and *In Vitro* Pharmacological Testing of Maltese Medicinal Plants

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1. Introduction

1.1 General background

The Maltese archipelago is composed of a small number of islands with a total surface area of approximately 457 km^2. Albeit this small size the Maltese islands host a vast number of plant and animal species. Plant biodiversity, with its 1264 vascular species, is mainly attributed to the strategic position of Malta within the Mediterranean, in which throughout the years several conquerors and civilisations sought to possess Malta particularly for military purposes. In part, the plant diversity of Malta is attributed to introductions brought about by various military forces, as an aid during injury and sickness. Naturally, the phytodiversity has an inclination towards the Mediterranean type of flora with an approximately 66% of the Maltese flora pertaining to this region (E. Attard, 2004). Typical Mediterranean medicinal plants include conifers (*Pinus halepensis* and *Cupressus sempervirens*), broad-leaved trees (*Laurus nobilis, Morus nigra* and *Tamarix gallica*), fruit trees (*Ceratonia siliqua, Citrus* trees, *Nerium oleander, Olea europaea* and *Punica granatum*), and others (*Allium sativum, Aloe ferox, Capparis spinosa, Opuntia ficus-indica, Origanum vulgare, Papaver somniferum, Phytolacca decandra* and *Pistacia lentiscus*). The other portion (34%) is attributed to plants originating from the warm North African (*Cynomorium coccineum, Ficus carica* and *Myrtus communis*) and the colder South Europaean regions (*Crataegus monogyna, Populus alba* and *Salix species*).

There are approximately 458 medicinal taxa, used in the past to treat one or more ailments (Lanfranco 1993; Lanfranco 1975). Most popular treatments were for the gastrointestinal system, nervous system, cardiovascular system and dermatological conditions. The most predominating plant family within this group is the Asteraceae family, followed by the Lamiaceae and Fabaceae families (Attard, 2004). In spite of their use, these medicinal plants were administered on a trial and error basis. Today, with the advent of modern scientific techniques, the ethnobotanical attributes of a medicinal plant can be challenged by phytochemical and pharmacological testing.

1.2 Scientific evaluation of local medicinal and aromatic plants in relation to pharmacology

Locally, only 8 % out of the 458 taxa have been studied scientifically. However, the studies conducted were rather fragmented and covering one or two extracts from a specific plant. Plants include *Ecballium elaterium* (E. Attard et al., 2005; E. Attard & H. Attard, 2008), *Crataegus monogyna* (E. Attard & H. Attard, 2006), *Olea eurpoaea* (Mangion Randon & E. Attard, 2007), *Ephedra fragilis* (E. Attard & Vella, 2009) *Urtica dubia* (Rossi & E. Attard, 2011), *Tetraclinis articulata* (Buhagiar et al., 1999) and *Ricinus communis* (Darmanin, 2003) amongst others.

1.3 Technical approaches

The evaluation of plant species using different solvent systems has been widely exploited in previous studies (Rodriguez-Lopez et al., 2003; Kumarasamy et al., 2002; Calderon et al., 2003; Konning et al., 2004). A wide spectrum of solvents may be employed when a small number of plants (1-15) are investigated, but when investigating larger numbers or a new group of plants for the first time, the solvents used in ethno-medicine are preferentially selected (Punjani and Kumar, 2003; Guarrera, 2003).

Phytochemical analysis for major classes of metabolites is an important first step in pharmacological evaluation of plant extracts. Some journals require that pharmacological studies be accompanied by a comprehensive phytochemical analysis. Details of such analysis are found in several text books (Harborne, 1984; Evans, 2009). The main secondary metabolite classes include flavonoids, terpenoids and alkaloids, which have been widely tested by the acidified vanillin test, the Salkowski test and the Dragendorff's test, respectively.

Bench top bioassays have been devised to facilitate screening of a large number of samples (Meyer et al., 1982; Carballo et al., 2002). They are based on the principle that pharmacology is simply toxicology at low doses, while toxicology is pharmacology at high doses. Several researchers have used these bioassays for primary pharmacological screening of medicinal plants (Franssen et al., 1997; Kanegusuku et al., 2001; Javidnia et al., 2003). The brine shrimp lethality test (BST), which involves the exposure of brine shrimps to different extract concentrations, is considered as a useful tool for preliminary assessment of cytotoxicity (Jaki et al., 1999). It is a rapid (24 hours), inexpensive and simple technique. A positive correlation has been found between the brine shrimp test and cytotoxicity of the 9KB human nasopharyngeal carcinoma, and other cell lines (Meyer et al., 1982; Kim et al., 2000).

The DNA methyl green bioassay is a simple and comprehensive technique with a high throughput. Methyl green, binds quantitatively to DNA forming a DNA-methyl green complex, hence identifying agents with a high affinity for the DNA. This affinity determines the displacement of methyl green, hence leading to a colourless carbinol (N. Kurnick, 1950; B. Kurnick and Foster, 1950; Krey and Hahn, 1975).

1.4 Aims of study

We believe that Maltese medicinal and aromatic plants have a great pharmacological potential. This is based on the concept that, in the past, these plants had important medicinal uses. Therefore, we aimed our study at ethnobotanical research by:

1. Identifying plants cited in ethnobotanical research as active medicinal plants
2. Preparing five extracts using different solvents from each medicinal plant, and the subsequent determination of the classes of metabolites present in the different extracts.
3. Determining whether or not, the extracts obtained eventually possess pharmacological activity employing a primary screening programme.
4. Identifying plant extracts that possess DNA binding.

2. Materials and methods

2.1 Plant materials

Fifty-five authenticated plant specimens were collected locally during different seasons of the year. The plants were selected on their relative abundance, and collected during their flowering period. The plants were further identified at the Rural Sciences and Food Systems Division, Institute of Earth Systems. Voucher specimens are stored within the Institute. The botanical and ethnobotanical details of the medicinal plants and their voucher specimen code numbers are listed in Table 1.

2.2 Preparation of plant extracts

Fresh plants were cut and oven-dried for 48 hours at 35-40°C in a hot air convection oven. Five 300g samples of the dried plants were ground in a heavy duty blender for 20 minutes. 500 ml of solvent (distilled water, distilled water and ethanol (1:1) mixture, ethanol or chloroform or petroleum ether) were added to the respective sample, shaken for 48 hours at 210 rpm, and filtered through a Buchner funnel. Each filtered extract was concentrated at 38 °C under reduced pressure, and finally dried in an oven at 38 °C.

2.3 Phytochemical analysis: quantitative colorimetric assays

Although most phytochemical analysis carried out may have a qualitative importance, the methods were modified according to other authors to read aborbance values at a wavelength of 405 nm rather than visual examination. The MTP reader gave more concrete results, in the form of absorbance values. Therefore semi-quantification is possible through this process.

Four colorimetric tests were quantitatively used to determine the presence or absence of metabolites:

1. The Salkowski test for terpenoids. After the addition of chloroform and concentrated sulphuric acid, a reddish brown colouration at the interface forms, hence showing a positive result for the presence of terpenoids (Edeoga et al., 2005);
2. The Dragendorff's test for alkaloids (Steinberg et al., 1997) gives a brown coloration;
3. The Acidified Vanillin test for flavonoids. Under acidic conditions, vanillaldehyde condenses to flavan-3,4-diols, flavan-3-ol monomers and proanthocyanidins to give a cherry-red product (Deshpande et al., 1986);
4. The ninhydrin test was used for proteins (Delhaye & Landry, 1992). The α-amino acids typically give a blue-purple product.

Voucher specimen number	Botanical Name, family	Maltese, (English) Names	Part/s used, preparation and Maltese Traditional uses
IOA-AMP-002	*Acanthus mollis* L., Acanthaceae	Ħannewija (Common bear's breeches)	Herb/Emollient as skin softener (Borg, 1927)
IOA-AMP-015	*Aloe vera* L., Liliaceae	Sabbara (Yellow aloe)	Leaf Juice in child weaning, laxative, increases menstruation (Penza, 1969; Cassar Pullicino, 1947; Cassar, 1964)
IOA-AMP-026	*Anagallis arvensis* L., Primulaceae	Ħarira ħamra or Ħarira kaħla (Scarlet pimpernel or Blue pimpernel)	Herb and seeds as sudorific and in rabies (Penza, 1969; Gulia, 1855)
IOA-AMP-037	*Antirrhinum siculum*, Mil., Scrophulariaceae	Papoċċi bojod (Sicilian snapdragon)	Leaves as astringent, diuretic and in chest problems (Penza, 1969; Borg, 1927)
IOA-AMP-036	*Antirrhinum tortuosum* L., Scrophulariaceae	Papoċċi ħomor (Red snapdragon)	Leaves as astringent, diuretic and in chest problems (Penza, 1969; Borg, 1927)
IOA-AMP-049	*Asparagus aphyllus* L., Liliaceae	Spraġ selvaġġ (Wild asparagus)	Herb as diuretic (Penza, 1969)
IOA-AMP-453	*Aster squamatus* (Sprengel) Hieron, Asteraceae	Settembrina selvaġġa (Narrow leaved aster)	A very abundant plant, said to be introduced to the Maltese Islands sometime around the 1930s
IOA-AMP-068	*Calendula arvensis* L., Asteraceae	Suffejra Selvaġġa (Wild or woody marigold)	Herb in coughs and colds, chiblains, sudorific, warts and calluses, jaundice (Lanfranco, 1993; Penza, 1969)
IOA-AMP-071	*Calendula suffruticosa* L., Asteraceae	Suffejra Selvaġġa (Wild or woody marigold)	Herb in jaundice (Penza, 1969)
IOA-AMP-081	*Carlina gummifera* (L.) Les., Asteraceae	Xewk tal-miskta (Stemless atractylis)	Herb is poisonous (Lanfranco, 1993)
IOA-AMP-091	*Ceratonia siliqua* L., Mimosaceae	Ħarruba (Carob)	Decoction of unripe pods as astringent for the gums and in cough (Penza, 1969; Lanfranco, 1980)
IOA-AMP-145	*Cynoglossum creticum* Miller, Boraginaceae	Ilsien il-kelb (Southern hound's tongue)	Root decoction and leaf poultice for joint pain and burn relief (Penza, 1969)
IOA-AMP-153	*Diplotaxis erucoides* (L.) DC., Brassicaceae	Ġarġir (White rocket)	Herb as a stimulant (Penza, 1969)
IOA-AMP-463	*Diplotaxis tenuifolia*, Brassicaceae	Ġarġir (perennial wall rocket)	Herb as a stimulant (Penza, 1969)
IOA-AMP-223	*Dittrichia viscosa* (L.) Greut., Asteraceae	Tulliera Komuni (Sticky Fleabane)	Leaf decoction, liquid preparation and oil as haemeostatic, wound healing, itching, improve eye sight; pain, depurative and venereal diseases (Penza, 1969; Lanfranco, 1980; Gulia, 1855; Cassar Pullicino, 1947)
IOA-AMP-460	*Eucalyptus globulus*, Myrtaceae	Ewkaliptus (Tasmanian Blue	Oil as astringent and expectorant (Lanfranco, 1993)

Voucher specimen number	**Botanical Name, family**	**Maltese, (English) Names**	**Part/s used, preparation and Maltese Traditional uses**
		Gum)	
IOA-AMP-459	*Ferula communis*, Apiaceae	Ferla (Giant fennel)	Herb (Penza, 1969)
IOA-AMP-185	*Foeniculum vulgare* Miller, Apiaceae	Busbies (fennel)	Seeds and herb as flavouring agent in liquid preparations and treatment of colic pain (Penza, 1969)
IOA-AMP-191	*Fumaria capreolata*, Fumariaceae	Daħnet l-art (Fumitory)	Herb infusion as tonic, taenifuge, stomachic, kidney stones, in bath for sick children (Borg, 1927; Penza, 1969; Gulia, 1855)
IOA-AMP-190	*Fumaria officinalis* L., Fumariaceae	Daħnet l-art (Fumitory)	Herb infusion as tonic, taenifuge, stomachic, kidney stones, in bath for sick children (Borg, 1927; Penza, 1969; Gulia, 1855)
IOA-AMP-454	*Galactites tomentosa* Moench, Asteraceae	Xewka bajda (Boar thistle)	Herb consumed as a monofloral boar thistle honey
IOA-AMP-197	*Gladiolus italicus* Gaud., Iridaceae	Gladjoli salvaġġ (Common cornflag)	Leaves and bulb as galactogogue, aphrodisiac and emmenagogue (Penza, 1969; Borg, 1927)
IOA-AMP-101	*Glebionis coronaria* Tzvelev, Asteraceae	Lellux or Żigland (Crown daisy)	Herb (Lanfranco, 1993)
IOA-AMP-202	*Hedera helix* L., Araliaceae	Liedna (Ivy)	Gum and leaves in wound healing and as astringent (Penza, 1969)
IOA-AMP-461	*Holoschoenus vulgaris*, *Cyperaceae*	Simar tal-boċċi (*roundhead bulrush*)	A common plant in halophytic environments
IOA-AMP-213	*Hyoscyamus albus* L., Solanaceae	Mammażejża (White henbane)	Leaf poultice and ointment as sedative, in haemorrhoids and wound healing (Penza, 1969)
IOA-AMP-217	*Hypericum aegyptiacum* L., Guttiferae	Fexfiex il-baħar (Egyptian St. John's wort)	Herb Juice in wound healing, urinary tract infections and increases menstrual flow (Penza, 1969)
IOA-AMP-450	*Inula crithmoides* L., Asteraceae	Xorbett (Golden samphire)	Herb (Gulia, 1855)
IOA-AMP-462	*Lactuca sativa*, Asteraceae	Ħassa salvaġġa (Wild lettuce)	Leaf poultice as sedative (Penza, 1969)
IOA-AMP-236	*Lactuca virosa*, Asteraceae	Ħassa salvaġġa (Wild lettuce)	White latex as sedative (Penza, 1969)
IOA-AMP-234	*Laurus nobilis* L., Lauracea	Rand (Laurel)	Seed oil and leaf decoction in rheumatic pain and neuralgia; stomachic; diaphoretic, depurative (Penza, 1969; Cassar Pullicino, 1947; Lanfranco, 1980; Cremona, 1971)
IOA-AMP-238	*Leontodon tuberosus*, Asteraceae	Żigland (Tuberous hawkbit)	Herb as diuretic and tonic (Lanfranco, 1993)
IOA-AMP-254	*Malva sylvestris* L., Malvaceae	Ħubbejża (Common mallow)	Leaf/flower poultices and root decoction in vaginitis, intestinal problems, depurative, skin and throat inflammation (Penza, 1969; Lanfranco, 1980)
IOA-AMP-268	*Mercurialis annua* L., Euphorbiaceae	Burikba (Annual mercury)	Juice as tonic and galactofuge (Penza, 1969; Lanfranco, 1975)

Voucher specimen number	Botanical Name, family	Maltese, (English) Names	Part/s used, preparation and Maltese Traditional uses
IOA-AMP-285	*Nerium oleander* L., Apocynaceae	Oljandru (Oleander)	Herb for skin itching (Cassar Pullicino, 1947)
IOA-AMP-290	*Olea europaea* L., Oleaceae	Żebbuġa (Olive)	Olive oil and leaves as laxative, wound healing, sunburn, antihypertensive, aching muscles (Penza 1969; Lanfranco, 1980)
IOA-AMP-286	*Opuntia ficus-indica* (L.) Mill., Cactaceae	Bgħajtar tax-xewk (Prickly pear)	Cladode/flower poultice in stomach pain, burnt skin, joint pain/headaches; astringent and antidiarrhoeal (Cassar Pullicino, 1947; Lanfranco, 1980; Lanfranco, 1975)
IOA-AMP-291	*Oxalis pes-caprae* L., Oxaliaceae	Ħaxixa ingliża, Cape sorrel	Herb juice as emetic and for acne (Lanfranco, 1975)
IOA-AMP-090	*Palaeocyanus crassifolius* (Bert.) Dost., Asteraceae	Widnet il-baħar (Maltese rock centaury)	National Plant of Malta
IOA-AMP-294	*Papaver somniferum* L. Papaveraceae	Xaħxieħ (Opium poppy)	Poppy heads and latex as sedative (Penza, 1969)
IOA-AMP-296	*Parietaria judaica*, Urticaceae	Xeħt ir-riħ (Pellitory of the wall)	Herb, decoction; herb boiled with garlic and chamomile in bronchitis, pharyngitis, pulmonitis and cough; catarrh; kidney stones; haemorrhoids (Borg, 1927; Penza, 1969; Cassar Pullicino, 1947)
IOA-AMP-304	*Phlomis fruticosa* L., Lamiaceae	Salvja tal-Madonna (Jerusalem sage)	Boiled leaves as cough remedy (Penza, 1969)
IOA-AMP-317	*Pinus halepensis* Miller, Pinaceae	Żnuber (Aleppo pine)	Inhalation and ointment for catarrh and as diuretic (Lanfranco, 1975)
IOA-AMP-319	*Pistacia lentiscus* L., Anacardiaceae	Deru (Mastic tree)	Mastic resin for filling of teeth (Gulia, 1855)
IOA-AMP-318	*Plantago lagopus* L., Plantaginaceae	Beżbula komuni (Hare's foot plantain)	Boiled leaves for wound healing, eye diseases and increases urination (Penza, 1969; Cassar Pullicino, 1947)
IOA-AMP-331	*Prasium majus* L., Lamiaceae	Te Sqalli (Mediterranean Prasium)	Infused leaves as diuretic (Penza, 1969; Gulia, 1855; Cremona, 1971)
IOA-AMP-345	*Psoralea bituminosa* L., Mimosaceae	Silla tal-blat (Bitumen pea)	Herb in rheumatic pain (Penza, 1969)
IOA-AMP-308	*Reicardia picroides*, Asteraceae	Kanċlita (Common reichardia)	Herb as diuretic and tonic (Lanfranco, 1993)
IOA-AMP-348	*Reseda alba* L., Resedaceae	Denb il-ħaruf (White mignonette)	Roots for painful gums (Borg, 1927; Penza, 1969)
IOA-AMP-360	*Ricinus communis* L., Euphorbiaceae	Riġnu (Castor oil tree)	Decoction of seeds, roots or leaves as laxative, rheumatism, neuralgic affections, ophthalmia; galactorrhoea (Penza, 1969)
IOA-AMP-374	*Schinus terebinthifolius*, Anacardiaceae	Bżar Falz (Drooping false pepper)	Ground fruit (Borg, 1927)
IOA-AMP-392	*Silybum marianum* (L.) Gaertn., Asteraceae	Xewk Bagħli (Milk thistle)	Herb as tonic, urinary tract, fever (Penza, 1969)

Voucher specimen number	Botanical Name, family	Maltese, (English) Names	Part/s used, preparation and Maltese Traditional uses
IOA-AMP-388	*Smyrnium olusatrum* L., Apiaceae	Karfus il-hmir (Alexanders)	Herb as stimulant (Penza, 1969)
IOA-AMP-393	*Sonchus oleraceus* L., Asteraceae	Tfief (Sow thistle)	Herb as diuretic and purgative (Penza, 1969)
IOA-AMP-443	*Verbena officinalis* L., Verbenaceae	Buqexrem (Vervain)	Poultice/decoction for wound healing, astringent, diarrhoea, dysentery, diabetes (Penza, 1969)

Table 1. Botanical, ethnobotanical and voucher specimen code numbers for the fifty-five plants studied.

2.4 Brine Shrimp Test (BST)

In a set of 12-well plates, each well contained 10 nauplii, 1 ml sea water and 1 ml of extract diluted to final concentrations of 1%, 0.1%, 0.01%, 0.001% and 0.0001% respectively. The tests were set out in triplicate so that a total of fifteen wells per extract were used. numbers of living nauplii were counted after 24 hours. The LC_{50} values and 95 % confidence intervals were determined in µg/ml, using the Finney probit analysis computer program. A median lethal concentration (LC_{50}) smaller than 1000 µg/ml (Alkofahi et al., 1997) indicates pharmacological activity.

2.5 DNA-methyl green (intercalation) tests

DNA intercalation assay for DNA activity. Samples were incubated with 200 µl of DNA-methyl green in the dark at 25 °C for 24 h. The decrease in absorbance at 650 nm was calculated as a percentage of the untreated DNA-methyl green absorbance value. The median inhibitory concentration (IC_{50}) was calculated (Desmarchelier et al., 1996) through regression analysis. Cucurbitacin E and Dexamethasone were used as potent and moderate positive controls, respectively. Data was analyzed using Student's t-test.

3. Results and discussion

In this study, 55 plant species from 31 plant families were studied. The plant families ranked in the following order: Asteraceae (15 species), Apiaceae (3 species), Liliaceae, Scrophulariaceae, Mimosaceae, Brassicaceae, Fumariaceae, Euphorbiaceae, Lamiaceae and Anacardiaceae (2 species), Acanthaceae, Primulaceae, Boraginaceae, Iridaceae, Araliaceae, Solanaceae, Guttiferae, Lauracea, Malvaceae, Apocynaceae, Cactaceae, Oleaceae, Oxaliaceae, Papaveraceae, Urticaceae, Pinaceae, Plantaginaceae, Resedaceae, Verbenaceae, Myrtaceae and Cyperaceae (1 species). The distribution of plants within families was as broad as possible. However, the most abundant plant family of the Maltese flora (Attard, 2004) was given more importance than the other families.

3.1 Phytochemical analysis

The results for the four phytochemical classes are illustrated in table 2 and a generalised picture of the number of extracts, containing phytochemicals for each solvent system used, is illustrated in figure 1.

PLANT NAME	P. N°	Aqueous	Aqueous-ethanol	Ethanol	Chloroform	Petroleum ether
Acanthus mollis	002	TP	TP	TFP	TP	T
Aloe vera	015	-	-	TF	AF	-
Anagallis arvensis	028	P	P	P	FP	P
Antirrhinum siculum	037	P	TP	TP	TP	T
Antirrhinum tortuosum	036	TFP	TFP	TFP	T	TP
Arum italicum	046	TFP	TFP	TFP	T	T
Asparagus aphyllus	049	TFP	TFP	TFP	TFP	TAFP
Aster squamatus	453	FP	AFP	P	-	F
Calendula arvensis	068	TAP	TFP	AFP	-	F
Calendula suffruticosa	073	F	TFP	TFP	-	P
Carlina gummifera	081	TAFP	FP	A	-	TF
Ceratonia siliqua	091	AF	F	FP	TAF	AF
Cynoglossum creticum	145	FP	TFP	TFP	-	TP
Diplotaxis erucoides	153	P	P	T	-	-
Diplotaxis tenuifolia	463	TF	FP	FP	TF	T
Dittrichia viscosa	223	TFP	FP	TF	FP	AFP
Eucalyptus globulus	460	TFP	TFP	TFP	TFP	-
Ferula communis	459	TP	TP	TP	-	FP
Foeniculum vulgare	185	T	TP	TP	-	F
Fumaria capreolata	191	TP	TFP	TFP	TF	TP
Fumaria officinalis	190	TP	TP	TP	F	P
Galactites tomentosa	454	TFP	TFP	AP	-	TFP
Gladiolus italicus	197	-	-	TAF	-	TF
Glebionis coronaria	101	TFP	T	TAFP	-	TF
Hedera helix	202	-	TP	T	-	TF
Holoschoenus vulgaris	461	TFP	FP	F	F	T
Hyoscyamus albus	213	P	A	TP	-	TP
Hypericum aegyptiacum	217	FP	F	-	F	TP
Inula crithmoides	450	TFP	TFP	TFP	F	TF
Lactuca sativa	462	T	T	P	P	F
Lactuca virosa	236	-	P	-	TP	F
Laurus nobilis	234	F	T	T	-	TAF
Leontodon tuberosus	238	TFP	TFP	TF	F	F
Malva sylvestris	254	T	TP	T	-	-
Mercurialis annua	268	TF	TF	F	TFP	TF
Nerium oleander	285	TF	TFP	TFP	P	TF
Olea europaea	290	TFP	TFP	TF	TF	TFP
Opuntia ficus- indica	286	T	TP	TFP	P	TP
Oxalis pes-caprae	291	TF	TFP	TF	TF	A
Palaeocyanus crassifolius	90	TFP	TP	TP	TP	TFP
Papaver somniferum	294	FP	TP	TP	FP	TF

PLANT NAME	P. N°	Aqueous	Aqueous-ethanol	Ethanol	Chloroform	Petroleum ether
Parietaria judaica	296	F	FP	TFP	FP	AP
Phlomis fruticosa	304	TP	TFP	TFP	T	TFP
Pinus halepensis	317	P	-	TF	F	AF
Pistacia lentiscus	319	TFP	TF	P	-	-
Plantago lagopus	318	-	FP	AFP	TP	AP
Prasium majus	331	TFP	TFP	TFP	TP	P
Psoralea bituminosa	345	TP	TFP	T	T	P
Reichardia picroides	308	TFP	TFP	TP	F	TFP
Ricinus communis	360	TFP	TP	TFP	TF	-
Schinus terebinthifolius	374	TFP	TP	TP	P	TF
Smyrnium olusatrum	388	TFP	P	TFP	F	P
Sonchus oleraceus	393	TF	TF	TF	-	TF
Verbena officinalis	443	P	TP	TP	-	P

Table 2. The phytochemical analysis of the extracts under investigation for the main phytochemical classes: Flavonoids (F), Terpenoids (T), Alkaloids (A) and Proteins (P)

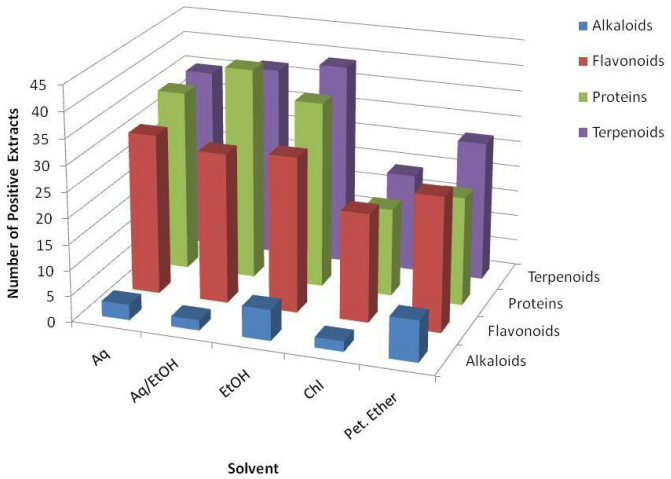

Fig. 1. A generalised profile of the number of extracts containing terpenoids, alkaloids, flavonoids and proteins for each solvent system used (n=280).

The predominating compound classes were terpenoids (56.07 %), followed by proteins (53.57 %) and flavonoids (48.93 %). Alkaloids were limited to a smaller number of extracts (7.50 %). The majority of the polar solvents, aqueous, aqueous-ethanol and ethanol contained terpenoids and proteins ($p<0.05$, $n=4$). The chloroform extract contained mainly flavonoids ($p<0.05$, $n=4$), while the petroleum ether extracts contained predominantly flavonoids and terpenoids.

The highest terpenoid contents were found in the ethanol and aqueous-ethanol extracts. In fact, it was observed that 70.70 % of the positive extracts were polar extracts, i.e. using

water, water-ethanol and ethanol as extracting solvents. This is due to the fact that most terpenoids are present in the glycosidic form rather than the non-polar or low polarity terpene aglycone form. Some plants exhibited the presence of terpenes and related compounds in all solvent systems. Typical examples included *Acanthus mollis*, which mainly contains β-sitosterol as the triterpene-like compound (Loukis & Philianos, 1980), *Antirrhinum tortuosum*, with mono and sesquiterpene volatile derivatives (Nagegowda et al., 2008), *Arum italicum*, with the tetraterpene carotenoids (Bonora et al., 2000), *Asparagus aphyllus* with saponins and sapogenins (Shao et al., 1996), *Olea europaea* containing mainly triterpenoids (Caputo et al., 1974; Elamrani, 2011), *Palaeocyanus crassifolius* containing sesquiterpene lactones (Koukoulitsa et al., 2002) and *Phlomis fruticosa*, mainly containing mono- and sesquiterpenes (Amor et al., 2009). In the case of *Fumaria capreolata* the main constituents mentioned in previous studies were the alkaloids (Soušek et al., 1999; Maiza-Benabdesselam et al., 2007). In this present study, there was the strong presence of terpenoids.

The distribution of alkaloids in polar and non-polar solvents was almost equal (52.38 % and 47.62 %, respectively). Alkaloids may be present either as the non-polar organic form or as the polar ionised alkaloid salt. The highest content was recorded in the ethanol extract of *Gladiolus italicus* and in the chloroform extract of Aloe vera. For *Gladiolus*, this result goes in accordance with that obtained by Ameh and coworkers (2011) and for *Aloe*, a similar result was obtained by Waller and coworkers (1978). Other plants with an alkaloidal content include *Asparagus aphyllus* (Negi et al., 2010), *Calendula arvensis* (Shamsa et al., 2008), *Glactites tomentosa*, *Glebionis coronaria*, *Oxalis pes-caprae*, *Parietaria judaica*, *Carlina gummifera*, *Hyoscyamus albus* (Doerk-Schmitz et al., 1993), *Laurus nobilis* (Nayak et al., 2006), *Pinus halepensis* (Tawara et al., 1993) and *Plantago lagopus* (Hultin & Torssell, 1965). *Fumaria* species are known to contain alkaloids (Soušek et al., 1999; Maiza-Benabdesselam et al., 2007). However, no alkaloids were detected for *Fumaria officinalis* and *Fumaria capreolata* in this present study. Although *Ceratonia siliqua* is claimed to contain no alkaloids (El Hajaji et al., 2011), in this present study, alkaloids were detected in the aqueous, chloroform and petroleum ether extracts. It was also observed that for *Papaver somniferum* no alkaloids were detected in the leaves. This depends on several factors. Primarily, the wild variety might have a low potential for the production of morphinan alkaloids, and other plant parts such as the stem, roots and capsules, tend to accumulate more alkaloids than the leaves (Williams & Ellis, 1989).

For the flavonoid group, out of the positive responses, 65.69 % were polar extracts while the rest (34.41 %) were extracts derived from non-polar solvents. Typically, flavonoids are polyphenolic compounds that are highly soluble in aqueous and aqueous-alcohol solvents. However, flavonoids have been reported to be also extracted by chloroform and petroleum ether (Gudej & Czapski, 2009; Rajendran & Krishnakumar, 2010). Plants containing flavonoids in all extracts, consistent with other studies, include *Asparagus aphyllus* (Sun et al., 2007), *Ceratonia siliqua* (Papagiannopoulos et al., 2004; Vaya & Mahmood, 2006), *Dittrichia viscosa* (M.J. Martin et al., 1988), *Leontodon tuberosus* (Zidorn & Stuppner, 2001), *Mercurialis annua* (Aquino et al., 1987) and *Olea europaea* (Benavente-García et al., 2000). Almost all plant species exhibited the presence of flavonoids in one or more extracts, except for four plants, namely, *Antirrhinum siculum*, *Diplotaxis erucoides*, *Malva sylvestris* and *Verbena officinalis*. Other studies report the presence of flavonoids in *Diplotaxis erucoides* (Bennett et al., 2006), *Malva sylvestris* (Billeter et al., 1991) and *Verbena officinalis* (Rehecho et al., 2011).

The absence of flavonoids in these species for the current study may be due to several factors that include a different chemotype, different environmental conditions and the presence of these compounds below the detection limit, amongst others. *Antirrhinum siculum* is palely pigmented and this may contribute to the insignificant content of flavonoids (C. Martin et al., 2010).

Proteins prevail in many plants. Within the positive response group, 74.67 % were polar extracts while 25.33 % were non-polar extracts. This indicated that three-forths of the detected proteins were functional proteins including enzymes. *Anagallis arvensis, Asparagus aphyllus, Palaeocyanus crassifolius* and *Prasium majus* exhibited the presence of proteins in all their extracts. This goes in accordance with previous studies carried out on these plants (Alignier et al., 2008; King et al., 1990). Plants that were devoid of proteins in all their extracts include *Aloe vera, Gladiolus italicus, Laurus nobilis* and *Sonchus oleraceus*. In previous studies, *Aloe vera* revealed the presence of glutathione peroxidase (Sabeh et al., 1993), *Gladiolus italicus* contained arabinogalactan-protein (Gleeson & Clarke, 1979) and *Laurus nobilis* contained lipase (Isbilir et al., 2008). Although most plant material was collected at flowering time, the inclusion of seed protein in the extract would have been possible in cases where fruit were harvested alongside the flowers.

3.2 The Brine Shrimp Test

The results for the tested extracts are given in Table 3. Primary screening involves the use of bench-top bioassays. Extracts exhibiting LC_{50} values above 1000 µg/ml are generally regarded as ineffective extracts. In this study, 42.26 % of the extracts were therefore inactive (Table 4). The most inactive were the petroleum ether extracts, while the most active were the ethanolic extracts. Correlating the BST lethal concentrations to phytochemical classes, it was observed that inactive extracts contained several phytochemicals. The reason may be due to the low concentration or possible antagonistic activity between the phytochemicals from the different classes. 55.68 % of the extracts exhibited LC_{50} values below 1000 µg/ml. The most active were the ethanolic extracts (72.97 %), while the least active were the petroleum ether extracts (35.14 %). Four plants exhibited activity for all their five extracts. These were *Nerium oleander*, *Olea europaea*, *Opuntia ficus-indica* and *Pinus halepensis*, all exhibiting LC_{50} values below 0.01 µg/ml. These four plant species are amongst the most popular Maltese traditional medicinal plants. It was also observed that some extracts with non-detectable phytochemicals exhibited significant LC_{50} values. Typical examples include the aqueous extract of *Lactuca virosa*, the aqueous-ethanol extract of *Pinus halepensis,* the ethanolic extracts of *Hypericum aegypticum* and *Lactuca virosa*, and the chloroform extracts of *Ferula communis, Foeniculum vulgare* and *Pistacia lentiscus.* On the other hand, there were extracts that exhibited significant LC_{50} values as opposed to other studies. For example, for *Fumaria officinalis* aqueous-ethanol and ethanol extracts, in the present study, exhibited significant effects on brine shrimps as opposed to the ethanol extract reported in the study by Erdoğan (2009).

3.3 The DNA-methyl green assay

Table 5 shows the IC_{50} values obtained for the DNA-methyl green assay. Although low IC_{50} values have been reported for pure compounds (Burres et al., 1992) , such as rubiflavin and

distamycin A (17 and 18 µg/ml, respectively), it is reasonable that in the case of extracts higher IC_{50} values are acceptable as for pyrido[2,3-d]pyrimidin-4(1H)-one and pyrido[2,3-d]triazolo[3,4-b]pyrimidine analogs (40 – 53 µg/ml) (Goda & Badria, 2005). Since plant extracts are complex matrices with several phytochemicals, IC_{50} values are expected to be higher than for pure compounds. Therefore, extracts with IC_{50} values below 70 µg/ml were considered as active (Figure 2). Only 15 % of the extracts displaced methyl green from the methyl green DNA complex. It is likely that these compounds act as intercalating agents at the DNA level. 86.67 % were active polar extracts with proteins predominating in these extracts. The other extracts either exhibited an IC_{50} value higher than 70 µg/ml or else a 50 % activity was never achieved. From the remaining 85 %, only one-third of the extracts exhibited values above 70 µg/ml. Alkaloids only featured in one active aqueous extract of *Ceratonia siliqua*. Terpenoids, flavonoids and proteins predominated mainly in aqueous and aqueous-ethanol extracts. For a few extracts, there was no correlation between the phytochemical class and DNA-methyl green activity. These include the aqueous-ethanol extract of *Gladiolus italicus,* the aqueous extract of *Hedera helix* and the ethanolic extract of *Hypericum aegyptiacum*. For example *H. aegyptiacum* contains hypericin that can inhibit DNA topoisomerase II (Peebles et al., 2001), but the naphthodianthrone was not detected by the phytochemical tests.

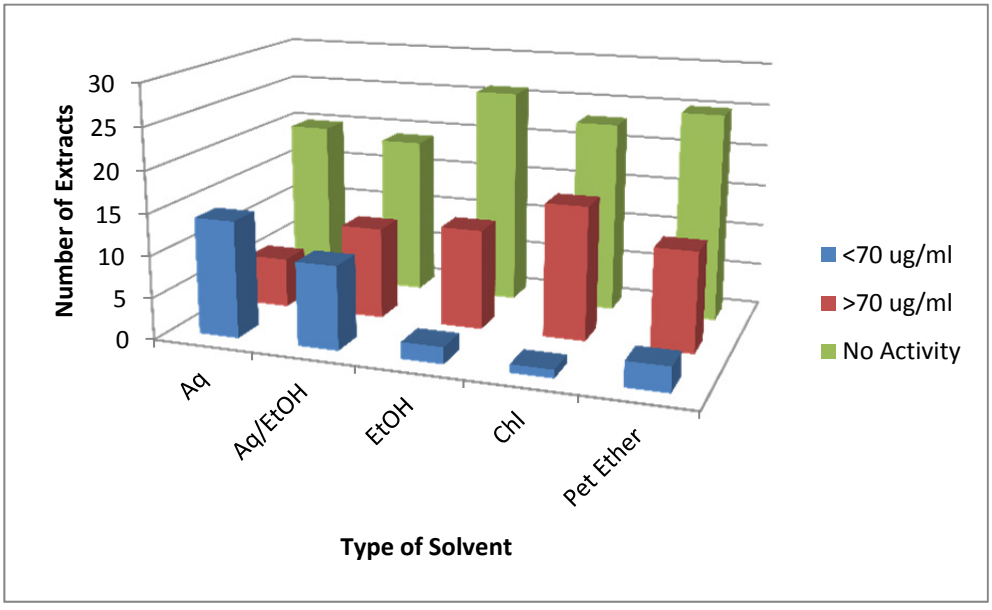

Fig. 2. The number of extracts classified as (a) below 70 µg/ml, (b) above 70 µg/ml range and (c) non-active extracts with the different solvent types for the DNA methyl green assay.

PLANT NAME	P. N°	Aqueous	Aqueous-ethanol	Ethanol	Chloroform	Petroleum ether
Acanthus mollis	002	<0.01	<0.01	<0.01	<0.01	>1000
Anagallis arvensis	028	460	<0.01	<0.01	>1000	>1000
Antirrhinum tortuosum	036	<0.01	>1000	<0.01	>1000	<0.01
Antirrhinum siculum	037	10	<0.01	<0.01	>1000	<0.01
Asparagus aphyllus	049	>1000	<0.01	<0.01	<0.01	>1000
Aster squamatus	453	>1000	>1000	>1000	>1000	>1000
Calendula arvensis	068	>1000	63	>1000	>1000	796
Carlina gummifera	081	>1000	>1000	>1000	>1000	>1000
Dittrichia viscosa	223	>1000	>1000	>1000	<0.01	>1000
Ferula communis	459	<0.01	<0.01	<0.01	<0.01	>1000
Foeniculum vulgare	185	>1000	<0.01	<0.01	<0.01	>1000
Fumaria officinalis	190	>1000	<0.01	<0.01	>1000	<0.01
Fumaria capreolata	191	>1000	>1000	<0.01	<0.01	>1000
Galactites tomentosa	454	>1000	>1000	>1000	>1000	>1000
Glebionis coronaria	101	>1000	>1000	93	131	>1000
Hyoscyamus albus	213	>1000	ND	<0.01	>1000	>1000
Hypericum aegyptiacum spreng	217	<0.01	<0.01	<0.01	>1000	>1000
Inula crithmoides	450	>1000	344	>1000	>1000	562
Lactuca sativa	462	<0.01	<0.01	<0.01	>1000	<0.01
Lactuca virosa	236	<0.01	<0.01	<0.01	>1000	>1000
Leontodon tuberosus	238	>1000	>1000	>1000	>1000	>1000
Nerium oleander	285	<0.01	<0.01	<0.01	<0.01	<0.01
Opuntia ficus- indica	286	<0.01	<0.01	<0.01	<0.01	<0.01
Olea europaea	290	<0.01	<0.01	<0.01	<0.01	<0.01
Oxalis pes-caprae	291	<0.01	<0.01	<0.01	<0.01	>1000
Palaeocyanus crassifolius	090	>1000	<0.01	<0.01	>1000	10
Papaver somniferum	294	<0.01	<0.01	<0.01	<0.01	>1000
Pinus halepensis	317	<0.01	<0.01	<0.01	<0.01	<0.01
Plantago lagopus	318	>1000	10	<0.01	>1000	0.07
Pistacia lentiscus	319	<0.01	<0.01	<0.01	<0.01	ND
Prasium majus	331	<0.01	<0.01	<0.01	<0.01	>1000
Psoralea bituminosa	345	<0.01	<0.01	<0.01	10	<0.01
Reichardia picroides	308	>1000	>1000	>1000	>1000	>1000
Reseda alba	348	>1000	<0.01	ND	10	>1000
Ricinus communis	360	>1000	<0.01	<0.01	<0.01	>1000
Schinus terebinthifolius	374	<0.01	<0.01	<0.01	<0.01	>1000
Sonchus oleraceus	393	>1000	>1000	>1000	>1000	>1000

Table 3. The result for the effect of extracts on the Brine Shrimp Test

	Percentage per extract type					Percentage of Total Extracts
BST result	Aqueous	Aqueous-ethanol	Ethanol	Chloroform	Petroleum ether	
>1000	51.35	27.03	24.32	48.65	59.46	42.16 %
0.01-1000	5.41	8.11	2.70	8.11	10.81	7.03 %
0.01	43.24	62.16	70.27	43.24	24.32	48.65 %
ND	0.00	2.70	2.70	0.00	5.41	2.16 %

Table 4. The percentage of results classified as (a) above 1000 µg/ml, (b) 0.01 – 1000 µg/ml range, (c) less than 0.01 µg/ml and (d) not determined (ND) with the different solvent types for the brine shrimp test.

PLANT NAME	P. N°	Aqueous	Aqueous-ethanol	Ethanol	Chloroform	Petroleum ether
Acanthus mollis	2	30.399	34.102	NA	131.005	34.354
Aloe vera	15	NA	278.589	270.983	NA	NA
Anagallis arvensis	28	28.658	43.534	NA	NA	NA
Antirrhinum tortuosum	36	63.354	156.171	314.838	38.065	354.278
Antirrhinum siculum	37	NA	NA	NA	NA	NA
Asparagus aphyllus	49	NA	52.985	NA	305.865	NA
Calendula suffruticosa	73	70.296	144.921	350.003	261.826	364.324
Ceratonia siliqua	91	23.230	NA	NA	134.563	NA
Cynoglossum creticum	145	45.941	50.063	NA	NA	NA
Eucalyptus globulus	460	NA	NA	NA	NA	NA
Ferula communis	459	NA	NA	NA	71.158	92.264
Foeniculum vulgare	185	NA	NA	NA	NA	NA
Fumaria capreolata	191	133.655	77.458	NA	596.272	NA
Fumaria officinalis	190	NA	346.108	NA	NA	79.496
Gladiolus italicus	197	NA	54.931	NA	143.109	251.228
Hedera helix	202	60.086	231.921	NA	NA	NA
Holoschoenus vulgaris	461	NA	91.229	NA	NA	NA
Hyoscyamus albus	213	NA	NA	195.094	NA	NA
Hypericum aegyptiacum	217	NA	NA	66.803	NA	443.799
Diplotaxis tenuifolia	463	NA	30.688	NA	NA	NA
Dittrichia viscosa	223	26.945	NA	108.418	171.989	NA
Laurus nobilis	234	50.835	NA	324.334	189.539	NA
Lactuca sativa	462	NA	166.722	326.359	NA	NA
Lactuca virosa	236	NA	NA	NA	NA	57.791
Malva sylvestris	254	NA	31.530	NA	NA	54.922
Mercurialis annua	268	NA	165.974	NA	NA	NA
Nerium oleander	285	NA	62.957	NA	105.401	163.761
Opuntia ficus- indica	286	41.899	85.339	NA	115.870	NA
Olea europaea	290	53.908	95.687	80.988	227.594	NA
Oxalis pes-caprae	291	NA	NA	64.089	223.200	171.288
Palaeocyanus crassifolius	90	122.931	NA	NA	NA	NA
Papaver somniferum	294	147.538	NA	NA	NA	131.727

Parietaria judaica	296	NA	NA	89.115	NA	NA
Phlomis fruticosa	304	137.404	NA	159.786	130.591	171.195
Pinus halepensis	317	NA	NA	NA	NA	NA
Plantago lagopus	318	NA	49.252	338.260	NA	NA
Pistacia lentiscus	319	NA	NA	NA	NA	NA
Prasium majus	331	48.142	103.372	273.826	92.489	279.194
Psoralea bituminosa	345	NA	59.332	NA	NA	177.895
Reseda alba	348	193.473	NA	NA	NA	NA
Ricinus communis	360	48.912	NA	NA	103.224	NA
Schinus terebinthifolius	374	NA	NA	NA	NA	181.849
Smyrnium olusatrum	388	104.605	NA	NA	NA	NA
Silybum marianum	392	NA	46.528	NA	NA	NA
Verbena officinalis	443	67.656	104.453	145.529	158.008	NA
Cucurbitacin E	-	19.12				
Dexamethasone	-	32.74				

Table 5. The median inhibitory concentration (IC_{50} in µg/ml) values obtained for the DNA-methyl green assay (NA no activity – 50% effect was never reached).

4. Conclusions

This study has confirmed the presence of useful phytochemicals and biological activities of several extracts from selected Mediterranean plants. It is expected that these results will serve as a stimulus for further investigations into the active phytochemicals.

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