

UNIVERSITY OF BELGRADE
FACULTY OF BIOLOGY

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**Micromorphological and cytological
analysis of trichomes and biological
effects of extracts of *Salvia aegyptiaca* L.,
S. fruticosa Mill. and *S. lanigera* Poir.
(Lamiaceae) from Libya**

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**Mikromorfološka i citološka analiza
trihoma i biološki efekti ekstrakata
Salvia aegyptiaca L., *S. fruticosa* Mill. i
S. lanigera Poir. (Lamiaceae) iz Libije**

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Micromorphological and cytological analysis of trichomes and biological effects of extracts of *Salvia aegyptiaca* L, *S. fruticosa* Mill. and *S. lanigera* Poir. (Lamiaceae) from Libya

ABSTRACT

Three Libyan *Salvia* species were subjected to comprehensive study of microscopic characteristics of trichomes and biological activities of their extracts, while the selected species have not been studied previously.

The micromorphology, ultrastructure and histochemistry of *S. aegyptiaca*, *S. fruticosa* and *S. lanigera* leaf trichomes were investigated using light, scanning and transmission electron microscopy. The leaves bear abundant unbranched non-glandular and two types of glandular trichomes - peltate and capitate, on both leaf surfaces. Since *Salvia* species are used for medicinal and cosmetic purposes, due to essential oils secreted in the glandular trichomes, the focus was on the analyses of the glandular trichomes.

In *S. aegyptiaca* leaves the peltate trichomes were composed of basal cell, short cylindrical stalk cell and broad head of eight to twelve secretory cells arranged in a single circle, or more in two circles. Capitate trichomes (hairs) consisted of one-celled glandular head, subtended by a stalk of variable length, and classified into two types: capitate type I or short-stalked capitate and capitate type II or long-stalked capitate trichomes. Histochemical tests showed that the secreted material in all types of *S. aegyptiaca* glandular trichomes was of a complex nature. Positive reactions to lipids for all types of glandular trichomes were obtained, with especially abundant secretion observed in peltate and capitate trichomes type II.

In *S. fruticosa* peltate hairs consisted of basal epidermal cell, very short stalk cell and large round head of eight secretory cells arranged in a circle. Capitate trichomes can be divided into two main types, short stalked and long stalked, and further into five subtypes according to the number of stalk cells, the morphology and the number of the glandular head cells. Digitiform trichomes consisted of one basal cell, one or two stalk cells and one apical secretory cell, which are of similar diameter and approximately

equal length. The abundant and diverse glandular trichomes and long stalks of the capitate trichomes of *S. fruticosa* show the xeromorphic character of the investigated plant species which is very drought resistant.

S. lanigera possessed peltate trichomes consisted of one basal epidermal cell, short cylindrical stalk cell with cutinized lateral walls and large round or slightly flattened secretory head of eight secretory cells arranged in a single circle. Capitate trichomes consisted of one-celled glandular head, subtended by a stalk of one cell, determined as short-stalked capitate trichomes.

It can be concluded that the investigated Libyan *Salvia* species showed differences in types, density and distribution of trichomes, with the most abundant and diverse glandular trichomes in *S. fruticosa*.

Considering the growing interest in substances of natural origin possessing various bioactivities, as well as the increasing concern about potentially harmful effects of synthetic additives, the potential pharmacological value of the investigated species was studied.

In the present study dichloromethane, ethyl acetate, methanol, acetone, chloroform, ethanol and water extracts prepared from three Libyan *Salvia* species were analyzed for their composition and the antioxidant activity measured by four parallel assays. For the antimicrobial, cytotoxic and enzyme inhibitory activities, water and ethanol extracts were chosen, because of their wide usage in phytotherapy.

Obtained results confirmed the high antioxidant capacity of *S. aegyptiaca*. Ethanol extract showed the strongest activity compared to other extracts in DPPH, ABTS and FRAP assays (IC_{50} 20.74 μ g/ml, 1.98 mg AEE/g, 1500.38 μ mol Fe(II)/g, respectively) while in the β -CB assay, ethanol extract showed low activity (9.04% of inhibition). The highest content of total phenolics was measured for ethanol extract (150.71 mg GAE/g), while flavonoids were the most abundant in the ethyl acetate extract (14.64 mg QE/g). All of assays were strongly correlated to total phenolic content (r value from ± 0.806 to ± 0.993 , respectively).

The ethanol extract of *S. fruticosa* showed the strongest antioxidant activity in all applied assays (DPPH: 29.55 IC_{50} , μ g/ml, ABTS: 2.31 mg AEE/g, FRAP: 834.10 μ mol Fe(II)/g, β -CB: 85.11%). Our study revealed that Libyan *S. fruticosa* have significant amount of phenolic compounds which are considered as powerful antioxidants (154.18 mg GAE/g dry ethanolic extract). The highest content of phenolic

compounds, mainly kaempferol glycosides, was present in the water, methanol and ethanol extracts. DPPH activity was strongly correlated with total phenolic content. In our study *S. fruticosa* ethanolic extract showed cytotoxic activity against HCT-116 cell line (375.96 $\mu\text{g/ml}$), while water extract performed stronger activity on K562 cells (386.00 $\mu\text{g/ml}$), which is considered as low cytotoxic activity. Ethanol extract showed stronger antibacterial activity than water extract, particularly against Gram-positive bacteria (MIC values 10-15 mg/ml). The antifungal activity of *S. fruticosa* extracts was estimated as poor comparing to values obtained for ketoconazole. In AChE inhibition assay, extracts exhibited weaker activity (16.28-26.82%) than standard galanthamine, while extracts performed stronger activity (57.89-74.66%) in tyrosinase inhibition assay in comparison with standard kojic acid at tested concentrations.

Methanol and water extracts showed the highest content of phenolic components, particularly kaempferol glycosides. Among *S. lanigera* extracts water extract exhibited better antioxidant activity in the applied assays, except in FRAP assay where methanol was better than water extract (DPPH: 205.45 IC_{50} , $\mu\text{g/ml}$, ABTS: 1.77 mg AEE/g, FRAP: 200.11 $\mu\text{mol Fe(II)/g}$). In β -CB assay, water and ethanol extracts exhibited activity (77.66% and 76.06%, respectively) higher than standards BHA and BHT. Antioxidant assays were more strongly correlated to total phenolic than to flavonoid content. *S. lanigera* extracts possessed very poor cytotoxic activity against HCT-116 cell line (IC_{50} values higher than the highest investigated concentration of 500 $\mu\text{g/ml}$), while water extract was stronger against K562 cells (383.29 $\mu\text{g/ml}$) Ethanol extract showed stronger inhibitory activity on bacterial growth (MICs 15-35 mg/ml) comparing to water extract (MICs from 30 to over 50 mg/ml). Ethanol extract showed better activity to *Aspergillus glaucus*, while the water extract showed the strongest effect on *Trichophyton mentagrophytes*. At tested concentrations, ethanol and water extracts exhibited inhibition of AChE about 30%. Both extracts performed stronger inhibition (55.26-70.20%) than standard kojic acid (33.73-51.81%) at tested concentrations in tyrosinase inhibition assay. The inhibitory effects of water extract was stronger compared to ethanol extract.

The investigated Libyan *Salvia* species showed differences in tested biological activities. In the cases of *S. aegyptiaca* and *S. frutocosa* antioxidant activity was high, but antimicrobial and cytotoxic activities were generally low. The water and ethanol

extracts of *S. fruticosa* and *S. lanigera* at tested concentrations performed stronger activity in tyrosinase inhibition assay compared to standard kojic acid.

Keywords:

Salvia aegyptiaca; *Salvia fruticosa*; *Salvia lanigera*; micromorphology; antioxidant activity; antimicrobial activity; cytotoxic activity; neurodegenerative enzymes.

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Mikromorfološka i citološka analiza trihoma i biološki efekti ekstrakata *Salvia aegyptiaca* L., *S. fruticosa* Mill. i *S. lanigera* Poir. (Lamiaceae) iz Libije

SAŽETAK

Tri vrste žalfije iz Libije su podvrgnute sveobuhvatnoj analizi mikroskopskih karakteristika lisnih trihoma (dlačica) i bioloških aktivnosti njihovih ekstrakata, s obzirom da nisu ranije proučavane.

Mikromorfologija, ultrastruktura i histohemija lisnih trihoma *S. aegyptiaca*, *S. fruticosa* i *S. lanigera* je proučavana svetlosnom, skenirajućom i transmisijom elektronskom mikroskopijom. Listovi na licu i naličju nose brojne negranate neglandularne trihome i dva tipa glandularnih trihoma – peltatne i kapitatne. S obzirom na korišćenje vrsta žalfije u lekovite i kozmetičke svrhe, zahvaljujući i etarskim uljima koja se sintetišu u glandularnim trihomama, u fokusu istraživanja je bila analiza glandularnih trihoma.

Na listovima *S. aegyptiaca* peltatne trihome su sastavljene od bazalne ćelije, kratke cilindrične ćelije vrata i široke glave od 8 do 12 ćelija u jednom krugu, ili više njih u dva kruga. Kapitatne trihome se sastoje od jedno ćelijske žlezdane glavice na dršci sastavljenoj od varijabilnog broja ćelija, klasifikovane u dva tipa: kapitatne trihome tipa I ili kratke kapitatne trihome i kapitatne trihome tipa II ili duge kapitatne trihome. Histohemijski testovi su pokazali da je sekretovani materijal u svim tipovima trihoma kompleksne prirode. Pozitivne reakcije na lipide su zapažene u svim tipovima trihoma, sa obilnom sekrecijom u peltatnim i kapitatnim trihomama tipa II.

Kod *S. fruticosa* peltatne trihome se sastoje od bazalne ćelije, vrlo kratke drške i krupne okrugle glave od osam ćelija kružno postavljenih. Kapitatne trihome se mogu podeliti u dve grupe, sa kratkim i sa dugim vratom, a zatim u pet podtipova, prema broju ćelija u vratu, morfologiji i broju ćelija u glavici. Digitiformne trihome se sastoje od jedne bazalne ćelije, jedne do dve ćelije drške i jedne apikalne sekretorne ćelije sličnog dijametra i dužine. Brojne i raznovrsne glandularne trihome i duge drške kapitatnih trihoma ukazuju na kseromorfnu prirodu ove vrste koja je otporna na sušu.

S. lanigera ima peltatne trihome sastavljene od jedne bazalne elije, kratke cilindrične elije vrata sa kutiniziranim zidovima i krupne okrugle ili malo ravnije sekretorne glave od osam elija u krugu. Kapitatne trihome se sastoje od jedno elijske žlezdane glavice i jedno elijske drške, a određene su kao kratke kapitatne trihome.

Može se zaključiti da su ispitivane libijske vrste *Salvia* pokazale razlike u tipovima, gustini i rasporedu trihoma, sa najvećom brojnošću i razlikama žlezdanih trihoma kod *S. fruticosa*.

S obzirom na rastuće interesovanje za supstance prirodnog porekla koje pokazuju različite biološke aktivnosti, kao i porast zabrinutosti zbog mogućih štetnih efekata sintetičkih aditiva, proučavana je potencijalna farmakološka vrednost ovih vrsta.

Analiziran je sastav dihlometanskih, etil acetatnih, metanolnih, acetonskih, hloroformskih, etanolnih i vodenih ekstrakata ovih vrsta, kao i njihova antioksidativna aktivnost pomoću četiri testa. Za antimikrobno, citotoksično dejstvo i antineurodegenerativnu aktivnost odabrani su vodeni i etanolni ekstrakti, s obzirom na njihovu široku upotrebu u fitoterapiji.

Dobijeni rezultati su potvrdili visok antioksidativni kapacitet *S. aegyptiaca*. Etanolni ekstrakt je pokazao najjaču aktivnost u poređenju sa ostalim ekstraktima, u DPPH, ABTS i FRAP testovima (IC_{50} 20.74 μ g/ml, 1.98 mg AEE/g, 1500.38 μ mol Fe(II)/g, respektivno), dok je u β -CB testu etanolni ekstrakt pokazao nisku aktivnost (9.04% inhibicije). Najveći sadržaj totalnih fenola je izmeren u etanolnom ekstraktu (150.71 mg GAE/g), dok je najviše flavonoida bilo u etil acetatnom ekstraktu (14.64 mg QE/g). Svi testovi su dobro korelisani sa totalnim sadržajem fenola (r vrednost od ± 0.806 do ± 0.993 , respektivno).

Etanolni ekstrakt *S. fruticosa* je pokazao najjaču antioksidativnu aktivnost u svim testovima (DPPH: 29.55 IC_{50} , μ g/ml, ABTS: 2.31 mg AEE/g, FRAP: 834.10 μ mol Fe(II)/g, β -CB: 85.11%). Pokazalo se da libijska *S. fruticosa* ima značajan sadržaj fenolnih komponenti koje se smatraju za jake antioksidanse (154.18 mg GAE/g suvog etanolnog ekstrakta). Najviši sadržaj fenolnih komponenti, pre svega kempferolnih glikozida je bio prisutan u vodenom, metanolnom i etanolnom ekstraktu. DPPH aktivnost je bila dobro korelisana sa sadržajem fenola. Etanolni ekstrakt je pokazao citotoksičnu aktivnost na elijsku liniju HCT-116 (375.96 μ g/ml), dok je vodeni ekstrakt pokazao jaču aktivnost na K562 elije (386.00 μ g/ml), što se smatra niskom

aktivnoš u. Etanolni ekstrakt pokazuje ja u antibakterijsku aktivnost od vodenog ekstrakta, naro ito protiv gram pozitivnih bakterija (MIC vrednosti 10-15 mg/ml). Antifungalno dejstvo ekstrakata *S. fruticosa* je ocenjeno kao slabo u pore enju sa ketokonazolom. U AChE testu inhibicije, ekstrakti su pokazali slabiju aktivnost od galantamina, dok su pokazali ja u aktivnost na testiranim koncentracijama (57.89-74.66%) u testu inhibicije tirozinaze u pore enju sa koji nom kiselinom.

Me u uzorcima *S. lanigera* metanolni i vodeni ekstrakti su pokazali najve i nivo fenolnih komponenti, posebno kempferolnih glikozida. Vodeni ekstrakt je pokazao bolju antioksidativnu aktivnost u primenjenim testovima, osim FRAP testa, gde je metanolni ekstrakt bolji od vodenog (DPPH: 205.45 IC₅₀, µg/ml, ABTS: 1.77 mg AEE/g, FRAP: 200.11 µmol Fe(II)/g). U β-CB testu vodeni i etanolni ekstrakti su ispoljili aktivnost (77.66% i 76.06%, respektivno) ve u od BHA i BHT standarda. Antioksidativni testovi su snažnije korelisani sa sadržajem totalnih fenola nego flavonoida. Ekstrakti su pokazali vrlo slabu citotoksi nost na HCT-116 liniju (IC₅₀ vrednosti ve e od najve e koncentracije od 500 µg/ml), dok je vodeni ekstrakt nešto ja e delovao na K562 elije (383.29 µg/ml). Etanolni ekstrakt je pokazao ja i inhibitorni efekat na bakterijski rast (vrednosti MIC 15-35 mg/ml) u pore enju sa vodenim ekstraktom (vrednosti MIC od 30 do preko 50 mg/ml). Etanolni ekstrakt je pokazao bolju aktivnost na *Aspergillus glaucus*, dok je vodeni ekstrakt pokazao najaj i efekat na *Trichophyton mentagrophytes*. Etanolni i vodeni ekstrakti su na testiranim koncentracijama pokazali inhibiciju AChE od oko 30%. Oba ekstrakta su na testiranim koncentracijama pokazala ja u inhibiciju (55.26-70.20%) od standarda koji ne kiseline (33.73-51.81%) u testu inhibicije tirozinaze. Inhibitorni efekat vodenih ekstrakata je ja i u pore enju sa etanolnim ekstraktom.

Ispitivane libijske vrste *Salvia* su pokazale razlike u testiranim biološkim aktivnostima. *S. aegyptiaca* and *S. fruticosa* su pokazale visoku antioksidativnu aktivnost, ali su antimikrobna i citotoksi na aktivnost uglavnom niske. Vodeni i etanolni ekstrakti *S. fruticosa* and *S. lanigera* su na testiranim koncentracijama u testu inhibicije tirozinaze pokazali ja u aktivnost u pore enju sa koji nom kiselinom kao standardom.

Cljučne riječi:

Salvia aegyptiaca; *Salvia fruticosa*; *Salvia lanigera*; mikromorfologija; antioksidativna aktivnost; antimikrobna aktivnost; citotoksična aktivnost; neurodegenerativni enzimi

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ABBREVIATIONS

ABTS	2,2'-azino-bis- (3-ethylbenzothiazoline –6-sulfonic acid
AChE	Acetylcholinesterase
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
DCM	Dichloromethane (DCM)
DPPH	2,2-diphenyl-1-picrylhydrazyl
ELISA	Enzyme-Linked Immunosorbent Assay
ETAC	Ethyl Acetate
ETOH	Ethanol
FRAP	Ferric-reducing ability of plasma assay
HPLC	High-Performance Liquid Chromatography
HW	Hot Water
LM	Light Microscopy
MBC	Minimum Bactericidal Concentration
MEOH	Methanol
MFC	Minimum Fungicidal Concentration
MIC	Minimum Inhibitory Concentration
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
ROS	Reactive Oxygen Species
SEM	Scanning Electron Microscopy
β-CB	β-carotene bleaching assay
TEM	Transmission Electron Microscopy
TFC	Total Flavonoid Content
TPC	Total Phenolic Content
TYR	Tyrosinase
UV-DAD	UV-Diode Array Detector

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1. INTRODUCTION

1.1. Medicinal plants

Medicinal plants represent the oldest and most widespread mode of medication. Medicinal plants have been used in treatments of numerous human diseases for thousands of years in many parts of the world. Despite the increasing use of synthetic drugs, natural organic healing materials have still persisted popular for treatment of various health problems in populations throughout the world. In rural areas of developing countries, herbal materials continue to be used as the primary source of medicines (Chitme et al., 2003).

Since the ancient times, in search for rescue for their disease, the people looked for drugs in nature. The beginnings of the medicinal plants use were instinctive, as is the case with animals. In view of the fact that at the time there was not sufficient information either concerning the reasons for the illnesses or concerning which plant and how it could be utilized as a cure, everything was based on experience. In time, the reasons for the usage of specific medicinal plants for treatment of certain diseases were being discovered; thus, the medicinal plants' usage gradually abandoned the empiric framework and became founded on explicatory facts. Although people may not understand the science behind these medicines, they knew that some medicinal plants are highly effective only when used at therapeutic doses (Maheshwari et al., 1986). The decreasing efficacy of synthetic drugs and the increasing contraindications of their usage make the usage of natural drugs topical again (Petrovska, 2012).

As civilizations grew from 3000 BC onwards in Egypt, the Middle East, India and China, the uses of herbs became more sophisticated and written records were prepared (Cragg et al., 1997). The specific plants to be used and the methods of application for particular ailments were passed down through oral history. Later on, information regarding medicinal plants was recorded in herbals (Balunas & Kinghorn, 2005). Historically, herbal drugs were used as tinctures, poultices, powders and teas followed by formulations, and lastly as pure compounds (Gurib-Fakim, 2006; Ramawat & Goyal, 2008).

The Babylonians, Assyrians and Sumerians comprise one of the oldest civilizations, and several plants were domesticated during this early period. Several

medicinal plants are mentioned in civil laws carved on stone and commissioned by the King of Babylon. In the ancient Western world, the Greeks contributed substantially to the rational development of the use of herbal drugs. Theophrastus in his “History of Plants” dealt with the medicinal qualities of herbs, while Dioscorides recorded the collection, storage, and use of medicinal herbs. Roman pharmacists Galen is well known for his complex prescriptions and formulas used in compounding drugs. During Middle Ages, the Arabs preserved the Greco-Roman knowledge, included use of their own resources, together with Chinese and Indian herbs, while in some European monasteries only the remnants of this knowledge were maintained (Cragg & Newman, 2002). The Arabs were the first to establish privately owned drug stores in the eighth century in Baghdad. The Persian scientist Abu Ali al-Husein ibn Sina (lat. Avicenna) described all Greco-Roman medicine in his book *Canon of Medicine*, which became a standard medical text at many medieval universities and remained in use as late as 1650. This text forms the basis of distinct Islamic healing system known today as Unani-Tibb (Theis & Lerda, 2003; Gurib-Fakim, 2006).

Medicinal plants or their extracts have been used by humans for different ailments and have provided valuable drugs such as analgesics (morphine), antitussives (codeine), antihypertensives (reserpine), cardiotonics (digoxin), antineoplastics (vinblastine and taxol) and antimalarials (quinine and artemisinin) (Ramawat et al., 2009).

According to World Health Organization (WHO) more than 80% of the populations living in the less developed countries rely almost exclusively on traditional medicine for their primary health care needs. Medicinal plants are the “backbone” of traditional medicine, which means more than 3.3 billion people in the less developed countries utilize medicinal plants on a regular basis. There are nearly 2000 ethnic groups in the world, and almost every group has its own traditional medical knowledge and experiences (Ahvazi et al., 2012).

Plant constituents may be isolated and used directly as therapeutic agents or as starting materials for drug synthesis or they may serve as models for pharmacologically active compounds in drug synthesis. The general research methods includes proper selection of medicinal plants, preparation of crude extracts, biological screening, detailed chemical and pharmacological investigation, toxicological and clinical studies,

standardization and use of active moiety as the lead molecule for drug design (Wink et al., 2005).

The medicinal value of plants lies in some chemical substances that produce a definite physiological action on the human body. The most important of these bioactive compounds of plants are alkaloids, tannins, steroids and phenolic compounds, including flavonoids. Leaves of many plants have antimicrobial principles such as tannins, essential oils and other aromatic compounds. These compounds protect the plant from microbial infection and deterioration. Some of these phytochemicals can significantly reduce the risk of cancer due to polyphenol antioxidant and anti-inflammatory effects (Karaka et al., 2012). The family Lamiaceae is rich in aromatic species which possess various biological activities, due to the essential oils produced in glandular hairs spread over the aerial vegetative and reproductive organs (Werker, 1993).

1.2. Family Lamiaceae

Lamiaceae Martinov (= Labiatae Adans., the mint family) has a world-wide distribution and comprises more than 7.200 species across approximately 240 genera which are classified in seven subfamilies: Ajugoideae Kostel., Lamioideae Harley, Nepetoideae (Dumort.) Luer., Prostantheroideae Luer., Scutellarioideae (Dumort.) Caruel, Symphorematoideae Briq. and Viticoideae Briq. (Harley et al., 2004). It is represented by 22 genera and 65 species in Libya (Jafri & El-Gadi, 1985). Lamiaceae comprises perennial herbs or undershrubs, rarely shrubs. The stems are usually quadrangular. The leaves are opposite or whorled usually are decussate, hairy, and often aromatic. The flowers are clustered into short stalked whorls or verticals in the axils of the upper leaves or bracts, flowers are hermaphrodite or sometimes are female only, zygomorphic (rarely actinomorphic as in *Mentha*). The corolla is tubular, 5-lobed, and strongly 2-lipped (sometimes with a single 5-lobed lip as in *Teucrium*). The gynoecium is bicarpellary, tetralocular when mature due to the formation of a false septum. The fruits are carcerulus with 4-1 seeded nutlets rarely are a drupe (as in *Prasium*). Seeds are non-endospermic or rarely with scanty endosperm (Jafri & El-Gadi, 1985).

Aerial parts of many Lamiaceae representatives are densely covered with glandular and non-glandular trichomes, which originate from the epidermal cells. Plant

species that contain glandular trichomes generally produce relatively large amounts of bioactive compounds. Reports indicate that the type and density of trichomes vary between species and may vary on organs of the same plant (Uphof, 1962). Non-glandular trichomes are thought to reduce the heat load of plants, increase tolerance to freezing, assist in seed dispersal, maintain water balance in leaves, deflect intense solar radiation and offer protection from insect herbivores (Mauricio & Rauscher, 1997; Werker, 2000), whereas glandular trichomes offer chemical protection against herbivorous insects as well as bacterial and fungal pathogens. The glandular trichomes, characteristic for the Lamiaceae family, are recognized as the site of essential oil biosynthesis, secretion and accumulation (Corsi & Bottega, 1999; Gershenzon et al., 1989; Ascensao et al., 1995). Some of the compounds produced by glandular trichomes are utilized in pharmaceuticals, nutraceuticals, natural pesticides, flavorings, fragrances, and for other non-food and fiber purposes (Duke, 1994).

1.3. Biological effects of plant derivatives

Plants are considered among the main sources of biologically active chemicals. It has been estimated that about 50% of the prescription products in Europe and USA are originating from natural products or their derivatives. Out of the 250.000–500.000 plant species on Earth, only 1-10% has been studied chemically and pharmacologically for their potential medicinal value. In Middle East region 700 species of identified plants are known for their medicinal values (Talib & Mahasneh, 2010). Numerous drugs from plant secondary metabolites are being developed and studied for their biological activities.

1.3.1. Antioxidant effects of plant derivatives

The field of antioxidants and free radicals is often perceived as focusing around the use of antioxidant supplements to prevent human disease. Antioxidants serve to keep down the levels of free radicals, permitting them to perform useful biological functions without too much damage (Halliwell, 2006).

The term reactive oxygen species (ROS), often used in the biomedical free radical literature is a collective term that includes not only oxygen-centered radicals

such as O_2^- and OH, but also some non-radical derivatives of oxygen, such as hydrogen peroxide (H_2O_2), and hypochlorous acid (HOCl). A similar term, reactive nitrogen species (RNS), is also becoming widely used. Some of these species are much less 'reactive' than others, e.g. O_2^- and NO react directly with few molecules in the human body, whereas OH can react with anything. When generated *in vivo*, OH will react at its site of formation (Halliwell, 2001a).

Radicals and other 'reactive oxygen/nitrogen/chlorine species' are widely believed to contribute to the development of several age-related diseases, species have been implicated in the pathology of over 100 human diseases (Halliwell, 2001a; Halliwell & Whiteman, 2004). These free radicals may oxidize nucleic acids, proteins, lipids, and can initiate degenerative process (Miller et al., 2000a,b; Nickavar et al., 2007). Persistent damage to DNA by ROS/RNS may play a role in the initiation of some human cancers, by creating mutagenic lesions such as 8-hydroxyguanine (Halliwell, 2001a). It can influence the aging process itself, by causing 'oxidative stress' and 'oxidative damage'. For example, many studies have shown increased oxidative damage to all the major classes of biomolecules in the brains of Alzheimer's patients. Other diseases in which oxidative damage has been implicated include cancer, atherosclerosis, other neurodegenerative diseases, diabetes, ulcerative colitis, haemorrhagic shock, cystic fibrosis and AIDS (Halliwell & Whiteman, 2004).

Antioxidant compounds in food play an important role as a health protecting factor. Scientific evidence suggests that antioxidants reduce the risk for chronic diseases including cancer and heart disease. Primary sources of naturally occurring antioxidants are whole grains, fruits and vegetables. Plant sourced food antioxidants like vitamin C, vitamin E, carotenes, phenolic acids, polyphenols, flavonoids, phytate and phytoestrogens have been recognized as having the potential to reduce disease risk. Some compounds, such as gallates, have strong antioxidant activity, while others, such as the mono-phenols are weak antioxidants (Miller et al., 2000a; Nickavar et al., 2007).

The actions of antioxidants include scavenging and neutralizing activity against free radical species, the inhibition of reactive species production, regulation of such antioxidant enzymes as superoxide dismutase and glutathione peroxidase, counteraction of the glutathione depletion, and prevention of the damage to lipids, proteins, and nucleic acids (Finley et al., 2011).

The Lamiaceae family includes a large number of plants that are well-known for their antioxidant properties (Capecka et al., 2005; Erdemoglu et al., 2006; Firuzi et al., 2010; Vladimir-Knežević et al., 2014). There are numerous reports dealing with the antioxidant potential of the Lamiaceae family representatives. In particular, many species of the genus *Salvia* have been widely studied and the majority of the antioxidant components have been identified (Lu & Foo, 2001; Bozan et al., 2002; Salah et al., 2006; Kamatou et al., 2008; enol, et al., 2010).

The antioxidant activities of four Lamiaceae plants, *Salvia viridis*, *S. multicaulis*, *Stachys byzantina* and *Eremostachys laciniata* growing in Turkey, which are often present in Turkish folk medicine, have been examined by Erdemoglu et al. (2006). The study showed that these plants are strong radical scavengers and may be considered as good sources of natural antioxidants for medicinal uses such as against aging and other diseases related to radical mechanisms (Capecka et al., 2005; Erdemoglu et al., 2006).

The methanolic extracts, infusions and decoctions of *Mentha piperita*, *Thymus vulgaris*, *Melissa officinalis*, *Ocimum basilicum*, *Rosmarinus officinalis* and *Salvia officinalis*, can be used as natural sources in food and the pharmaceutical industry due to their strong antimicrobial and antioxidant activities. Phenolic acids and flavonoids have been shown to be responsible for the antioxidant activity of plant materials against oxidative deterioration (Albayrak et al., 2013).

Medicinal plants have been used in folk medicine in Libyan rural areas at relatively cheaper expenses than modern medicine. They have been widely used as diuretics, topical anti-inflammants, and haemostatics (Alghazeer et al., 2012).

Libyan plants belonging to Lamiaceae were rarely studied for their antioxidant potential. The essential oil isolated from *Satureja thymbra*, growing wild in Libya exhibited strong antioxidant activity (Giweli et al., 2012). The essential oil isolated from *Thymus algeriensis* growing wild in Libya was screened for antioxidant activity using DPPH assay and findings showed that the *Th. algeriensis* essential oil possessed a strong antioxidant activity (Giweli et al., 2013a). The antioxidant activity of essentials oil of four Libyan medicinal plants from family Lamiaceae, *Ajuga iva*, *Marrubium vulgare*, *Rosmarinus officinalis* and *Thymus capitatus* was tested, and the results showed that the four essential oils have potent antioxidant activity, while the oil of *M. vulgare* had the most powerful antioxidant activity (El-Hawary et al., 2013). Eight

Libyan medicinal plants belonging to different families were studied for their antioxidant activities by Alsabri et al. (2012). Three extracts of each plant were *in vitro* evaluated for their antioxidant activity using DPPH assay. The methanolic extract of *Arbutus pavarii* was the most active. Five Libyan medicinal plants *Thapsia garganica*, *Hammada scoparia*, *Euphorbia serrata*, *Hyoscymus albus* and *Retama rateam* were selected for evaluation of their biological activities. Their crude extracts showed reducing potential proportional to their concentration (Alghazeer et al., 2012).

1.3.2. Cytotoxic effects of plant derivatives

Cancer is a term for diseases in which abnormal cells divide without control and can invade nearby tissues. Cancer is caused by defects in the mechanisms underlying cell proliferation and cell death. The development of tumors results from excessive cell proliferation combined with inhibition of cell apoptosis, which eventually leads to imbalances in tissue homeostasis and uncontrolled proliferation (Hanahan & Weinberg, 2000). Proliferation and apoptosis involve different pathways and molecular actors, proliferation relying on cyclin-dependent protein kinases - regulators of the cell division cycle (Nigg, 1995) and apoptosis primarily depending on caspases - cysteine proteases executing a cell death program (Nicholson, 1999).

Cancer cells that keep dividing and growing over time under laboratory conditions are cancer cell lines and are used to study the biology of cancer and to test cancer treatments. Cell lines derived from tumors are the most frequently utilized models in cancer research and their use has advanced the understanding of cancer biology tremendously over the past decades (Domcke et al., 2013).

The potential of bioactive plants or their extracts to provide new and novel products for disease treatment and prevention is still enormous. Plants have an almost unlimited capacity to produce substances that attract researchers in the quest for new and novel chemotherapeutics. The continuing search for new anticancer compounds in plant medicines and traditional foods is a realistic and promising strategy for its prevention. Numerous groups with antitumor properties are plant derived natural products including alkaloids, phenylpropanoids, and terpenoids. According to the American National Cancer Institute (NCI), the criteria of cytotoxic activity for the crude

extracts is an $IC_{50} < 30 \mu\text{g/ml}$ (Talib & Mahasneh, 2010). Compounds from plants are important source of anti-cancer agents and although plant natural products have long history of use in the treatment of cancer, there is continuing need for development of new anticancer drugs (Hartwell, 1982; Mukherjee et al., 2001; Cragg & Newman, 2005a,b).

Cancer incidence and mortality increased by approximately 22% up until 1990. In 2000, there were 10 million new cases and over 6 million deaths worldwide. Plant derived drugs are playing an important role in the upgrowth of cancer therapy. Most of the active compounds in these extracts remain unidentified, and their presence is only detected by biological tests. The structure and mechanism of action in others have been elucidated, and some compounds such as rubomycin, vinblastine, vincristine, colchamine, and camptothecin have been investigated for their activities in chemotherapy (Karaka et al., 2012).

Various Lamiaceae plants were investigated for their potential cytotoxic effect. Results from testing antitumor activity of essential oils from *Thymus serpyllum*, *Thymus algeriensis* and *Thymus vulgaris* on growth of four human tumor cell lines (NCI-H460, MCF-7, HCT-15 and AGS) showed that *T. serpyllum* oil was the most effective one against all tested cell lines. Moreover, none of the essential oils showed toxicity at tested concentrations ($>400 \text{ g/ml}$) for porcine liver primary cell culture (Nikoli et al., 2014).

Antitumoral activity of Moroccan thyme species (*Thymus maroccanus*, *Th. leptobotris*, *Th. pallidus*, *Th. zygis*), extracts as well as that of two pure compounds (carvacrol and thymol) was evaluated against P815 tumor cell line. The results show that Moroccan thyme extracts have an important cytotoxic effect and that carvacrol is the most important cytotoxic agent (Jaafari et al., 2007).

Sixteen plants grown in Bolu, Turkey, including aqueous, ethanol and methanol extracts of *Mentha pulegium*, were tested for antitumor activity. Among all extracts of *M. pulegium*, the best antitumor activity was observed with aqueous extracts (94%) (Karaka et al., 2012).

Methanolic extract of *Teucrium persicum* native to Iran was investigated for possible antitumor effects on different cell lines. The study showed that HeLa cells, originating from epitheloid cervix carcinoma, was mostly affected by this extract. In a

previous study, the antitumor activity of *Teucrium* diterpenoids against P 388 lymphocytic leukemia in mice has been reported, *T. marum* has also been shown to have cytotoxic properties (Amirghofran et al., 2010).

Different extracts obtained from several Libyan plants were studied for their possible cytotoxic effects, such as *Ferula hermonis* (Elouzi et al., 2008), *Ballota pseudodictamnus*, *Hedera helix* and *Thapsia garganica* (Elmezogi et al., 2013), *Capparis spinosa*, *Juniperus phoenicea*, *Ruta graveolanse* and *Artimisia herba alba* (Aljaiyash et al., 2014).

1.3.3. Antimicrobial effects of plant derivatives

It is of great interest to search for new and hopefully more potent antimicrobial drugs of natural origin to complement the existing antibiotics that are gradually becoming less potent against human pathogenic microorganisms. To reach this goal, one of the first steps is the *in vitro* antimicrobial activity assay.

Common types of microorganisms, such as bacteria, viruses and fungi, have been found in water, air, food, blood/body fluids on nonliving environmental surfaces, humans and animals. Pathogens can enter human bodies through four primary routes. Swallowing contaminated food, beverages or water into the digestive tract can ingest harmful microorganisms. Breathing contaminated air or vapors into the respiratory tract can cause us to inhale harmful microbes. Puncturing our skin through a wound or an insect bite can infect our blood stream, and also microbes can be absorbed through the skin (McFadden et al., 2003).

The contamination of food products with microorganisms presents a problem of global concern, since the growth and metabolism of microorganisms can cause serious foodborne intoxications and a rapid spoilage of the food products. Thus, the acceptance and safety of a food product for the consumers depends in great part on the presence and nature of microorganisms. Besides molds and yeasts, bacteria are the principle responsible for various types of food spoilage and foodborne intoxications. Spoilage bacteria cause the deterioration of food and develop unpleasant odors, tastes, and textures. The microbial spoilage of food products constitutes an important economic problem (Blackburn, 2006). As it is well known, *Staphylococcus aureus*, *Escherichia*

coli and *Bacillus* species, especially *B. cereus*, are the most frequent agents of food poisoning. The most interesting area of application for plant extracts and oils is the inhibition of growth and reduction in numbers food-borne pathogens (Vila, 2002).

In the recent years, many possible sources of natural antibiotics have been in use for several infectious diseases, mostly bacterial and fungal. In view of this, the searches for new antimicrobial agents from medicinal plants are even more urgent in the countries where infectious diseases of bacterial origin are not only rampant, but the causative agents are also developing an increasing resistance against many of the commonly used antibiotics. Considering the high costs of the synthetic drugs and their various side effects, the search for alternative products from plants used in folklore medicine is further justified (Kamaraj et al., 2012). Essential oils obtained from various plants are potentially important for their antimicrobial activity (Burt, 2004).

Species of the Lamiaceae family are important for their antimicrobial activities. Several studies indicate that the essential oils of the plants belonging to Lamiaceae family possess biological activity against certain bacteria and yeast. Most of the essential oils, in the case of *Thymus vulgaris*, *Salvia officinalis*, *Lavandula officinalis*, *Mentha x piperita*, *Rosmarinus officinalis* and *Mellisa officinalis*, showed strong antimicrobial activity (both bacteriostatic and bactericidal effect) against *Staphylococcus aureus*, *Salmonella enteritidis* and *E. coli* (Karaka et al., 2012). The essential oil of *Lavandula multifida* L. showed a remarkable effect on the different Gram-positive and Gram-negative pathogenic bacterial strains (Benbelaid et al., 2012).

The aqueous and organic solvents extracts of *Mentha piperita* leaves were found to possess strong antibacterial activity against *B. subtilis*, *P. aureus*, *P. aerogenosa*, *Serratia marcesens* and *Streptococcus aureus* (Bupesh et al., 2007). The methanolic extracts, infusions, decoctions and hydrosols of *Mentha piperita*, *Thymus vulgaris*, *Melissa officinalis*, *Ocimum basilicum*, *Rosmarinus officinalis* and *Salvia officinalis* were investigated for their antimicrobial activity. Comparing the six plants studied, *S. officinalis* extracts were the most effective (Albayrak et al., 2013).

The essential oil isolated from *Satureja thymbra*, growing wild in Libya was screened for its antimicrobial activity against eight bacterial and eight fungal species, showing excellent antimicrobial activity against the microorganisms used, in particular against studied fungi (Giweli et al., 2012). Essential oil isolated from *Thymus*

algeriensis growing wild in Libya was screened for antimicrobial activity against bacterial and fungal species, and findings showed that the *Th. algeriensis* essential oil possessed a high antimicrobial activity (Giweli et al., 2013a).

The extracts of other Libyan plants, *Thapsia garganica*, *Hammada scoparia*, *Euphorbia serrata*, *Hyoscyamus albus* and *Retama rateam* (Alghazeer et al., 2012), *Capparis spinosa*, *Juniperus phoenicea*, *Ruta graveolans* and *Artimisia herba alba* (Aljaiyash et al., 2014) were evaluated for their antimicrobial activity.

1.3.4. Acetylcholinesterase and tyrosinase inhibitory activities of plant derivatives

Neurodegenerative diseases comprise a condition in which nerve cells from brain and spinal cord are lost leading to either functional loss (ataxia) or sensory dysfunction (dementia). Loss of cognitive function and the development of dementia are among the greatest concerns confronting older individuals. As populations around the world age, the global prevalence of dementia is predicted to increase substantially (Gill & Seitz, 2015).

Mitochondrial (Mt) dysfunctions and excitotoxicity and finally apoptosis have been reported as pathological cause for aging and neurodegenerative diseases such as Parkinson's disease (PD), Alzheimer's disease (AD), Multiple sclerosis (MS) and amyotrophic lateral sclerosis (ALS). Neurodegeneration have been speculated to be interplay of a number of factors including environmental and genetic predisposition, but redox metal abuse occupies central role as most of symptoms stems out from abnormal metal metabolism (Uttara et al., 2009).

Parkinson's disease (PD) was first described by James Parkinson (Thomas & Flint Beal, 2007) and afflicts nearly 1% of the population above the age of 60. It is a multisystem neurodegenerative disorder in which progressive loss of midbrain dopamine (DA) neurons, with resulting dopaminergic deafferentation of the basal ganglia, gives rise to characteristic motor disturbances that include slowing of movement, muscular rigidity, and resting tremor. These signs of motor dysfunction, if lateralized, can be clinically diagnostic of PD (Gelb et al., 1999).

Alzheimer's disease (AD) was first described in 1906 by a Bavarian neuropsychiatrist Alois Alzheimer (Hostettmann et al., 2006). It is a complex,

multifactoral, progressive, neurodegenerative disease primarily affecting the elderly population and is estimated to account for 50-60% of dementia cases in persons over 65 years of age (Frank & Gupta, 2005; Adewusi et al., 2010; Tali et al., 2014). The number of individuals worldwide with AD is expected to rise to 34 million in the next three decades, a dramatic increase from 7.3 million today (Dhivya et al., 2014). AD is characterized by loss of cognitive function leading to dementia (Tali et al., 2014), showing difficulty performing familiar tasks, problems with language, disorientation of time and place, poor or decreased judgment, problems with abstract thinking, misplacing things, changes in mood or behavior, changes in personality, and loss of initiative (Topcu & Kusman, 2014). The pathological features identified in the central nervous system (CNS) in AD are amyloid- β -peptide (A β) plaques, neurofibrillary tangles of hyperphosphorylated tau protein, inflammatory processes and disturbance of neurotransmitters (Adewusi et al., 2010).

Many findings suggest that oxidative stress play an important role in the pathogenesis of AD. Antioxidants may decrease the level of oxidative stress in the brain and thereby reduce the amount of DNA damage, neuronal cell death, and aggregation of A β within the brain (Demirezer et al., 2014).

The most remarkable biochemical change in AD patients is a reduction of acetylcholine (ACh) levels in the hippocampus and cortex of the brain (Tali et al., 2014). Acetylcholine has a functional key role in cognitive functions including learning and memory, arousal, and attentional processes in the brain (Demirezer et al., 2014). It is an organic molecule liberated at the nerve endings as a neurotransmitter. It is produced by the synthetic enzyme choline acetyltransferase which uses acetyl coenzyme-A and choline as substrates for the formation of acetylcholine in specific cells known as cholinergic neurons (Dhivya et al., 2014). In mammalian brain, there are two major forms of cholinesterases, namely, acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) (Giacobini, 2003; Tali et al., 2014). In patients with AD, released ACh has a very short half-life due to the presence of large amounts of the enzymes AChE and BChE (Orhan et al., 2004; Adewusi et al., 2010).

AChE is the key enzyme in the breakdown of acetylcholine. The inhibition of AChE is considered one of the treatment strategies against several neurological disorders such as AD, senile dementia, ataxia, and myasthenia gravis (Demirezer et al.,

2014). AChE is found in all excitable tissue, whether nerve or muscle, in most erythrocytes and in placental tissue (Tali et al., 2014).

Currently, several AChE inhibitors, such as Donepezil, Galanthamine and Rivastigmine are available for the symptomatic treatment of patients with mild to moderate Alzheimer's disease (Dhivya et al., 2014). However, none of them have the ability to stop the disease. Thus, there is still great urgency for finding new drug candidates for AD treatment (Demirezer et al., 2014). Some reports propound that acetylcholinesterase inhibitors have inclement side effects such as anorexia, diarrhoea, fatigue, nausea, muscle cramps as well as gastrointestinal, cardiorespiratory, genitourinary and sleep disturbances (Chattipakorn et al., 2007), short duration of biological action, low bioavailability, hepatotoxicity. Therefore, the search for new inhibitors with low toxicity from natural plants continues (Aminudin et al., 2015).

In order to find new natural agents for the treatment of AD based on the cholinesterase inhibitory mechanism, many research groups in the world study different plants from various families, including Amaryllidaceae, Fumariaceae, Papaveraceae, and Lamiaceae families. So far, some potential triterpenoids, such as ursolic acid and oleanolic acid, ginsenosides, ginkgolides, and cannabinoids have been studied as potential cholinesterase inhibitors in AD treatment, but they are still awaiting clinical trials, as well as some plants that contain different types of alkaloids (Topcu & Kusman, 2014).

The essential oils and extracts of different Lamiaceae plants were studied for their cholinesterase inhibition action. The extracts of *Thymus lotocephalus*, exhibited high anticholinesterase activity, and the essential oil of *T. lotocephalus* also strongly inhibited cholinesterases (Costa et al., 2012). Ferreira et al. (2006) reported the AChE inhibitory activity of the essential oil, ethanol extract and decoction of ten plant species from Portugal. *Melissa officinalis* and *Mentha suaveolens* showed AChE inhibitory capacity higher than 50% in the essential oil fraction. High inhibition of the AChE was obtained for decoctions of *Lavandula pedunculata* (68%) and *Mentha suaveolens* (69%). Among *Stachys lavandulifolia* extracts, the most active extract against AChE was the *n*-hexane extract (Tundis et al., 2015). Acetyl- and butyryl-cholinesterase inhibitory activities of extracts of the aerial parts of *Micromeria juliana* were

investigated, and exhibited only weak inhibition against the AChE, and moderate to high inhibition against BChE (Topcu & Kusman, 2014).

Tyrosinase is a multifunctional copper-containing enzyme involved in the biosynthesis of melanin. It is also known as polyphenol oxidase (PPO) (Tel et al., 2013). This enzyme uses molecular oxygen to catalyze the oxidation of monophenols to their corresponding *o*-diphenols (monophenolase or cresolase activity) and their subsequent oxidation to *o*-quinones (diphenolase or catecholase activity) (Fais et al., 2009). The enzyme seems to be almost universally distributed in animals, plants, fungi and bacteria. Moreover, tyrosinase is responsible for browning of certain fruits and vegetables during their handling, processing and storage after harvest (Xu et al., 1998; Asanuma et al., 2003; Fais et al., 2009).

In mammals tyrosinase activity can cause dermatological disorders such as hyperpigmentation of skin, melasma, age spots and freckles. Furthermore, formation of neuromelanin in the mammalian brain may be related to neurodegeneration associated with Parkinson's disease (Asanuma et al. 2003; Aminudin et al., 2015).

Therefore, the development of safe and effective tyrosinase inhibitors has become important for improving food quality and preventing pigmentation disorders and other melanin related human health issues. Additionally, tyrosinase inhibitors are supposed to have broad applications as cosmetics whitening agents. As plants are a rich source of bioactive chemicals that are mostly free from harmful side effects, interest in finding natural tyrosinase inhibitors in bioactive chemicals is also increasing (Karina et al, 2013; Aminudin et al., 2015). Many inhibitors and enhancers of melanin biosynthesis have been described and some are obtained from natural sources, kojic acid, for example, is a well-known tyrosinase inhibitor produced by *Aspergillus* and *Penicillium* fungi (Karina et al, 2013). Some potent tyrosinase inhibitors, such as anisaldehyde, quercetin, myricetin, and glycoside of myricetin, and recently dalenin have been isolated from various plants (Jennifer et al., 2012; Karina et al, 2013; Aminudin et al., 2015).

The search for tyrosinase inhibitors included different Lamiaceae plants. Aqueous and organic solvent extracts obtained from 19 plants native to the Amazon rain forest and to the Atlantic forest, including *Salvia dorisiana*, *S. elegans*, and *S. officinalis*, were evaluated as tyrosinase inhibitors. Nine out of 49 extracts showed

inhibitory activity in the screening process. The 50% inhibitory activity was calculated, revealing that the most active extracts were the organic extracts from the leaves and stem of *Ruprechtia* sp. and *Rapanea parviflora* (Macrini et al., 2009).

The tyrosinase inhibition activities of methanol, acetone and water extract of *Ocimum americanum* were studied by Khanom et al. (2000), and results indicated that the methanol and water extract of this plant have tyrosinase inhibition activity. The other *Ocimum* species, *O. gratissimum* has shown high inhibition of dopachrome formation (Lin et al., 2011).

The tyrosinase inhibitory effects of *Stachys lavandulifolia* extracts increased in a linear concentration-dependent manner, ethanol and methanol extracts were the most active. These extracts were characterized by the presence of known tyrosinase inhibitors, such as acteoside and arbutin (Tundis et al., 2015).

Flavonoids and phenylethanoid glycosides from *Marrubium velutinum* and *M. cylleneum* showed moderate inhibitory activity against tyrosinase, while methanolic extract of both plants showed higher activity, probably due to synergistic effect of different compounds (Karioti et al., 2007).

The Libyan plants were rarely studied for their possible neuropharmacological properties, only ethanol extract of *Launaea resedifolia* (Auzi et al., 2007), and methanol extract of *Retama raetam* (Al-Tubuly et al., 2011) were examined for effects on CNS of mice.

1.4. Genus *Salvia* L.

The genus name, *Salvia*, was first used by Pliny the Elder, Roman scientist and historian, for a plant that was likely *S. officinalis* (common sage) and is derived from the Latin *salvere* ("to feel well and healthy, be well, in good health, health, heal") (Dweck, 2002).

The genus was known to the Egyptians as “anusi”, to the Greeks as “elelisphakon” to the Romans as “herb sacra” the “salve” (saviour) and to Spanish and Moroccan Arab herbalists as “salima” or “asphacus”. There may be confusion in the older (herbal) literature as to which particular *Salvia* species is denoted by each of these

names. Thus assignment of medicinal value to a particular species is not always clear cut when its ancient use is mentioned (Perry et al., 2000a).

Salvia L. (sage) is the largest genus of Lamiaceae, composed of nearly 1000 species distributed extensively in three regions of the world, Central and South America (500 spp.), Western Asia (250 spp.) and Eastern Asia (100 spp.) (Walker et al., 2004). Species of the genus are widely distributed in tropical and temperate areas of both Old and New World. Western Asia and Mediterranean regions are considered to be its two distributional centers (Zhiyun et al., 2004; Kahraman, 2010). The genus has a sub-cosmopolitan distribution, but it is largely absent in the North Amazon basin and Central and West Africa (Dyubeni & Buwa, 2011).

Salvia species are herbs or undershrubs. The leaves are entire, toothed or deeply lobed; bracts are small or large, whorls two to many flowered, in spikes, racemes or panicles; the flowers are large and showy or sometimes are small and inconspicuous variously colored; the calyx is tubular, campanulate or ovoid, bilabiate; upper lip is entire or toothed; the corolla is tubular included or exserted, equal, ventricose or enlarged above; the stamens are two, anticus, arcuate; filaments are short; connective jointed to the filaments, linear, elongated. *Salvia* is distinguished from other genera of the tribe Mentheae by only two fertile stamens in which two thecae of each stamen are divided by an elongate connective tissue which are modified lever-like stamens playing a central role in the process of pollen transfer. Some revised classification of certain genera in the Lamiaceae was done on the basis of palynological features. The genus *Salvia* is placed within the subfamily Nepetoideae since it had hexacolpate pollen grains. *Salvia* pollen is small to large (Ozler et al., 2011; Jafri & El-Gadi, 1985). The style is shortly two-fid at the apex; the nutlets are ovoid triquetrous or compressed, smooth (Jafri & El-Gadi, 1985).

1.4.1. Micromorphological and histochemical characteristics of sage

Several *Salvia* species have been investigated from the point of morphological and anatomical features, and histochemical analyses. The majority of papers are dealing with characteristics of glandular trichomes distributed on vegetative and reproductive organs.

The leaves of numerous plants, including *Salvia* species, are densely covered with glandular and non-glandular trichomes, which originate from epidermal cells (Werker, 2000), and vary considerably in morphology, location, ability to secrete, mode of secretion, composition of secreted material, etc. between species and even on the organs of the same individual. Plant species that develop glandular trichomes generally produce relatively large amounts of bioactive compounds which include highly concentrated phytochemicals possessing biological activity and potential applications in the food or pharmaceutical industries (Duke, 1994; Turner et al. 2000; Burt, 2004; Giuliani & Maleci Bini, 2008; Miguel, 2010). There are a number of studies on foliar micromorphology of the representatives of the genus *Salvia* (Serrato-Valenti et al. 1997; Corsi and Bottega, 1999; Kaya et al. 2003; Siebert, 2004; Kamatou et al. 2007a; Özkan, 2008; Kahraman et al. 2009; Kahraman et al. 2010; Celep et al. 2011; Al Sheef et al., 2013). Tissier (2012) emphasized that plant glandular trichomes represent a highly active area of research, mainly due to interests in the elucidation of the biosynthetic pathways of industrially relevant compounds such as essential oils, which play role in the plant defense and may be used as pharmaceutical ingredients or substances.

Glandular trichomes are found on the organs of many *Salvia* species. These trichomes are the main secretory parts of the plants, and their structures can vary widely among species (Werker et al., 1985; Serrato-Valenti et al., 1997). Two main types of glandular trichomes – capitate and peltate are known on *Salvia* species. They can be distinguished by head size and stalk length. The capitate trichomes are very variable in stalk length, head shape and can be subdivided into I–IV types (Corsi & Bottega, 1999). Generally, they consist of one to two-celled head supported by one to four stalk cells. The peltate trichomes are composed of a short stalk, a basal cell and a large two to four-celled head. In the Lamiaceae family, the morphology, localization and frequency of glandular trichomes are used as distinguishing taxonomic characters (Ascensao et al.,

1995). Based on their morphology and number of cells, Cantino (1990) divided non-glandular trichomes into four types: simple unicellular, simple multicellular, branched unicellular and branched multicellular. The observation that non-glandular trichomes did not give any positive reaction supports the argument that their role is protection from water loss, the regulation of temperature through their reflective capacity and mechanical protection from herbivores (Ascensão et al., 1995, 1999).

Histochemical tests of secretion products in glandular trichomes of *Salvia* species were done aimed to reveal the composition of secretions and to evaluate the possible functional significance of the various trichomes.

The structure, site and histochemistry of glandular hairs on the vegetative and reproductive parts of *S. officinalis* were investigated by Corsi & Bottega (1999). Five distinct types of glandular hair (one peltate and four capitate) with different sites, secretory modes and secretions, were identified. All hair types show mixed secretions, i.e., hydrophilic and lipophilic, except type I capitate hairs, which have hydrophilic secretions only. In peltate hairs and in type II capitate hairs hydrophilic secretion prevails; in the remaining types, lipophilic secretion dominates (Corsi & Bottega, 1999).

Anatomical and histological investigations of the secretory hairs of *Salvia aurea* leaves, and identification of the main components of the essential oil were carried out by Serrato-Valenti et al (1997). Two types of glandular trichome were found: peltate trichome, and capitate trichomes of two kinds, the first with a short monocellular stalk and two-cellular head (type I), and the second with a multicellular stalk, a neck cell and a small globose unicellular head (type II). The histochemical study suggested an 'endodermal' role for the stalk cell (peltate and capitate type I) as well as for the neck cell (capitate type II), preventing the loss of essential oil. Histological reactions also revealed the complex nature of the material secreted by all types of *S. aurea* trichomes, including polysaccharides, polyphenols and proteins, in addition to the essential oil (Serrato-Valenti et al. 1997).

Morphological and histological investigations of cuticle and indumentum, as well as identification of the main components of the secreted material, were carried out for *S. blepharophylla*. Besides nonglandular hairs, three types of glandular trichomes (peltate and capitate) are described. The histological findings and chemical analysis of the

essential oil and leaf surface extracts revealed a complex secretion product (Bisio et al., 1999).

1.4.2. Medicinal properties and uses of sage

The genus has been valued since ancient times for its medicinal properties. Since *Salvia* is the largest genus in the Lamiaceae family, there are still numerous species which have not yet been pharmacologically studied.

Members of this genus produce many useful secondary metabolites, including terpenes and phenolics and their derivatives which have been in the center of pharmacopoeias of many countries, confirming that plants of this genus are particularly useful as natural preservative ingredients and have great potential for the food and cosmetic industries (Tenore et al., 2011). The percentage of essential oil corresponds remarkably well with Erdtman's two subfamilies: the Nepetoideae which are oil-rich (> 0.5% of dry weight) and the Lamioideae which are oil-poor (< 0.1% of dry weight). Iridoid glycosides occur in the Lamioideae but are absent in the Nepetoideae. *Salvia* is an exception to those general rules. It belongs to the subfamily Nepetoideae, but many *Salvia* species are oil-poor and contain iridoids (Basaif, 2004).

Plants belonging to the genus *Salvia* are rich source of essential oils, diterpenes, diterpenequinones and triterpenes (Ghulam et al., 1985). The species are rich source of polyphenols, with an excess of 160 polyphenols having been identified, some of which are unique to the genus. A large number of these polyphenolic compounds are apparently constructed from the caffeic acid building block via a variety of condensation reactions. The species are widely used in traditional medicine and are a rich source of polyphenolic, flavonoids and phenolic acids (Lu & Foo, 2001).

Salvia species are used in traditional medicines all around the world, possessing various effects on human health. Recently, many studies have focused on the biological properties of the essential oils and extracts of different *Salvia* species, which have shown antimicrobial, antioxidant, antidiabetic, antitumor, antiplasmodial, antiinflammatory, and anticholinesterase activities (Sivropolou et al., 1997; Dorman & Deans, 2000; Ulubelen, 2003; Tepe et al., 2004; 2006; Orhan et al., 2007; Kamatou et

al., 2008; Tosun et al., 2009; enol et al., 2010; Chan et al., 2011; Abu-Dahab et al., 2012).

Many *Salvia* species are used as herbal tea and as food additive, seasoning, as condiment, in cosmetics, perfumery and the pharmaceutical industry. Besides their medicinal value, *Salvia* species are grown in parks and gardens as ornamental plants (Flamini, 2007; Kahraman, 2010). Sage tea has been traditionally used for the treatment of digestive and circulation disturbances, bronchitis, cough, asthma, angina, mouth and throat inflammations, depression, excessive sweating, skin diseases, and many other diseases, while essential oils have been used in the treatment of a wide range of diseases like those of the nervous system, heart and blood circulation, respiratory system, digestive system, and metabolic and endocrine diseases (Hamidpour et al., 2014).

The seeds of *Salvia* species often produce mucilage on wetting, which is used for lacquerware, or mixed with fruit juices for producing drinks in Mexico, or in the Eastern countries for the treatment of eye diseases (Ozkan et al., 2008).

Salvia species were reported to be used for memory-enhancing purposes in European folk medicine (Aktas et al., 2009). The inhibition of cholinesterase *in vitro* and *in vivo* by *S. lavandulaefolia* oil may help in explaining its traditional use for ailing memory (Perry et al., 2002). Sage is also used in baths to treat skin problems, and also other external properties, astringent, antiseptic, tonic, antirheumatic in baths, for atonic wounds, sores, ulcers, dermatosis (eczemas), etc. (Dweck, 2002).

1.4.2.1. Antioxidant activity of sage

Commercially available extracts of sage are mainly utilised by the food processing industry, but may be applicable in human health. Main sage phenolic diterpenes, which show high antioxidative activity are carnosic acid which is known for its instability, and its degradation derivatives carnosol rosmanol, its isomer epirosmanol, 7-methyl-epirosmanol as well as rosmanol 9-ethyl ether. Rosmarinic acid also accounts for the antioxidant activity of sage. When measuring the radical scavenger effect on (DPPH) free radical, the antioxidative effect of rosmarinic acid was comparable to that of ascorbic acid (Bari evi & Bartol, 2000).

Different *Salvia* species were subjected to the analysis of their antioxidant activity. The antioxidant activities of *S. viridis* and *S. multicaulis* have been determined by using DPPH radical scavenging assay, and the results showed that the extracts are a potential source of antioxidants of natural origin (Bari evi & Bartol, 2000). The total antioxidant capacities of *S. satureioides* methanolic extract was determined by FRAP, and all the concentrations were average compared to controls used (BHA, BHT) (Belmekki & Bendimerad, 2012). The methanol extracts of *S. verticillata* ssp. *verticillata* and spp. *amasiaca* were found to possess strong antioxidant activities (Yumrutas et al., 2011). About 165 extracts prepared with dichloromethane, ethyl acetate, and methanol from 55 taxa of *Salvia* species of Turkish origin were screened for the antioxidant activity using DPPH radical scavenging method. Among the dichloromethane extracts screened herein, the best scavenging activity against DPPH radical was caused by *S. fruticosa* (89.23%) and *S. russelli* (86.36%) at 100 µg/ml. Only the ethyl acetate extracts of twelve tested *Salvia* species showed DPPH radical scavenging effect over 90%, while the methanol extracts exerted very high scavenging effect at all concentrations tested (Enol et al., 2010).

The radical scavenging activity of ethanolic extracts from five *Salvia* species including *S. hypoleuca*, *S. reuterana*, *S. verticillata*, *S. virgata* and *S. officinalis* was evaluated by DPPH free radical test. All of extracts showed radical scavenging effects, especially *S. verticillata* and *S. virgata* were found to be the most active species (Nickavar et al., 2007). Ethanolic extracts of leaves and stems of *S. amplexicaulis* showed stronger antioxidant activity compared to the methanolic extracts. The ethanol extract of leaves showed a noticeably strong DPPH activity, stronger than that of the synthetic antioxidant BHT and similar to BHA (Alimpi et al., 2014). Among the extracts of twenty-four plants of the family Lamiaceae, the extracts of *Salvia* in general were the most active radical scavengers among which *S. eremophila* and *S. santolinifolia* were the most active ones (Firuzi et al., 2010).

The essential oil and methanolic extract of *S. eremophila* were subjected to DPPH assay. Only the methanolic extract of the plant significantly reduced the reagent (Ebrahimabadi et al., 2010). Examination of antioxidant and total phenol contents of six *Salvia* species has resulted in determination of *S. xanthocheila* and *S. sclarea* as the weakest radical scavengers in those plant series (Asadi et al., 2010). Fourteen Turkish

sage species were examined for their antioxidant activity using DPPH reagent, among which different extracts of *S. sclarea* and *S. syriaca* showed similar inhibition percentage; however, these two plant extracts had the lowest antioxidant activity (Orhan et al., 2007).

The antioxidant activity of methanolic extracts of oregano and *S. officinalis* and *S. fruticosa* was tested by Pizzale et al. (2002). Some samples of sage had a very high antioxidant activity, with induction times more than 10-fold higher than that of lard used as the reference sample.

Erbil and Digrak (2015) tested the DPPH and ABTS scavenging ability of methanolic extracts of *S. verticillata* var. *amasiaca* and *S. microstegia* aerial parts and revealed the antioxidant effects.

The study of antioxidant activities of four *Salvia* species methanolic extracts (*S. officinalis*, *S. verbenaca*, *S. aegyptiaca*, *S. argentea*) growing in various habitats in Tunisia showed that *S. officinalis* revealed the greatest free radical-scavenging activity in three applied assays (Ben Farhat et al., 2013a).

In evaluation of the antioxidant activity of various extracts of Egyptian *S. officinalis* by two complementary test systems (DPPH free radical scavenging and α -carotene/linoleic acid systems), the results revealed that water and ethanolic extract were more efficient in scavenging free radicals than essential oil. In α -carotene/linoleic acid test system, ethanolic extract was superior to the other extracts studied and also contained the high level of total phenolic compounds (Rasmy et al., 2012). Tepe et al. (2005) reported that oxidation of linoleic acid was effectively inhibited by the polar subfraction of methanol/water extract (90.6%) of *S. tomentosa*, close to the synthetic antioxidant reagent BHT, while the essential oil was less effective.

1.4.2.2. Cytotoxic activity of sage

Many *Salvia* species have been reported to have anti-proliferative and cytotoxic effects on several cancer cell lines (Fiore et al., 2006; Gali-Muhtasib, 2006; Ozcelik et al., 2011). A diterpenequinone (4,5-seco-5,10-friedoabieta-3,5(10),6,8,13-pentaene-11,12-dione) isolated from *S. prionitis* exhibited not only antibacterial, antitubercular, and antiphlogistic activity, but even cytotoxic property against cell lines *in vitro* (Zhang

et al., 1999). Successively the natural product has been modified structurally to give a novel compound, named salvicine that displayed a marked antitumor activity on human solid tumor cell lines (Qing et al., 2001). Salvicine was considered a promising compound to develop a new anticancer drug and it has been chosen for preclinical studies. On the basis of these findings, *Salvia* species could be considered as a source of potential antitumor agents. Many diterpenes, isolated from plants of several species of the genus *Salvia*, have been demonstrated to possess interesting pharmacological properties, such as antioxidant, antimicrobial, antiinflammatory, analgesic, antipyretic, hemostatic hypoglycemic and antitumor (Fiore et al., 2006).

The cytotoxicity of the crude methanol extract of *S. chorassanica* on HeLa cells (human cervix carcinoma) were studied by MTS tetrazolium assay, and confirmed that the extract possesses anti-proliferative qualities, showing dose-dependent growth inhibition of malignant and non-malignant cell lines by different fractions. Dichloromethane fraction showed most potent inhibitory effects on the proliferation of HeLa cells (Parsaee et al., 2013). The methanolic extracts of selected Iranian plants including *S. santolinifolia*, *S. eremophil*, *S. macrosiphon*, and *S. reuterana*, were examined on various tumor cell lines, with the strongest inhibitory effect of *S. reuterana* extract. This extract showed a strong cytotoxic effect on the Raji lymphoma cell line. More than 50% of Raji cells growth was inhibited by 21 µg/ml of this extract. *S. macrosiphon* extract also showed a strong inhibitory effect on this tumor cell line ($IC_{50}=77$ µg/ml). Several reports showed the existence of various cytotoxic compounds in different *Salvia* species, i.e. salvicine, salvinal and tanshinone (Meng et al., 2001; Chang et al., 2004). The ability of *Salvia* species including *S. perfoliata* and *S. officinalis* in inhibition of human tumor cell growth has also been shown, while *S. mirzayan* extract demonstrated an activity against proliferative lymphocyte in the growth inhibition assay (Amirghofran et al., 2010).

The *S. leriifolia* hexane and dichloromethane extracts showed the strongest cytotoxic activity against the C32 cell line and the ethyl acetate extract was the most active extract against COR-L23 cell line. Buchariol, a sesquiterpene obtained by biofractionation of the dichloromethane extract, exhibited a higher activity than the positive control vinblastine against the C32 and A549 cell lines. Interesting results were also obtained for naringenin, which exhibited a strong cytotoxic activity against the

C32, LNCaP, and COR-L23 cell lines, compared to vinblastine. None of the tested compounds affected the proliferation of skin fibroblasts (142BR), suggesting a selective activity against tumor cells (Tundis et al., 2011).

Abu-Dahab et al. (2012) investigated ethanol extracts from nine *Salvia* species grown in Jordan for their antiproliferative activity using a panel of breast cancer cell. The results indicated that the extracts of three *Salvia* species, among them *S. fruticosa*, exhibit selective antiproliferative activity against estrogen positive cell lines and with minimum toxicity against normal human periodontal fibroblasts.

The chloroform and methanol extracts obtained from 14 Turkish *Salvia* species were tested against Herpes simplex (type-1, HSV-1) and Parainfluenza (type-3, PI- 3) using Madin-Darby Bovine Kidney and Vero cell lines. The chloroform extracts of *S. cyanescens* and *S. microstegia* were found to inhibit both HSV-1 and PI-3 effectively (Ozcelik et al., 2011).

Antitumor activity was evaluated on various species of the genus *Salvia*. Using *Agrobacterium tumefaciens* induced potato disk tumor assay, the highest antitumor activity of *S. tomentosa* (88%) and *S. verticillata* (86%) was shown with methanolic extracts (Karaka et al., 2012).

1.4.2.3. Antimicrobial activity of sage

Salvia species possess antimicrobial principles such as tannins, thujon, camphor and 1,8-cineole (eucalyptol) in the essential oils and extracts, which are well-known chemicals with their pronounced antimicrobial potentials and other aromatic compounds. These compounds protect the plant from microbial infection and deterioration (Shahidi & Wanasundara, 1992; Pattnaik et al., 1997; Xu et al., 2007; Tenore et al., 2011; Karaka et al., 2012).

The studies of antimicrobial activity of *Salvia* species oils or extracts are numerous. Investigation of Javidnia et al. (2008) on the antibacterial activity of the essential oils of *S. reuterana* and *S. multicaulis* showed that essential oil are more active against Gram-negative bacteria, while in investigations of Tepe et al. (2004) and Karatas & Ertekin (2010) showed that essential oil and extract of *S. multicaulis* represented higher activities against Gram-positive bacteria. The extracts and essential

oils of *S. multicaulis* and *S. reuterana* may be used in the treatment of diseases caused by microorganisms tested (Karamian et al., 2013). Other study was designed to examine the *in vitro* antimicrobial and antioxidant activities of the essential oil and various extracts of *S. tomentosa* and the essential oil was particularly found to possess strong antimicrobial activity, while non-polar extracts and subfractions showed moderate activities while polar extracts remained almost inactive (Tepe et al 2005; Karakas et al., 2012).

According to Deans and Ritchie (1987), who tested 50 essential oils against 25 genera of bacteria, *S. officinalis* essential oil (undiluted) was moderately effective. When tested against eight bacteria and five fungi commercial sage essential oil (probably issued from a mixture of *S. triloba* and of *S. lavandulifolia*) had almost no effect. Contrary to this result, by measuring the antifungal property of sage essential oil against *Alternaria alternata* and against *Aspergillus parasiticus*, a strong fungistatic effect was observed. Volatile oils showed much stronger fungistatic properties than tested extracts. The essential oil of *S. plebeia* is also reported to have fungitoxic potential, inhibiting the growth of storage fungus *Aspergillus flavus* (Bari evi & Bartol, 2000). Sage oil turned out to exhibit inhibitory effects on many of oral bacteria, such an obligate anaerobs and some capnophilic micro aerophiles (Shapiro et al., 1994).

Several *Salvia* extracts showed good antifungal and antibacterial activities against human and animal skin pathogenic microflora and, therefore, may have some value in the treatment of skin mycosis, or of unpleasant corporal odors (Sabri, 1989; Salah et al., 2006).

An acetone extract from *S. jaminiana*, containing the sterols campesterol, stigmasterol and sitosterol, as well as five diterpenoids, was found to markedly inhibit the growth of bacteria (Kabouche et al., 2005).

It was found that the dichloromethane fractions from acetone extracts of *S. sclarea* roots as well as four pure diterpenes isolated from hairy root cultures presented antimicrobial activity against Gram-positive bacteria. Additionally, a methanolic extract from *S. pisidica* showed high antimicrobial activity against Gram-positive bacteria and a moderate effect against Gram-negative species, while extract of *S. cabulica* showed moderate antifungal activity against *Trichophyton longifusus* and *Trichophyton longifusus* and *Microsporum canis* (Rashid et al., 2009). Methanolic extracts from *S.*

cryptantha and *S. multicaulis* also exhibited antimicrobial potential (Ibrahim, 2012). Ethanolic and methanolic extracts of *S. tomentosa* had stronger antimicrobial activity than erythromycin (positive control) against *Proteus vulgaris* and *E. cloacae*. Aqueous extract of *S. verticillata* was more effective than carbenicillin and erythromycin against *Klebsiella pneumoniae* (Karaka et al., 2012). An antimicrobial analysis of *S. chamelaeagnea* extracts showed strong antibacterial activity against both Gram-positive and Gram-negative bacteria (Kamatou et al, 2007b). The extracts obtained from aerial parts of *S. cabulica* possessed *in vitro* antibacterial activity (Rashid et al., 2009).

1.4.2.4. Effects of sage to the neurodegenerative disorders

According to Perry et al. (2000a) sage (*Salvia* spp.) was classified in the European herbal encyclopedias as enhancing-memory plant. The current memory-enhancing antidementia drugs are based on enhancing cholinergic activity by inhibiting cholinesterase.

The essential oil of Anatolian *S. chionantha* showed moderate inhibitory activity against both cholinesterases AChE, while the hexane extract only exhibited activity against BChE enzyme. *S. chrysophylla* showed high anticholinesterase activity against both AChE and BChE enzymes (Topcu & Kusman, 2014).

Many studies support cholinesterase inhibition by the monoterpenoids of the essential oil and the extracts from *S. officinalis* and *S. lavandulifolia* (enol et al., 2010). In clinical trials the ethanolic extracts and volatile oils of *S. officinalis* and *S. lavandulifolia* have been shown to be effective in mild scaled AD patients, even in low doses and anti-inflammatory activity of *S. lavandulifolia* oil and di- and triterpenoids from *S. fruticosa*, as well as diterpenoids with acetylcholinesterase inhibitory activity from a Chinese sage, *S. miltiorrhiza*. The dried root of *S. miltiorrhiza* has been used for the treatment of cerebrovascular disease and centralnervous system deterioration in old age for over 1000 years (Topcu & Kusman, 2014). Four diterpenes, dihydrotanshinone, cryptotanshinone, tanshinone I, and tanshinone IIA, were isolated from the acetone extract of the dried root of *S. miltiorrhiza* and it was concluded that these compounds contributed to the anticholinesterase activity of the plant (Ren et al., 2004; Orhan & Aslan, 2009). In the study of 56 extracts prepared by organic solvents from 14 Turkish

Salvia species for anticholinesterase activity, the most active extracts for AChE inhibition were observed to be a petroleum ether extract of *S. albimaculata* (89.4%) and a chloroform extract of *S. cyanescens* (80.2%) (Orhan et al., 2007).

The ethyl acetate and methanol extracts from 16 *Salvia* species were screened for their inhibitory activity against AChE, BuChE, lipoxygenase, and tyrosinase. The extracts exerted weak cholinesterase and tyrosinase inhibition, and remarkable inhibition against lipoxygenase (Orhan et al., 2012).

In another study aqueous and methanolic extracts of five plants including *S. officinalis* used in traditional medicine to treat different disorders of the central nervous system were tested for the AChE and BuChE inhibitory activity. The results showed that the lowest inhibition effect for the investigated enzymes was reported for sage extracts (Tali et al., 2014).

The dichloromethane, ethyl acetate, and methanol extracts prepared from 55 Turkish *Salvia* taxa were tested for their AChE inhibitory activity. Among 165 *Salvia* extracts screened, only the dichloromethane extract of *S. fruticosa* showed inhibition towards AChE (Enol et al., 2010). Cultivated *S. fruticosa* showed higher acetylcholinesterase activity compared to wild growing samples (Enol et al., 2011). All the extracts of the wild-grown species had a lower inhibitory effect than those of the cultivated species. Lebanese *S. triloba* showed weak activity in the acetylcholinesterase assay (Salah & Jäger, 2005).

1.4.3. The genus *Salvia* in Libyan flora

Libya occupies an area of about 1.7 million km², but most of which is Sahara desert. The most important areas for plant diversity are the coastal strip and mountains of the Mediterranean coastline which is 1900 km long, the longest of any African country bordering the Mediterranean coastline. The Libyan Mediterranean Coast is characterized by rich biodiversity. Several multi purposes plants are used for extraction of essential oils for cosmetic and pharmaceutical uses, honey-bees pasture, ornament, feed, food, etc. During collection missions for inventorying and collecting seeds of multi-purpose plant species, only 27 species with pharmaceutical benefits were

encountered. Among the medicinal plant Fabaceae and Lamiaceae families were more prevailing with 30% and 12% respectively (Louhaichi et al., 2011).

Libya comprises three main provinces: Tripolitania, Cyrenaica and Fezzan. Tripolitania extends over the north western corner of the country and Fezzan south of Tripolitania (Hegazy et al., 2011). Cyrenaica, the largest geographic region, covers the entire eastern half of the country. The region holds Al-Jabal Al-Akhdar (the Green Mountain) landscape with the richest vegetation and highest species diversity in the country. Tripolitania holds the Nafusah plateau. Fezzan is home to desert lands, including Sahara (Johnson, 1973). The geographical affinity of the flora is mainly East Mediterranean rather than neighboring regions of North Africa (Hegazy et al., 2011). Much of the indigenous vegetation of Libya consists of surviving remnants of a more favourable climatic age (Keith, 1965). The flora of Libya is not rich in the number of species, except the Green Mt. landscape comprising the richest vegetation and the highest number of species known in Libya (Boulos, 1972, 1997). This mountainous landscape confines about 50% of the endemic species in Libya (Qaiser & El- Gadi, 1984).

Five confirmed Important Plant Areas (IPAs) have been identified in Libya to date: Al Jabal Al Akhdar, Tawuorgha Sebka, Jabal Nafusah, Jabal Aweinat and Messak Mountain (Fig. 1). IPAs in Libya are found in the coastal, mountain and desert habitat types. Al Jabal Al Akhdar IPA (The Green Mountain) in the Cyrenaica region of north east Libya is the largest and most significant IPA in Libya. Libyan IPAs face a number of threats including development of tourism infrastructure, overgrazing of livestock, forest cutting for wood and charcoal and the spread of invasive alien species (Radford et al., 2011).

In total there are approximately 1750 plant species in Libya, 4% of which are Libyan endemics. Phytogeographically, the flora is predominantly Mediterranean, with strong links to the Eastern Mediterranean (Palestine to Greece), more so than with the rest of North Africa; particularly strong are the links to Crete. Approximately 50% of the Libyan endemics are endemic to Cyrenaica (Radford et al., 2011).

Genus *Salvia* is represented by ten species in Libya, *S. aegyptiaca*, *S. fruticosa*, *S. lanigera*, *S. verbenaca*, *S. chudaei*, *S. spinosa*, *S. viridis*, *S. officinalis*, *S. coccinea*, *S.*

splendens, out of which three (*S. fruticosa*, *S. officinalis*, *S. splendens*) are cultivated (Jafri & El-Gadi, 1985).

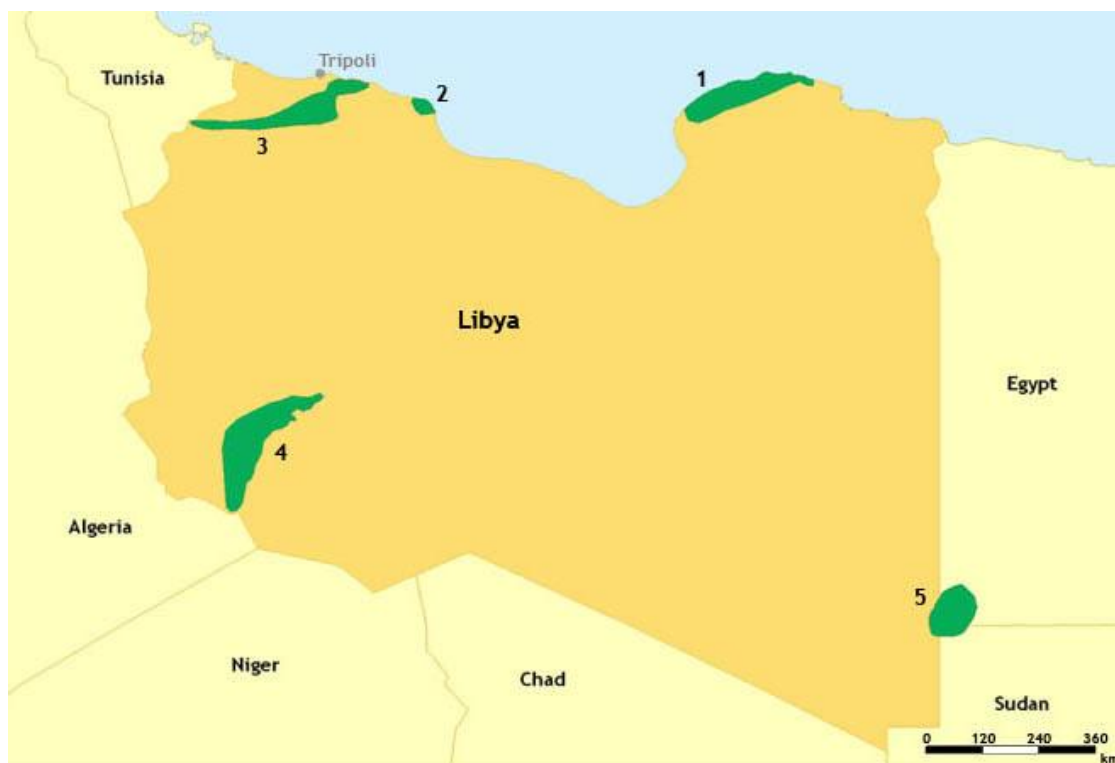


Figure 1. Map of Libya with Important Plant Areas (IPAs) according to Radford et al. (2011).

1.4.4. *Salvia aegyptiaca* L.

S. aegyptiaca (Egyptian sage) grows in various locations in the Arabian peninsula, Egypt, Palestine, Iran and Afghanistan, Canary Island, Morocco, Mauritania, Niger, Chad, Sudan, Ethiopia, Arabia, Pakistan and India. It occurs on dry rocky or gypsum soils of the sunny and well drained piedmont, hamada and deserted rangelands (Jafri & El- Gadi, 1985; Boulos, 2002).

The plant (Fig. 2) is pubescent low shrub, 15-40 cm tall; the stems are erect or ascending, very much branched with stiff and almost spinescent branches having retrorse eglandular hairs; the leaves are narrow linear-elliptic to oblong-linear, tapering to a short petiole, crenate to serrate, with short and long eglandular hairs; floral leaves are ovate-lanceolate, bracts are present; pedicels 5-6 mm; the calyx is ovate to tubular

campanulate, enlarging in fruit to 7 mm; the corolla is pale lilac-blue or lavender (rarely is white); staminal connective, filaments lower theca fertile. Nutlets are trigonous, smooth, black (Jafri & El-Gadi, 1985; Boulos, 2002). Stamen type of *S. aegyptiaca* is posterior thecae expressed and not fused to one another (Walker and Sytsma, 2007).



Figure 2. *Salvia aegyptiaca* L.

The whole plant contains flavonoids, tannins, sterols/triterpenes and coumarins, terpenes, pinene, cineol, borneol, ursolic acid. Several flavonoids and one phenolic acid have been isolated from the plant, including flavonoid aglycones luteolin and apigenin and its glycosides, rosmarinic acid and the bitter principle picrosalvin (Hussein, 1985; Salah et al., 2006).

The study of the flavonoids in *S. aegyptiaca* revealed the isolation and identification of luteolin 7-*O*-glycoside, apigenin and apigenin 7-*O*-glucoside.

Investigation of the lipid fraction resulted in the isolation and identification of α - and β -amyrins. The fatty acids were identified as undecanoic, dodecanoic, tridecanoic, myristic, pentadecanoic, palmitic, heptadecanoic, stearic, oleic and linoleic acid (El Missiry, 1994).

In the first report on the chemical composition of the essential oil analyzed by GC and GC–MS of *S. aegyptiaca* from Algeria the identified components constituted 91.1% of the oil. Thirty five components were identified. The main constituents were limonene (3.2%), bornyl acetate (8.5%), α -bourbonene (2.9%), α -caryophyllene (10.2%), α -gurjunene (7.6%), selina-4,11-diene (9.7%), germacrene D (7.0%), bicyclogermacrene (2.3%), α -cadinene (3.4%), germacrene B (4.8%), spatulenol (3.1%), α -caryophyllene oxide (6.2%) and α -cadinol (2.9%) (Mohammadi et al., 2014).

The outer surface of nutlets of *S. aegyptiaca* contains a pectinaceous mucilage layer that can imbibe a large amount of water when wetted, and it is commonly used in local folk medical practices and in cosmetic. The seeds are used as a demulcent for diarrhoea and for piles and the whole plant is used in gonorrhoea and haemorrhoids, eye diseases, and as an antispasmodic and stomachic. The plant is also used in case of nervous disorders, dizziness and trembling (Jafri & El-Gadi, 1985; Al-Yousuf et al., 2002; Gorai et al., 2011) occurring in Parkinson's disease, Huntington's chorea and other diseases. Huntington's chorea involves the loss of some cholinergic neurons. Because of the plant has a cholinergic (muscarinic) activity, it can be used in the treatment of such diseases (Al-Yahya et al., 1990). *S. aegyptiaca* mixed with *Trigonella foenum graecum* seeds showed decrease in some parameters of liver in mice. In kidney function, the administration of mixed extracts showed decrease in urea, while non-significantly changed in uric acid and creatinine, indicates that it improve the liver and kidney function (Tohamy et al., 2012).

The Egyptian sage essential oil, composed mostly of thujone (41.5%) and of limonene (14.7%), shows antibacterial activity against Gram-positive *Sarcina* spp., *Staphylococcus aureus*, *Bacillus subtilis* and against yeast *Saccharomyces cerevisiae* (Bari evi & Bartol, 2000).

The examination of antioxidant of methanolic extracts of *S. aegyptiaca* growing wild in Tunisia, showed high antioxidant activity (Ben Farhat et al., 2013a). The methanolic extracts of three *Salvia* species from Tunisia: *S. aegyptiaca*, *S. argentea* and *S. verbenaca* ssp. *clandestina*, inhibited the growth of dermatophytes and of bacteria responsible for unpleasant odors to varying degrees; the pathogenic yeasts *Candida albicans* and *Cryptococcus neoformans*, the filamentous fungi *Aspergillus fumigatus* and selected dog otitis bacteria were all resistant to each of the extracts. The petrol extract of *S. aegyptiaca* roots showed potent inhibitory activity against *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Staphylococcus aureus* and *Candida albicans* (Salah et al., 2006).

The study of Baka et al. (2014) was carried out to evaluate the antifungal potential of water and ethanol extracts from aerial parts of five wild medicinal plants, including *S. aegyptiaca*, collected from Sinai Peninsula, Egypt, and the extracts were tested *in vitro* and *in vivo* against *Phytophthora infestans*, the causal agent of late blight

disease of tomato. Ethanol extracts were more effective on the pathogen than water extracts at all concentrations used. Water and ethanol extracts of all plants tested reduced mycelial growth and inhibited spore germination of the pathogen with varying degrees. Water and ethanolic extracts also reduced the disease infection with pathogen comparing with control in detached leaves technique (Baka et al., 2014).

Crude aqueous extracts of eleven *Salvia* spp. grown and sold in Jordan (Lamiaceae) including *S. aegyptiaca*, tested for general cytotoxicity against a panel of colorectal cancer cell lines (HT29, HCT116, SW620 and Caco2), none of the tested extracts were found to possess cisplatin- or doxorobocin-like antiproliferative capacities in comparison to non induced basal incubations (Kasabri et al., 2014).

The extract of *S. aegyptiaca* caused an increase in acetylcholine content and this increase may be due to the decrease in the activity of acetylcholine esterase enzyme which play an important role in the treatment of Alzheimer's disease (Abdel Kader, 2004).

1.4.5. *Salvia fruticosa* Mill.

The Greek sage, *S. fruticosa*, was known until recently as *S. triloba* L. The specific epithet "*triloba*" (= three lobes) describes a species with three-lobed leaf blades. However, the Greek sage plants are very variable, having either entire or three-lobed leaves. This variation often causes confusion in the taxonomic identification of the wild growing plants (Sivropoulou et al., 1997; Pitarokili et al., 2003; Karousou et al., 2000).

S. fruticosa is an endemic species of the Eastern Mediterranean region ranging from Southern Italy and Sicily to Palestine its total native range extends from Cyrenaica, through the Southern part of the Balkan Peninsula to West Syria. Furthermore, it is found as a naturalized plant in parts of the Western Mediterranean region in Malta, Spain and Portugal. The species was probably introduced for cultivation in the Iberian Peninsula by the ancient Phoenicians and Greeks; remnants of these cultivations are found today in several coastal areas (Jafri & El-Gadi, 1985; Karousou & Kokkini, 1997; Karousou et al., 2000). It is found in dry rocky limestone

soils or the edges of pine forests, riverbeds, and roadsides, growing in an altitude from 100 to 800 m (Gali-Muhtasib, 2006).

The plant (Fig. 3) is subshrub, 50-120 mm tall; the stem is much branched below with appressed white glandular tomentum above and englandular tomentum below; the leaves are simple with 1-2 pairs of ovate lateral segments and large oblong - elliptical terminal segment; the calyx is 5-8 mm, campanulate, toothed; often purple glandular or englandular hairy; the corolla is lilac, pink or rarely white and 10 -25 mm long (Jafri & El-Gadi, 1985).



Figure 3. *Salvia fruticosa* Mill.

Often referred to as Greek sage, and is one of the most commercially exploited sage plants (Skoula et al., 1999, 2000). The leaves are covered with numerous non-glandular unbranched trichomes, and peltate, capitate and digitiform glandular trichomes (Al Sheef et al., 2013).

The leaves give the essential oil because of the glandular hairs present on leaves. The oil yield is supreme in the post-flowering period, when the weather is dry in the Mediterranean region (Bellomaria et al., 1992). The quality and quantity of the *S. fruticosa* oil differs by geographic region and by the part of the plant used (Bellomaria et al., 1992; Arnold & Bellomaria, 1993). The content of β -thujone and borneol is higher in the full-blooming period (Gali-Muhtasib, 2006). Due to the essential oil *S. fruticosa* has a camphor-like scent and its aromatic taste is very bitter (Gali-Muhtasib, 2006). The composition of essential oil isolated from *S. fruticosa* wild growing in Libya, was analyzed by GC and GC-MS. Forty-five compounds were identified. The essential oil contained 1.8-cineole (49.34%), camphor (7.53%), -pinene (7.38%),

myrcene (7.38%), -pinene (5.15%), -caryophyllene (4.13%) and -terpineol (3.25%) (Giweli et al., 2013b).

S. fruticosa is considered economically as one of the most important *Salvia* species cultivated as culinary and medicinal herbs. Cultivation of medicinal plants for the purpose of extraction of active constituents may face certain limitations such as climate, season, water availability, diseases, pests, and scarcity of naturally growing plant (Arikat et al., 2004). The plant is widely used in folk medicine, cosmetics, perfumes and the flavoring of food products (Sivropoulou et al., 1997). The leaves of the plant are boiled and used as a herbal tea for treatment of bloating, gastric disorders, abdominal pain, oral infections, gum and tooth pains, headaches, cough, influenza and cold, feminine sterility, skin disorders, nervous conditions, asthma, rheumatism and diabetes, and the extracts have been shown to possess antioxidant, anti-inflammatory, anticancer and antimicrobial activities (Ibrahim and Aqel, 2010).

S. fruticosa has been used not only in therapy but also as a spice to flavor many foods. The dried leaves and essential oil of *S. fruticosa* and other species of sage are employed as seasonings for sausages, ground meats, fish, honey, salads, soups, and stews. Sage is used along with rosemary and thyme to preserve a number of foods, including meats and cheeses. It is also used as a flavoring and antioxidant in cheeses, pickles, vegetables, processed foods, and beverages. The oil of *S. fruticosa* is used to extend the keeping quality of fats and meats (Leung & Foster, 1995). In the Lebanese traditional medicine, the oil and water extract of *S. fruticosa* are used in several towns and villages, the water extract of the plant is either internally used as infusions or is inhaled in steam baths or applied externally to heal fractured bones (Karim & Al-Quraan, 1986). The herbalists of Lebanon, Syria, and Jordan consider this species as a panacea that is a universal drug, as it is sold in the market. The plant is used in Jordan for the treatment of ulcer pains and indigestion (Karim & Al-Quran, 1986). In Turkey, the same species is used for kidney and gall bladder stones and for the relief from colds, coughs, and influenza (Baser et al., 1986). It has been reported for *S. fruticosa* to act as a bactericide and it is used in mouth washes and gargles. The plant is also used as an agitator and antisecretory agent, and as salvin (Murakami et al., 1990).

Bioactive carnosic acid was identified as a new class of lipid absorption inhibitor from *S. fruticosa* with an anti-pancreatic lipase. Besides it could effectively reduce the

gain of body weight and the accumulation of epididymal fat weight in high fat diet-fed mice after 14 days. Thus, it was closely proven for its safe and effective clinical antihyperlipidemic effects in hypercholesterolemic and/or hypertriglyceridemic patients (Kasabri et al. 2014).

Because the sage plant is widely used in traditional medicine, any wrong use could cause various complications, due to the established toxicity of the essential oil of this plant. The herb should be avoided during pregnancy because it is a uterine stimulant. Patients suffering from epilepsy are not supposed to use sage. Women who are breast-feeding should only use sage in medicinal amounts if they want to dry up the flow of milk. There has been no reported toxicity of the water extract of this plant (Gali-Muhtasib, 2006).

Bozan et al. (2002) investigated antioxidant properties of several *Salvia* species, and found that *S. fruticosa* has moderate antioxidant activity in comparison to the other species. They also estimated that *S. fruticosa* provided almost 15% inhibitions of DPPH radicals. Antioxidant activity of Libyan *S. fruticosa* oil was analyzed using the DPPH free radical scavenging method and low antioxidant activity was found ($IC_{50} = 15.53$ mg/ml), compared to BHA (Giweli et al., 2013b).

The hydroalcoholic extracts of three traditionally used Lamiaceae species for memory-enhancement; *S. triloba*, *Melissa officinalis*, and *Teucrium polium*, were assessed for their scopolamine-induced antiamnesic and antioxidant activities, and *S. triloba* was the most effective in antiamnesic experiment (Orhan & Aslan, 2009).

In the study of inhibitory potentials against acetylcholinesterase and antioxidant capacity which comprised 165 extracts of 55 Turkish *Salvia* taxa, most of the extracts showed remarkable scavenging effect against DPPH radical (enol et al., 2010). enol et al. (2011) examined the antioxidant activities of different extracts of three wild-growing and one cultivated sample of *S. fruticosa*. All extracts of cultivated *S. fruticosa* showed better scavenging activity in DPPH and FRAP tests than the wild samples.

Dincer et al. (2012) compared wild and cultivated *S. fruticosa* in two seasons using different methods, and obtained higher activity for samples in the second harvesting year.

Sage oil has been shown to be effective against both Gram-positive and Gram-negative bacteria including *Escherichia coli* and *Salmonella* species, and against filamentous fungi (Valnet, 1986) and yeast such as *Candida albicans*. Such antimicrobial properties have been shown to reduce plaque growth, inhibit gingival inflammation and exert positive effects on caries prophylaxis (Willershausen et al., 1991). The essential oil of Libyan *S. fruticosa* was screened for its antimicrobial activity against eight bacteria (four Gram-negative and four Gram-positive) and eight fungi, and showed minimal inhibitory concentration (MIC) at 0.125-1.5 mg/ml and bactericidal (MBC) at 0.5-2.0 mg/ml. In addition, it exhibited fungistatic (MIC) at 0.125-1.0 mg/ml and fungicidal effect (MFC) at 0.125-1.5 mg/ml (Giweli et al., 2013b).

The essential oil of *S. fruticosa* collected in Greece was investigated for the antifungal activity. The main components, 1,8-cineole and camphor, were evaluated *in vitro* against five phytopathogenic fungi. The oils exhibited high antifungal activities against certain soil borne pathogens (Pitarokili et al., 2003).

Gonaid et al. (2012) analyzed the ethanol extract obtained from Libyan *S. fruticosa* and their results showed a moderately high activity against *E. coli* and *Pseudomonas aeruginosa*. *S. fruticosa* possess higher inhibitory effects against *Bacillus subtilis* and a moderate effect on *Staphylococcus aureus*. In the analysis of antimicrobial activities of methanol extracts of Turkish medicinal plants, Askun et al. (2009) found that *S. fruticosa* displayed high activity on Gram-negative bacteria, with MIC 640 µg/ml against *S. typhimurium* and *E. aerogenes*, which could be probably attributed to the significant amount of carvacrol in the extract.

The essential oils of *Origanum vulgare* subsp. *hirtum*, *Mentha spicata*, *Lavandula angustifolia*, and *S. fruticosa* exhibited antifungal properties against the human pathogens *Malassezia furfur*, *Trichophyton rubrum*, and *Trichosporon beigelii* (Adam et al., 1998).

The essential oils of *S. officinalis* and *S. triloba* cultivated in South Brazil exhibited remarkable bacteriostatic and bactericidal activities against *Bacillus cereus*, *Bacillus megatherium*, *Bacillus subtilis*, *Aeromonas hydrophila*, *Aeromonas sobria*, and *Klebsiella oxytoca*. Moreover, the essential oil of *S. triloba* efficiently inhibited the growth of *Staphylococcus aureus*. *S. aureus* and *A. hydrophila* growth were drastically reduced (Delamare et al., 2007).

The anti-inflammatory activity of Libyan *S. fruticosa* extracts was evaluated and results demonstrated significant anti-inflammatory properties of the test extracts. The anti-inflammatory activity of *S. fruticosa* could be assumed to be related to high levels of phenolic compounds, e.g., flavonoids, present in this plants. *S. fruticosa* could be a potential source for the discovery and development of new anti-inflammatory drugs (Elmezogi et al., 2012).

Xavier et al. (2009) examined the antiproliferative and proapoptotic effect of water extracts of *S. fruticosa*, *S. officinalis* and rosmarinic acid on human colorectal carcinoma cell lines, and obtained the apoptosis in both cell lines. *S. fruticosa* has cytotoxic effect against some cancer cell lines (Gorai et al., 2011).

The essential oil of *S. fructicosa* and the three main components exhibited cytotoxic activity against African Green Monkey kidney (Vero) cells and high levels of virucidal activity against herpes simplex virus, a ubiquitous humanvirus. Thujone was the most biologically active compound of the oil, since it exhibited high levels of antibacterial, cytotoxic, and antiviral activities (Sivropoulou et al., 1997). The essential oil extracted from the Lebanese sage plant has potent chemopreventive effects against skin tumor promotion in mice (Gali-Muhtasib, 2006). *S. fruticosa* was also reported to possess antiproliferative properties against colon cancer cell lines (Elmezogi et al., 2013).

S. fruticosa was among five *Salvia* collected from different Greek islands which were evaluated for cytotoxic activities against brine shrimps and four human cancer cell lines, human colon adenocarcinoma (HCA), HepG2, MCF-7, and human pancreatic carcinoma (HPC).The obtained results showed high activity of *S. fruticosa* against human cancer cell lines (Badisa et al., 2005).

Kaileh et al. (2007) tested the organic extracts of 24 selected Palestinian medicinal plant species for their potential cytotoxic effect on the murine fibrosarcoma L929sA cells, and on the human breast cancer cells MDA-MB231 and MCF7. Among examined plant extracts using MTT colorimetric assay, *S. fruticosa* displayed a remarkable activity. The essential oil was found to be cytotoxic at concentrations that range between 180 µg/ml in L929sA mouse fibrosarcoma cells to 290 µg/ml in MDA-MB 231 metastatic human breast carcinoma cells, indicating differential sensitivities of various cancer cell lines to the oil.

Abu-Dahab et al. (2012) investigated ethanol extracts from nine *Salvia* species grown in Jordan for their antiproliferative activity using a panel of breast cancer cell. The results indicated that the extracts of three *Salvia* species, among them *S. fruticosa*, exhibit selective antiproliferative activity against estrogen positive cell lines and with minimum toxicity against normal human periodontal fibroblasts. *S. fruticosa* showed an inhibitory concentration of 50% of cells against four cell lines.

In the study of Alimpi et al. (2015) of cytotoxic effects of water extracts of five *Salvia* species, *S. fruticosa* extract exhibited cytotoxic effect on K562 cells (IC_{50} 200-400 $\mu\text{g/ml}$). Sivropoulou et al (1997) report on the high virucidal activity of essential oil of *S. triloba* against HSV. Water and alcoholic extracts of *S. triloba* were active against influenza, herpes simplex and vaccinia viruses. This preparation was officially approved for clinical use in Bulgaria (Bari et al. & Bartol, 2000). In Anatolian folk medicine, the leaves of *S. fruticosa* are used as infusion (1-5%) for simple disorders, and it is one of the plants used for memory enhancing and is neuroprotective in Anatolia (Topcu & Kusman, 2014). Aqueous, ethanol and ethyl acetate extracts of seven Lebanese plants that are used traditionally for neurological disorders as Alzheimer's disease, epilepsy and affective disorders as depression were tested for inhibition of acetylcholinesterase. *S. triloba* extract exhibited weak activity in the acetylcholinesterase assay (Salah & Jäger, 2005).

The hydroalcoholic extracts of three traditionally used Lamiaceae species for memory-enhancement; *S. triloba* L., *Melissa officinalis* L., and *Teucrium polium* L., were assessed for their anticholinesterase and activities the extracts showed similar inhibitions against acetylcholinesterase (Orhan & Aslan, 2009). The dichloromethane, ethyl acetate, and methanol extracts prepared from 55 *Salvia* taxa were tested for their AChE inhibitory activity 165 *Salvia* extracts screened, only the dichloromethane extract of *S. fruticosa* showed inhibition towards AChE at 100 $\mu\text{g/ml}$ having 51.07% of inhibition, while only the dichloromethane and ethyl acetate extracts of *Salvia cilicica* had a notable iron-chelating capacity at 100 $\mu\text{g/ml}$ having 54.71% of chelating capacity (Enol et al., 2010). Enol et al. (2011) were tested the inhibitory activity of dichloromethane, ethyl acetate and ethanol extracts of three wild-grown and one cultivated *S. fruticosa* samples against acetylcholinesterase and butyrylcholinesterase enzymes. Among the extracts, the most active ones were the essential oil,

dichloromethane and ethyl acetate extracts of cultivated sample. All the extracts of the wild-grown species had a lower inhibitory effect than those of the cultivated species.

1.4.6. *Salvia lanigera* Poir.

S. lanigera (wooly sage) characterized by the hairs that cover all parts of the plant, grows in sandy loam and chalky sandstone soils (Flamini et al., 2007). The plant is distributed in North Africa (coastal Mediterranean region from Morocco to Egypt), Cyprus, Iraq, Syria, Lebanon, Palestine, Iran and Jordan (Jafri & El-Gadi, 1985; Boulos, 2002).

It is strongly aromatic perennial herb (Fig. 4), 20-40 cm tall with thick woody rootstock; the stem is erect, simple, or branched with short and long eglandular hairs; the leaves are deeply pinnatisect, lobes irregular, linear with eglandular hairs and oil globules; the floral leaves are broadly ovate. Bracts are absent; the calyx is tubular covered with short capitate glandular hairs; the corolla is deep violet or dark purple; the stamens with sterile lower theca; the nutlets are smooth, black, oblong – trigonus (Jafri & El-Gadi, 1985; Boulos, 2002).



Figure 4. *Salvia lanigera* Poir.

The leaves are used as an aromatic tea for a variety of abdominal troubles (Al-Howiriny, 2003). It is used by Bedouins as a condiment for tea (Flamini et al., 2007). *S. lanigera* essential oil could be considered as a natural alternative to traditional food preservatives and be used to enhance food safety and shelf life.

The plants originated from different countries were studied for the chemical composition and characteristics, as well as for antioxidant, antimicrobial and cytotoxic properties.

Several papers report only non-volatile compounds of the plant, such as sterols, triterpenes and tannins, diterpene endowed with antimicrobial activity named lanigerol which showed activity against Gram-positive bacteria (El-Lakany et al. 1996; Hawas & El-Ansari, 2006; Flamini et al., 2007; Tenore et al., 2011).

The composition of the essential oils of three Jordanian *Salvia* species, *S. lanigera* and *S. spinosa* from a desert climate, and *S. syriaca* from a Mediterranean habitat, were studied. All examined oils were found to be rich in monoterpene derivatives (68-73%), *S. lanigera* and *S. spinosa* showed a very high content of thymol, 54.9% and 68.9%, respectively (Flamini et al., 2007). The essential oil composition of *S. langiera* growing wild in Egyptian desert was characterized by a high percentage of oxygenated sesquiterpene, with major constituent spathulenol (Ibrahim et al., 2013).

The chloroform extract of the roots of the Egyptian *S. lanigera* afforded two new orthoquinones, lanigerone and salvigerone together with two known diterpenoids, arucadiol and pisiferol (Lee et al., 1998). Twelve flavonoid compounds were isolated and identified as five flavones glycosides, two aglycones and five methylated flavones (Hawas & El-Ansari, 2006).

The n-butanol extract of *S. lanigera* contained two known diterpenes - horminone and 7-*O*-ethylhorminone, three new diterpenes - salviatane B, salvianol A and salviaclerodan A, ursolic and oleanolic acid, -sitosterol, salvigenin and apigenin, and one new caffeic acid dimmer; 3, 3 - dehydrodicaffeic acid (Shaheen et al., 2011).

The antioxidant activity of the essential oil of aerial parts of *S. lanigera* from Cyprus, in DPPH and FRAP assays was higher than that of all of standards used at the same dose (Tenore et al., 2011). Results of the investigation of the antioxidant activity of the different extracts (methanol, acetone and n-butanol) of *S. lanigera* and *S. splendens* showed the highest activity in DPPH quenching (Shaheen et al., 2011).

Antimicrobial activity of the essential oil of *S. lanigera* collected in Cyprus evaluated using the microdilution method, resulted in higher activity against Gram-positive bacteria than the other strains tested (Tenore et al., 2011). The study of antimicrobial activity of the essential oil of *S. lanigera* growing wild in Egyptian desert

showed that the essential oil was more active against *Saccharomyces cerevisiae*, intermediate sensitive with *Bacillus megaterium*, while it was the least effective against *Bacillus subtilis* (Ibrahim et al., 2013). By inhibiting the growth of almost all the human pathogenic and/or food spoilage bacteria, moulds and the yeast tested, *S. lanigera* essential oil exerted a broad antimicrobial spectrum (Ibrahim et al., 2013). In the study of Fiore et al. (2006) methanol extracts of six Jordanian *Salvia* species were screened in human cancer cell lines from different histological types. The extract of *S. lanigera* did not show a dose response effect.

2. AIMS OF THE STUDY

Based on the above described background related to micromorphology and biological activities of *Salvia* species, **the aim of this study is to investigate the micromorphological and cytological characteristics of the leaf trichomes, and biological activities of Libyan *S. aegyptiaca*, *S. fruticosa* and *S. lanigera*.**

For that purpose, following specific assignments are given:

1. micromorphological and histo-cytological characterization of leaf trichomes
2. chemical analysis of dichloromethane, ethyl acetate, methanol, acetone, chloroform, ethanol and water extracts prepared from Libyan species
3. investigation of possible antioxidant, antimicrobial, cytotoxic and antineurodegenerative effects of the extracts

To these aims,

1. the light and electron microscopes were used
2. HPLC analysis of extracts was performed
3. Four antioxidant assays were applied, microdilution method for antimicrobial activity, MTT cell viability assay for cytotoxicity, and acetylcholinesterase and tyrosinase inhibitory activities of the extracts were tested.

It is expected to obtain results about leaf secretory structures and data that could enable evaluation of biological activities of analyzed plants and determination of their possible pharmacological application.

3. MATERIAL AND METHODS

3.1. Plant material

Aerial parts of the *Salvia aegyptiaca* L. plants cultivated in the glasshouse of the Institute of Biological Research “Siniša Stankovi ” were used for microscopical and chemical analyses. The microscopic analysis was performed on the fresh leaves, while the material for antioxidant activity was collected in January 2014, air-dried and kept in shadow at room temperature for further processing. The plants were propagated from seeds obtained from the Seed bank in Tripoli, which were collected in Om-Jersan on Western Mountain in Libya. Voucher samples were stored in the Herbarium of the Institute of Botany and Botanical Garden “Jevremovac”, Faculty of Biology, University of Belgrade (BEOU: 16881).

Leaves of the *Salvia fruticosa* Mill., collected from plants growing in pots, were used for microscopical analysis. The plants were propagated from seeds obtained from the Seed bank in Tripoli. For the extraction procedure aerial parts of the *S. fruticosa* were collected at the flowering stage from natural populations in Biadda, which is located on the Green Mountain in eastern Libya, in March 2010. Plant material was dried and kept in shadow at room temperature for further processing. Voucher samples are deposited in the Herbarium of the Institute of Botany and Botanical Garden “Jevremovac”, Faculty of Biology, University of Belgrade (BEOU: 16702).

Leaves of the *Salvia lanigera* Poir., collected from plants growing in pots, were used for microscopical analysis. The plants were propagated from seeds obtained from the Seed bank in Tripoli. For the extraction procedure aerial parts of *S. lanigera* were collected during flowering period from the natural populations in Zintan (Libya), which is located on the top of Western Mt. (Aljabel Algarbi) at altitude about 700 m a.s.l. in March 2010. Plant material was dried and kept in shadow at room temperature for further processing. Voucher samples were stored in the Herbarium of Institute of Botany and Botanical Garden “Jevremovac”, Faculty of Biology, University of Belgrade (BEOU: 16880).

3.2. Microscopical analyses

3.2.1. Light Microscopy

For light microscopy (LM) small segments (5 mm²) cut from the leaf near midrib, were fixed with 3% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.2, for 24 h at 4°C. Subsequently, material was washed in sodium phosphate buffer, 3 times over 2 h, post-fixed in 1% osmium tetroxide in same buffer, for 24 h at 4°C. The fixed material was washed with distilled water, dehydrated in a graded ethanol series and embedded in Araldite resin CY 212 (Agar Scientific Ltd. England). Semithin cross sections (1–1.5 µm thick) were cut on a LKB III ultramicrotome and stained with 0.1% methylene blue in 1% borax. Sections were photographed under a Zeiss Axiovert microscope (Carl Zeiss GmbH, Göttingen, Germany).

3.2.2. Histochemical analyses

Histochemical characterization of the secreted material was performed on fresh free-hand sections of leaves using the following histochemical tests: Sudan IV and Sudan black B for total lipids (Jensen, 1962); osmium tetroxide (OsO₄) for unsaturated lipids (Jensen, 1962; Gahan, 1984); Nile blue A for neutral and acidic lipids (Cain, 1947); Nadi reagent for terpenes (David and Carde, 1964); ferric trichloride for phenolic compounds (Gahan, 1984); Toluidine blue O for tannins (Baker, 1966); periodic acid-Schiff (PAS) reagent for polysaccharides (Jensen, 1962); Ruthenium Red for pectins (Johansen, 1940); Sudan red 7B/hematoxylin (Liebman, 1942) for lipophilic and hydrophilic secretions, simultaneously.

Fluorescence microscopy examination under ultraviolet light was utilized for phenolic substances detection (Mabry et al., 1970). For flavonoids detection induction of fluorescence with the fluorochrome aluminium trichloride under ultra violet light was employed (Charrière-Ladreix, 1973).

Standard control procedures were carried out simultaneously. For the control, free-hand sections were kept in a solution of methanol, chloroform, water, and HCl, in the relation 66: 33: 4: 1, for 3 hours, before staining.

All sections were mounted in glycerine under a coverslip, examined and photographed using Zeiss Axiovert microscope (Carl Zeiss GmbH, Göttingen, Germany).

3.2.3. Transmission electron microscopy

For transmission electron microscopy (TEM) ultrathin sections were stained with uranyl acetate and lead citrate and examined with a MORGAGNI 268 (FEI Company, Eindhoven, The Netherlands) transmission electron microscope operated at 100 kV.

3.2.4. Scanning electron microscopy

For scanning electron microscopy (SEM) segments of fully developed fresh leaves were coated with a thin layer of gold and paladium in BAL-TEC SCD 005 sputter coater. Both adaxial and abaxial surfaces were examined with a JEOL JSM-6390 LV at an acceleration voltage of 13 kV.

3.3. Chemicals

Methanol, ethanol, distilled water, glacial acetic acid, hydrochloric acid, dichloromethane, acetone, chloroform and ethyl acetate were purchased from Zorka Pharma, Šabac (Serbia). Gallic acid, quercetin, ascorbic acid, 2(3)-t-butyl-4-hydroxyanisole (BHA), 3,5-di-tert-butyl-4 hydroxytoluene (BHT) 2,2-dyphenyl-1-picrylhydrazyl (DPPH), 2,2 -azino-bis(3-ethylbenzothiazoline-6-sulfonic acid diammonium salt (ABTS), 2,4,6-tripyridyl-s-triazine (TPTZ), potassium acetate ($C_2H_3KO_2$), potassium-persulfate ($K_2S_2O_8$), dimethylsulfoxide (DMSO), sodium carbonate anhydrous (Na_2CO_3), aluminum nitrate nonahydrate ($Al(NO_3)_3 \cdot 9H_2O$), sodium acetate ($C_2H_3NaO_2$), iron (III) chloride ($FeCl_3$), iron (II)-sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$), beta-carotene, Folin-Ciocalteu phenol reagent, sodium phosphate monobasic (NaH_2PO_4), sodium phosphate dibasic (Na_2HPO_4), 5,5 -dithiobis(2-nitrobenzoic acid) (DTNB), acetylcholinesterase from *Electrophorus electricus* (electric eel) (AChE), acetylcholine iodide, galanthamine hydrobromide from *Lycoris sp.*, kojic

acid, tyrosinase from mushroom and 3,4-dihydroxy-L-phenylalanine (L-DOPA) were purchased from Sigma Chemicals Co. (USA) while Tween 40 and linoleic acid were purchased from Acros Organics (Belgium). The phenolic compounds standards (gallic acid, caffeic acid, rosmarinic acid, apigenin, luteolin, genkwanin, quercetin, hyperoside, rutin, coumarin), were purchased from Merck (Germany).

3.4. Preparation of plant extracts

Extracts were prepared of whole aerial plant parts using two parallel extraction procedures. Dry plant material was grounded in small pieces (2-6 mm) in the cylindrical crusher. First, 10 g of plant material was exposed to successive extraction by 100 ml of dichloromethane, ethyl acetate and methanol (Orhan et al., 2013). Second, portions of 10 g of plant material were individually extracted by 100 ml of solvent (ethanol, distilled water, chloroform and acetone). In both cases, extraction was performed by classic maceration during 24h at room temperature (10% w/v). The mixture was exposed to ultrasound 1h before and after 24h-maceration. Subsequently, extracts were filtered through a paper filter (Whatman No.1) and evaporated under reduced pressure by the rotary evaporator (Buchi rotavapor R-114). After evaporation of the solvent, the obtained crude extracts were stored in the fridge at +4 °C for further experiments.

3.5. HPLC analysis of extracts

The HPLC analyses of phenolic components were performed using the Agilent 1100 Series and UV-DAD (UV-Diode Array Detector) according to procedure of Veit et al. (1995). The column was an Agilent Eclipse XDB-C18 (5 μ m, 150*4.6 mm, 80 Å). Injection volume was 15 μ L of extracts in concentration of 10 mg/ml. Peak detection in UV region at 350 nm was used. The mobile phase was composed of solvent (A) 0.15% (w/v) phosphoric acid in water:methanol mixture (77:23, v/v, pH 2) and solvent (B) methanol as follows: isocratic 0-3.6 min 100% A; 3.6-24 min 80.5 % A; 24-30 min isocratic; linear 30-60 min 51.8 % A; 60-67.2 min 100% B. The flow rate of mobile phase was set to the 1 cm³/min and temperature to 15°C. Phenolic compounds in the samples were identified by comparing their retention times and spectra with retention

time and spectrum of standards for each component. Quantification was done using calibration curves of referent substances. Glycosides are expressed as total glycosides of corresponding aglycone.

3.6. Determination of total phenolic content

The total phenolic content of extracts was measured using spectrophotometric method (Singleton & Rossi, 1965). The reaction mixture was prepared by mixing 0.2 ml of extract (1 mg/ml) and 1 ml of 10% Folin-Ciocalteu reagents, and after six minutes 0.8 ml of 7.5% Na_2CO_3 was added. Blank was prepared to contain distilled water instead of extract. Absorbance was recorded at 740 nm after two hours incubation at room temperature using JENWAY 6305UV/Vis spectrophotometer. The same procedure was repeated for standard solution of water solution of gallic acid in order to construct calibration curve. Phenolic content in samples was calculated from standard curve equation and expressed as gallic acid equivalents (mg GAE/g dry extract) averaged from three measurements.

3.7. Determination of flavonoid concentration

Flavonoid concentrations of samples were measured spectrophotometrically according to procedure of Park et al. (1997). The reaction mixture was prepared by mixing 1 ml of extract (1 mg/ml), 4.1 ml of 80% ethanol, 0.1 ml of 10 % $\text{Al}(\text{NO}_3)_3 \cdot 9 \text{H}_2\text{O}$, and 0.1 ml 1M dillution CH_3COOK . Blank was prepared to contain 96% ethanol instead of extract. After 40 min of incubation at room temperature, absorbance was measured at 415 nm using JENWAY 6305UV/Vis spectrophotometer. The same procedure was repeated for 96% ethanol solution of standard antioxidant quercetin in order to construct calibration curve. Concentration of flavonoids in samples was calculated from standard curve equation and expressed as quercetin equivalents (mg QE/g dry extract) averaged from three measurements.

3.8. Evaluation of antioxidant activity

3.8.1. DPPH assay

For evaluation of antioxidant activity of extracts, 2,2-dyphenyl-1-picrylhydrazyl (DPPH) free radical scavenging method (Blois, 1958) with slight modifications was used. This assay is spectrophotometric and uses stable DPPH radical as reagent. Stock extract solution (10 mg/ml) was mixed with methanolic solution of DPPH (40 µg/ml) to adjust the final volume of reaction mixture (2000 µl) of the test tube. Methanol was used as a blank, while methanol with DPPH solution was used as a control. BHA, BHT and ascorbic acid were used as standards. All measurements were performed in triplicate. Absorbance of the reaction mixture was measured after 30 minutes in the dark at room temperature at 517 nm using the JENWAY 6305UV/Vis spectrophotometer. The inhibition of DPPH radical at 517 nm was calculated using equation:

$$\text{Inhibition of DPPH radical (\%)} = [(A_c - A_s)/A_c] * 100\%,$$

where A_c is the absorbance of control (without test sample) and A_s is the absorbance of the test, samples at different concentrations. Concentrations of the test samples and standard antioxidants providing 50% inhibition of DPPH radicals, IC_{50} values (µg/ml), were calculated from DPPH absorption curve at 517 nm.

3.8.2. ABTS assay

In this test, antioxidant activity of samples was tracked spectrophotometrically, using change of ABTS solution color in presence of antioxidants. ABTS assay is performed according to procedure Miller et al. (1993) with some modifications. Stock $ABTS^+$ solution (7 mM) was prepared 12-16 hours before experiment in 2.46 mM potassium-persulfate and stored in the dark at room temperature. Stock $ABTS^+$ solution was diluted by distilled water to obtain an absorbance of working solution 0.700 ± 0.020 at 734 nm. 50 µl of extract (1 mg/ml) and/or standard solutions (0.1 mg/ml) were mixed with 2 ml of working $ABTS^+$ solution and incubated for 30 min at 30 °C. Absorbance was recorded at 734 nm using JENWAY 6305UV/Vis spectrophotometer. Distilled water was used as blank. BHA and BHT dissolved in methanol in concentration 0.1 mg/ml were used as standards. ABTS activity was calculated from ascorbic acid

calibration curve (0-2 mg/l) and expressed as ascorbic acid equivalents per gram of dry extract (mg AAE/g). All experimental measurements were carried out three times were presented as average \pm standard deviation.

3.8.3. FRAP assay

Ferric-reducing ability of plasma (FRAP) assay evaluates total antioxidant power of the sample using reduction of ferric tripyridyltriazine (Fe (III)-TPTZ) complex to the ferrous tripyridyltriazine (Fe(II)-TPTZ) by a test sample at low pH. The FRAP assay was performed according to Benzie & Strain (1996) procedure with slight modifications. FRAP reagent was prepared freshly to contain sodium acetate buffer (300 mmol/l, pH 3.6), 10 mmol/l TPTZ in 40 mmol/l HCl and $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ solution (20 mmol/l), i.e. in proportion 10:1:1 (v/v/v), respectively. Working FRAP solution was warmed to 37 °C prior to use. 100 μl of test sample (0.5 mg/ml) were added to 3 ml of working FRAP reagent and absorbance was recorded at 593 nm after 4 minutes using the JENWAY 6305UV/Vis spectrophotometer. Blank was prepared to contain distilled water instead of extract. Ascorbic acid, BHA and BHT (concentration of 0.1 mg/ml) were used as standards. The same procedure was repeated for standard solution of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (0.2-1.6 mmol/l) in order to construct calibration curve. FRAP values of sample was calculated from standard curve equation and expressed as $\mu\text{mol FeSO}_4 \cdot 7\text{H}_2\text{O}$ /g dry extract) averaged from three measurements.

3.8.4. β -carotene bleaching (β -CB) assay

β -CB assay, designed to evaluate the capacity of the antioxidants to reduce the oxidative loss of beta-carotene in a beta-carotene linoleic acid emulsion, was performed according to slightly modified procedure of Dapkevicius et al. (1998). β -carotene (2 mg), linoleic acid (50 μl) and Tween 40 (400 mg) were dissolved in 2 ml of chloroform. Chloroform was removed using a rotary evaporator at 40 °C, and 200 ml of distilled water were added with vigorous shaking. Crude extracts and standards BHA, BHT and ascorbic acid were dissolved in ethanol in concentration of 0.5 mg/ml (w/v). Aliquots of 150 μl of the emulsion and 21 μl of sample (extract/standard) were added to 96-well plate, while control contained 96% ethanol instead of sample. The absorbances were

measured immediately (t=0 min) at 490 nm using Tecan Sunrise SN microplate reader equipped by XFluor4 software. After 2 hours incubation, absorbances were measured again (t=120 min). The antioxidant activity of the samples was evaluated in terms of inhibition of β -carotene bleaching using the following equation:

$$\% \text{ Inhibition} = [(A_{120} - C_{120}) / (C_0 - C_{120})] * 100,$$

where A_{120} and C_{120} are the absorbances measured in t=120 minutes for sample and control, respectively, while C_0 is absorbance of control in t=0 min.

3.9. Cytotoxic activity

Cytotoxic activity of extracts was assessed against HCT-116 and K562 cell lines using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay in two parallel experimental designs. It is a colorimetric assay which detects conversion of yellow tetrazolium salt into purple formazan. The conversion is catalyzed by cellular enzymes and its rate represents measure of cells viability.

Experimental design 1: MTT assay was performed according to Mosmann (1985). HCT-116 cell line was obtained from American Type Culture Collection. Cells were maintained in DMEM supplemented with 10% FBS, with 100 units/ml penicillin and 100 μ g/ml of streptomycin. Cells were cultured in a humidified atmosphere with 5% CO_2 at 37 °C. Cells were grown in 75 cm^2 culture bottles supplied with 15 ml DMEM, and after a few passages cells were seeded in 96-well plate (10^4 cells per well). After 24 h of cells incubation, the medium was replaced with 100 μ l medium containing various doses of extract at different concentrations (1-500 μ g/ml). Untreated cells were used as the control. Solvent of MTT (final concentration 5 mg/ml in PBS) was added to each well and incubated at 37 °C in 5% CO_2 for 2-4 h. The colored crystals of produced formazan were dissolved in 150 μ l of DMSO. The absorbance was measured at 570 nm on Microplate Reader (ELISA 2100 C) after 24 and 72 h. Cell proliferation was calculated as the ratio of absorbance of treated group divided by the absorbance of control group, multiplied by 100 to give a percentage proliferation.

Experimental design 2: MTT assay was performed according to Drakuli et al. (2012) using K562 cells (human immortalized myelogenous leukemia line, erythroleukemia type). The line is derived from a CML patient in blast crisis. K562 cells

were maintained in essential minimal medium (MEM) supplemented with 10% FCS. Cells were treated in 96 well plates for 48 h with (50-400 µg/ml) water extracts in MEM. After addition of MTT solution (0.5 mg/ml), plate was incubated additionally for 3 h. Acidified isopropanol was added to dissolve tetrazolium salt. Absorbance was measured at 620 nm.

3.10. Antimicrobial activity

3.10.1. Antibacterial assay

The antibacterial activity was tested against six Gram-negative: *Escherichia coli* (ATCC 25922), *Salmonella typhimurium* (ATCC 14028), *Salmonella enteritidis* (ATCC 13076), *Pseudomonas tolaasii* (NCTC 387), *Pseudomonas aeruginosa* (ATCC 27853), *Proteus mirabilis* (ATCC 14273) and five Gram-positive bacteria: *Staphylococcus aureus* (ATCC 25923), *Bacillus cereus* (ATCC 10876), *Micrococcus flavus* (ATCC 14452), *Sarcina lutea* (ATCC 10054) and *Listeria monocytogenes* (ATCC 15313). In order to investigate the antimicrobial activity of extracts, a modified version of the microdilution itory concentrations) was performed by a microdilution technique using 96-well microtiter plate technique was used (Daouk et al., 1995; Sarker et al., 2007). Determination of MIC (minimum inhibitory concentration) was performed by a microdilution technique using 96-well microtiter plates. Serial dilutions of stock solutions of extracts in broth medium (Muller–Hinton broth for bacteria) were prepared in a 96-well plates. The microbial suspensions were adjusted with sterile saline to a concentration of 1.0×10^5 CFU/ml. The microplates were incubated at 37 °C during 48 h. The lowest concentrations without visible growth were defined as concentrations that completely inhibited bacterial growth (MICs). The standard antibiotic streptomycin (1 mg/ml DMSO) was used to control the sensitivity of the tested bacteria.

3.10.2. Antifungal assay

Antifungal activity of extracts was tested against pathogenic micromycetes (human isolates): *Candida krusei*, *Candida albicans*, *Candida parapsilosis*, *Aspergillus glaucus*, *Aspergillus fumigatus*, *Aspergillus flavus* and *Trichophyton mentagrophytes*.

Cultures were maintained on Sabouraud Dextrose Agar (SDA) at 4 °C in the culture collection of the Institute of Botany, Faculty of Biology, University of Belgrade (BEOFB). Antifungal activity of extracts was studied by microdilution method using 96-well plates (Hanel & Raether, 1988; Daouk et al., 1995; Sarker et al., 2007). Spore suspensions were prepared by washing of SDA surface using sterile 0.9% saline containing 0.1% Tween 20 (v/v). Turbidity was determined spectrophotometrically at 530 nm and spore number was adjusted to 10^6 CFU/ml (NCCLS, 1998). Ethanol and water extracts were dissolved in 5% DMSO in stock concentration. Series of double extract and essential oil dilutions (64-0.25 mg/ml) in Sabouraud liquid medium were analyzed. Each well contained Sabouraud liquid medium, spore suspension, resazurine, and extract of defined concentration. The mixture without extract was used as the negative control, while the positive control contained commercial antimycotic, ketoconazole, instead of extract. Incubation was continued for another 48 h and results were recorded using binocular microscope. The lowest concentration of extract or essential oil without visible fungal growth was defined as minimal inhibitory concentration (MIC). The lowest concentration of extract which inhibited fungal growth after re-inoculation on SDA was defined as minimal fungicidal concentration (MFC).

3.11. Enzyme inhibiting activity

3.11.1. Acetylcholinesterase (AChE) inhibitory activity assay

AChE inhibitory activity assay was performed according to spectrophotometric method (Ellman et al., 1961) using 96-well plates as described before (Orhan et al., 2007, 2013; enol et al., 2010) with slight modifications. The AChE activity was measured by monitoring of increase of yellow color produced from tiiocholine when it reacts with DTNB ion. The test reaction mixture was prepared by adding 140 µl of sodium phosphate buffer (0.1 M, pH 7.0), 20 µl of DTNB, 20 µl of extract-buffer solution containing 5% DMSO (concentration of 25, 50 and 100 µg/ml) and 20 µl of AChE solution (5 units/ml). The mixture without extract was used as the control, while the commercial anticholinesterase alkaloid-type of drug galanthamine was used as reference. After incubation (15 min, 25 °C), the reaction was initiated with the addition of 10 µl of acetylthiocholine iodide and absorbance was measured at wavelength of 412

nm using Tecan Sunrise SN microplate reader equipped by XFluor4 software.

Percentage of inhibition of AChE was determined using the formula $(C \cdot S)/C \cdot 100\%$, where C is the activity of enzyme in control (without test sample) and S is the activity of enzyme with test sample.

3.11.2. Tyrosinase inhibitory activity assay

Tyrosinase inhibitory activity assay was performed according to slightly modified spectrophotometric method of Masuda et al. (2005) using 96-well plates. Samples (extracts and reference kojic acid) were dissolved in sodium phosphate buffer (0.1 M, pH 7.0) containing 5% DMSO and phosphate buffer, respectively, in concentration of 25, 50 and 100 µg/ml. The wells were designed as: A (containing 120 µl of sodium buffer and 40 µl of tyrosinase in the same buffer (46 units/l)), B (containing only buffer), C (containing 80 µl of buffer, 40 µl of tyrosinase and 40 µl of sample and D (containing 120 µl of buffer and 40 µl of sample). After addition of 40 µl of L-DOPA and incubation (30 min, 25 °C), absorbance was measured at wavelength of 475 nm using Tecan Sunrise SN microplate reader equipped by XFluor4 software.

Percentage of inhibition of tyrosinase was determined using the formula: $[(A-B)-(C-D)/(A-B)] \cdot 100\%$.

3.12. Statistical analysis

All experimental measurements were carried out in triplicate and are expressed as average of three measurements \pm standard deviation. Pearson's correlation coefficients were calculated among phenolics, flavonoids and antioxidant activities, and interpreted according to Taylor (1990). Calculations and constructing of the charts were performed using the MS Office Excel, 2007.

4. RESULTS AND DISCUSSION

4.1. *Salvia aegyptiaca*

In spite of the large number and wide distribution of *Salvia* species (Kintzios, 2000), there is a lack of agronomic and morphological comparative information among the different species, which may be relevant for species characterisation and industrial applications. Furthermore, it is well known that edaphic and climatic variations and genetic characteristics may have a strong influence on the morphological, agronomic and essential oil chemical characteristics (Mossi et al., 2011).

S. aegyptiaca is commonly used for various medicinal and cosmetic purposes (Gorai et al., 2011), and it has been examined phytochemically (Al-Yahya et al. 1990; Bouchra et al. 2003; Basaif, 2004), but the micromorphology and histochemistry have not been studied previously. Considering the potential pharmacological value of this species, due to essential oils produced in glandular trichomes, and lack of data about the leaf trichomes characteristics, the comprehensive study of distribution, morphology and structure of the foliar appendages, emphasizing glandular trichomes, as well as histochemical characterization of the secreted products and nature, were carried out in order to evaluate the possible functional significance of the various trichomes.

Since there is no data about biological activities of *S. aegyptiaca* originated from Lybia, the plant material was subjected to the analysis of antioxidant activity using different methods.

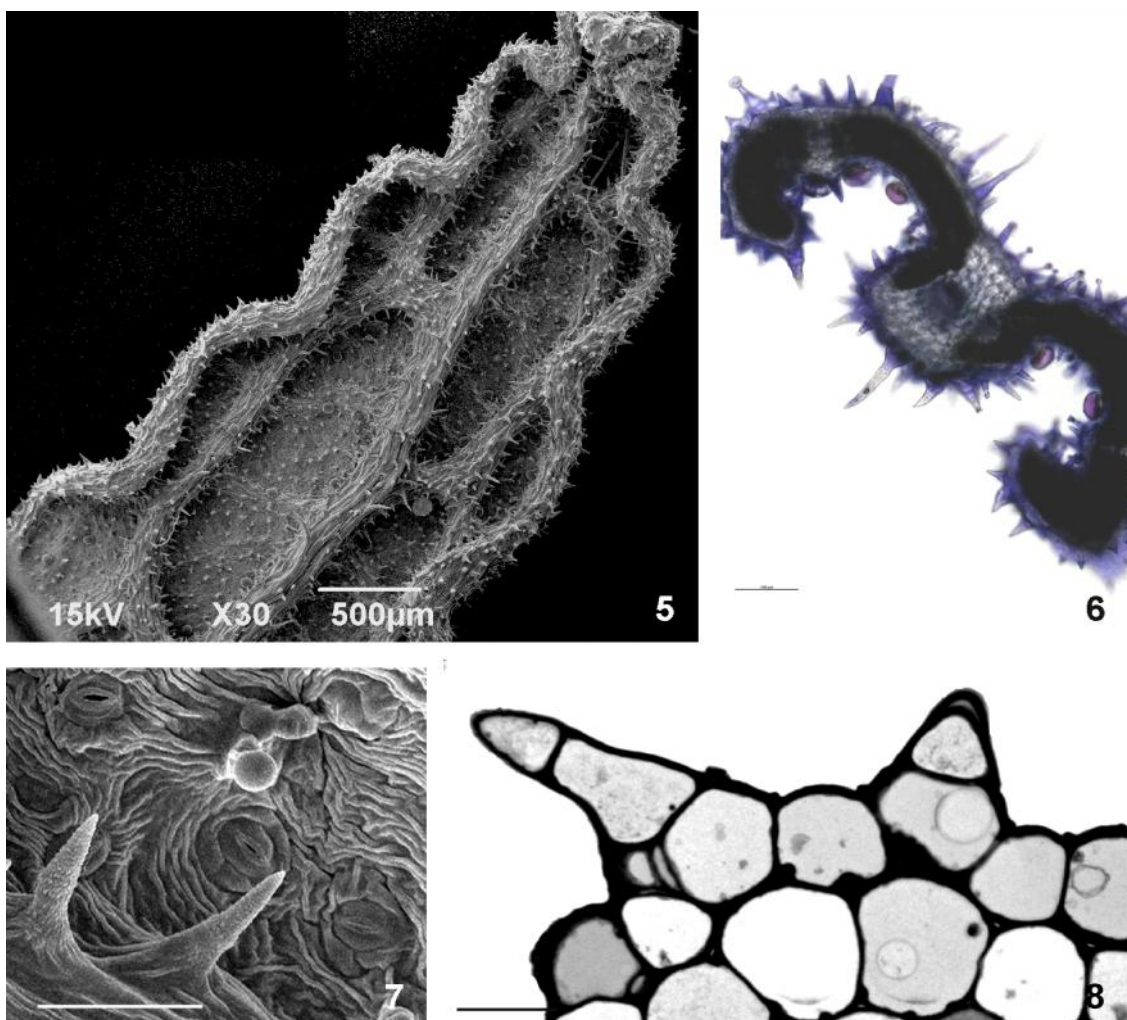
4.1.1. Micromorphological and histological analysis of leaf trichomes of *S. aegyptiaca*

The leaves of *S. aegyptiaca* are oblong, crenate to serrate, basal leaves are stalkless, upper with short stalk, and covered with an indumentum of non-glandular and glandular trichomes on both surfaces (Figs. 5 and 6). The abaxial leaf side is covered with numerous peltate and capitate trichomes (Fig. 6), while capitate trichomes are more abundant on adaxial leaf side, where peltate trichomes are rare.

Non-glandular trichomes are of two types: unicellular papillae and multicellular, uniseriate, two to six-celled, erect or slightly leaned toward the epidermis. Non-

glandular trichomes are characterized by a swollen basal cell, acute apices and thick, warty, cell walls (Figs. 7 and 8). Non-glandular trichomes can be characterized as arrect type according to Payne's classification (Payne, 1978).

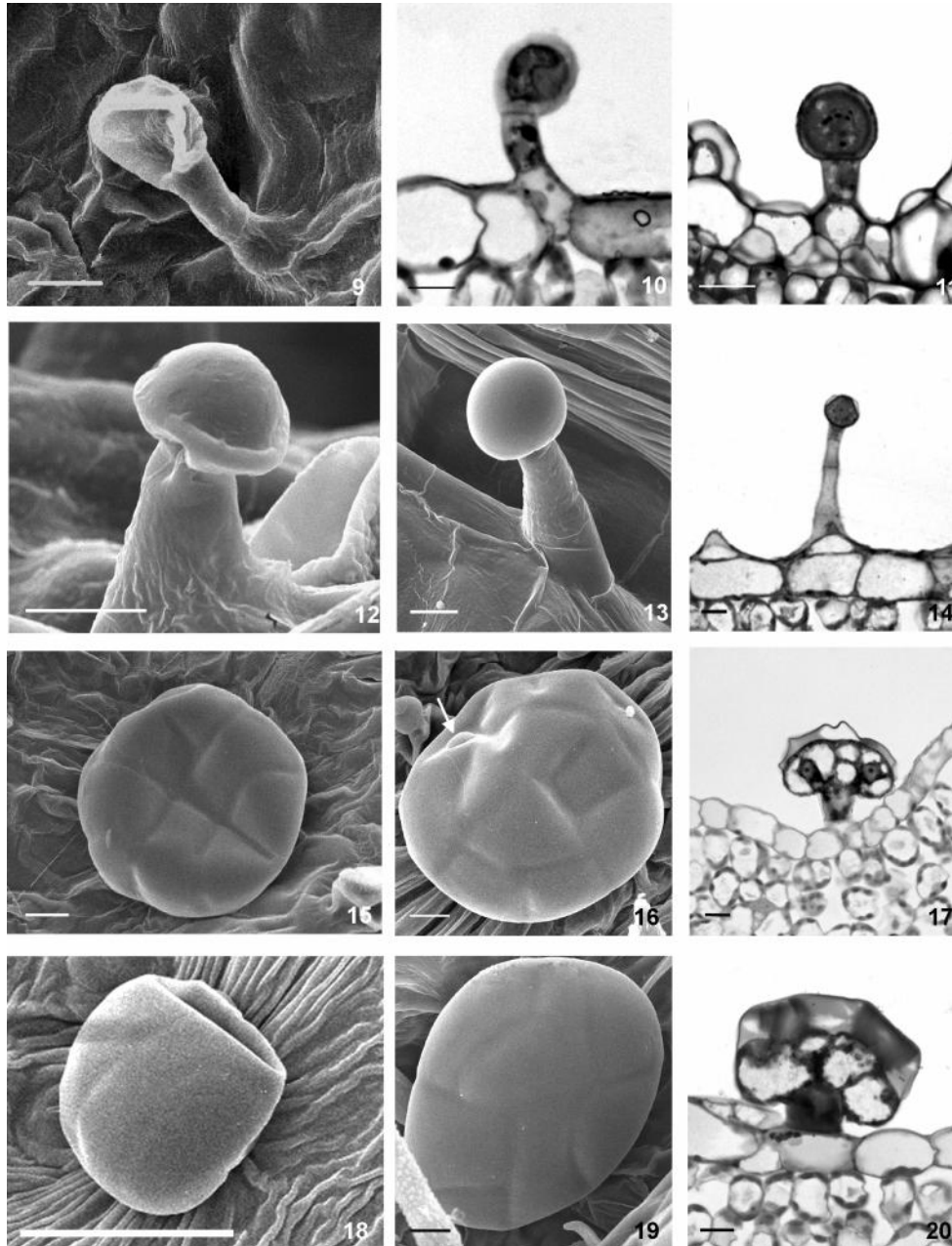
The glandular trichomes include capitate and peltate types. Capitate trichomes consist of one-celled glandular head, subtended by a stalk of variable length, facilitating their classification into two types: capitate trichomes type I or short-stalked glandular trichomes (Figs. 9, 10 and 11) and capitate trichomes type II or long-stalked glandular trichomes (Figs. 12, 13 and 14). The capitate trichomes of both types were observed on both leaf surfaces, but were more abundant on the adaxial epidermis and along the veins. With leaf expansion, density of trichomes decreases.



Figures 5–8. SEM and LM micrographs of *S. aegyptiaca* leaves.

- 5. SEM micrograph showing trichome indumentum on abaxial leaf surface.
- 6. Hand cross-section of a leaf with non-glandular and glandular trichomes.

7. SEM micrograph showing morphology of non-glandular trichomes.
8. Semi-fine section of non-glandular trichomes in longitudinal direction.
- Bar = 500 μ m (5) 100 μ m (6), 50 μ m (7), 20 μ m (8).



Figures 9–20. SEM and LM micrographs showing types, morphology and structure of glandular trichomes on leaf of *S. aegyptiaca*.

9. SEM micrograph of capitate glandular trichome type I; note shrunken head cell after releasing the secretion.

10–11. LM micrographs showing the morphology of capitate trichomes type I; note thick cuticle (6), rich and dense cytoplasm with dark vesicles and small vacuoles in secretory head cell (7).

12–13. SEM micrographs of mature capitate glandular trichomes type II with a large spherical secretory head.

14. Semi-fine section of capitate glandular trichomes type II showing secretory head cell with dense cytoplasm, small vacuoles and dark vesicles.

15. SEM micrograph of juvenile peltate trichome with cuticle closely attached to the secretory head cell walls;

16. SEM micrograph of peltate trichome with wrinkled surface; note the drop of secreted material ().

17. Semi-fine longitudinal section of peltate glandular trichome with wrinkled cuticle and small subcuticular space.

18. SEM micrograph of peltate glandular trichome with cuticle detached from the secretory head cells.

19. SEM micrograph of mature, balloon shaped, peltate trichome with smooth cuticle.

20. Semi-fine longitudinal section of mature peltate glandular trichome with osmiophilic secretory product in subcuticular space.

Bar = 10 μm (9-20), 50 μm (18)

Capitate trichomes type I consist of a cubical or cylindrical basal cell, embedded in epidermis, unicellular stalk with thick cutinized anticlinal cell walls and unicellular globoid to ovoid head (Figs. 10 and 11). Cytoplasm of stalk cell is dense and rich in dark stained organelles and vesicles, and small, translucent vacuoles. The head cell is characterized by dense cytoplasm, dark vesicles and minor vacuoles. The thick cuticle tightly covers the cell of secretory head in young trichomes and at maturity small, often laterally positioned subcuticular space is present (Figs. 23 and 24). The secreted material, which temporarily accumulates in the subcuticular chamber, probably exudes through the intact cuticle. Following the release of secretion, shrinking of head cell and leaning of trichome toward the epidermis was observed, ending with degradation of short trichome (Fig. 9).

Capitate trichomes type II are composed of basal vacuolated cubical or cylindrical cell, the stalk of three-four cylindrical cells covered with thick cuticle and oval secretory head (Figs. 14 and 30). The neck cell (top stalk cell) is rich in cytoplasm and small vacuoles. Secretory head cell is rich in cytoplasm, small vacuoles and dark stained vesicles. The thick cuticle tightly covers the cell of secretory head in young trichomes (Figs. 14, 27 and 32). Subsequently, cuticle detaches from the cell wall forming the subcuticular space and secretory product accumulates inside the subcuticular space (Figs. 12, 13 and 31). At the end of secretory stage, the shrinkage of the head cuticle occurs and secretory head become deflated and cup-shaped (Fig. 28).

Peltate trichomes are balloon-shaped and composed of basal cell, short cylindrical stalk cell and broad head of eight to twelve secretory cells arranged in a one or two circles (Figs. 15, 16 and 20). The basal cell is slightly larger than the other epidermal cells. The most dominant organelle in basal cell is large, translucent vacuole. Stalk cell have dense cytoplasm, dark organelles and small, light vacuoles. The head cells volume occupies large vacuole and osmiophilic dark deposits that are stored in the space between vacuole and plasma membrane (Fig. 20). Cuticle of young trichomes, in the presecretory stage of development, is closely attached to the secretory cells emphasizing the cell outlines (Fig. 15). Subsequently, after secretory product start to accumulate inside the subcuticular space, cuticle detaches from the secretory cells and it becomes wrinkled in appearance (Figs. 16 and 17). At maturity, when subcuticular space that increased considerably in volume is filled with secreted material, cuticle becomes smooth (Figs. 18 and 19). SEM images revealed drops of secretory material visible below cuticle (Fig. 16). Light microscopy investigation indicated that cuticle rupture is frequent along the equatorial plane of the head following secretion release and detachment of the cuticular cup (not shown).

Leaves of *S. aegyptiaca* possess both peltate and capitate glandular trichomes, as well as non-glandular ones, which is characteristic feature for Lamiaceae species (Werker et al. 1985). Two types of non-glandular trichomes were observed, unicellular and multicellular. Non-glandular trichomes, by covering the epidermal layer, provide protection of the plant against insects herbivores and airborne propagules of fungi and also increase tolerance to freezing, assist in seed dispersal, maintain water balance in leaves, deflect intense solar radiation, etc. (Werker, 2000; Mayekiso et al. 2008; 2009;

Dyubeni & Buwa, 2012). Since *S. aegyptiaca* grows in arid habitats, the function of these non-glandular trichomes appeared to have a protective function by covering the layer of epidermis in response to the environmental conditions. It is also believed that the non-glandular trichomes could affect the transpiration process by influencing the water diffusion boundary layer of the transpiring leaf (Mayekiso et al. 2009). Non-glandular trichomes of *S. aegyptiaca* were warty, and these structures are believed to arise either from the cell wall or the cuticle (Werker, 2000), which can thicken the walls of the trichomes and contribute to their function.

The unique characteristics of trichomes have traditionally been used as reliable characters for diagnostic key in plant taxonomy (Wagner, 1991; Xiang et al., 2010). In plant parts where secondary development has not yet occurred, the plant retained the similar arrangement of high distribution of non-glandular and glandular trichomes mechanical strategy (Mayekiso et al., 2009).

Glandular trichomes are epidermal structures specialized for particular metabolic functions, usually the biosynthesis and secretion of particular secretory products performing different functions in plants (Lange & Turner, 2013). Glandular trichomes vary in morphology, structure and also in number per unit area of epidermis, among species and organs (Serrato-Valenti et al. 1997; Ascensao et al. 1999). Two main types, peltate with large secretory head, and capitate trichomes with stalk and small head, were found in majority of studied leaves and flowers of different Lamiaceae species. Different variations have been observed in studied species, in stalk length, head shape and number of cells, release of secretion products, etc.

The compounds secreted by capitate glandular trichomes are mostly excreted to the surrounding environment, apparently through pores in the cuticle of the head cell(s). On the contrary, in peltate glandular trichomes the secretions accumulate in a capacious subcuticular space formed by the separation of the head cell walls from the cuticular dome that encloses them, and remain there until the cuticle is physically ruptured. Thus, peltate glandular trichomes function as repositories for the specialized phytochemicals that they secrete (Siebert, 2004).

Peltate trichomes are usually composed of basal epidermal cell, a short wide stalk cell and large round head of several secretory cells (Hallahan, 2000). The number of secretory cells could vary in the peltate trichomes of Lamiaceae taxa, from *Nepeta*

species (Metcalf & Chalk, 1950), *Ocimum basilicum* (Werker et al. 1993), where four cells were noticed, up to 16 cells in *Origanum* (Bosabalidis and Tsekos, 1984), *Satureja thymbra* (Bosabalidis, 1990), and *S. chamelaeagnea* (Kamatou et al., 2006), arranged in two concentric circles (Kamatou et al., 2007a). The number of head-cells also vary among *Salvia* species, from four cells in *S. blepharophylla* (Bisio et al. 1999), *S. divinorum* (Siebert, 2004), *S. verticillata* (Krsti et al. 2006) to six or eight cells in a single disc in *S. aurea* (Serrato-Valenti et al. 1997), *S. officinalis* (Werker et al. 1985), or by 12-18 cells arranged in two concentric circles with four central and eight or more peripheral cells (Corsi & Bottega, 1999; Kamatou et al. 2006; Özkan, 2008; Baran et al. 2010; Kahraman et al. 2010).

The present study showed that *S. aegyptiaca* has the peltate glandular trichomes consisted of the basal epidermal cell, short cylindrical stalk cell with cutinized lateral walls and large round head of 8-12 secretory cells arranged in one or two circles. During the secretion phase, peltate trichomes have a characteristic spherically shaped or slightly flattened head due to the development of a large subcuticular space where secretory products accumulate (Ascensao et al. 1999; Gairola et al. 2009).

In our research we have noticed two main types, short with one-celled cutinized stalk, and long stalked capitate trichomes composed of three to four cylindrical cells, with more or less oval secretory head. Capitate glandular trichomes constitute a significant taxonomic character of the Lamiaceae and form part of the floral specialized properties for pollination (Navarro & El Oualidi, 2000; Kahraman et al. 2009).

Capitate trichomes are very common in many species of *Salvia*, but variability in stalk length and head shape is noticed (Corsi and Bottega, 1999; Siebert 2004; Kamatou et al. 2006; Kahraman et al. 2009). They generally consist of one to two stalk cell(s) and one to two cell(s) forming a rounded to pear-shaped secretory head (Werker et al. 1985). Considering the variations in capitate trichomes, Werker et al. (1985), for the first time classified capitate hairs as type I, II and III according to their morphology and secretion mode. Following their classification, type IV capitate hairs were described in *S. officinalis* by Corsi & Bottega (1999). Several researchers divided capitate trichomes only into two types according to the dimensions of the stalk, the morphology of glandular head and the secretion process (Ascensao et al. 1999; Serrato-Valenti et al. 1997; Bisio et al 1999). Serrato-Valenti et al. (1997) in *S. aurea* described the capitate

trichomes type I consisting of short stalk and bicellular head, and capitate trichomes type II consisting of 1-4 stalk cells, narrow neck cell and globose unicellular head, and similar types were found in *S. blepharophylla* (Bisio et al. 1999), *S. albicaulis*, *S. dolomitica* (Kamatou et al. 2007a), etc.

The obtained results in presented study showed that *S. aegyptiaca* is very rich from the point of glandular trichome diversity and quantity. The secretions of the glandular trichomes could have different roles, such as the chemical defense of plants, guide for insects, or act as floral rewards to pollinators, but the specific function of each trichome type is not clear (Ascensao et al. 1999).

Detailed studies of morphology, anatomy and ultrastructure may be useful in interpretation of their functions. The abundant non-glandular hairs are involved in mechanical defense, and protect the plant from excessive transpiration and insolation (Corsi & Bottega, 1999). Baran et al. (2010) assumed that the features like abundance and diversity of glandular trichomes on plant organs, presence or absence of neck cells, the thickness of their side walls, and the stalk length, could show variation according to xeromorphic character of plants. The abundant and diverse glandular trichomes and long-length stalks of capitate trichomes of *S. aegyptiaca* show the xeromorphic character of the investigated plant species which in its native environment grows in dry habitats and it is drought resistant.

In order to clarify the specific secretory products and possible function of each glandular trichome type of *S. aegyptiaca*, further research on histochemical characterization was done.

4.1.2. Histochemistry of leaf glandular trichomes of *S. aegyptiaca*

Most studies on glandular trichomes use histochemical methods (Ascensão et al., 1999; Bisio et al., 1999; Corsi & Bottega 1999) because they are considered useful for an initial investigation of the presence of some substances. The characteristics of trichomes and the composition of secretory substances secreted in trichomes, vary greatly among species and are used in plant classification to distinguish between the related genera or species (Xiang et al., 2010).

Peltate trichomes produce most of the essential oil, with terpenes comprising the main component (Huang et al., 2008). In addition to small amounts of essential oil, capitate trichomes normally secrete varying amounts of polysaccharides (Werker, 1993). A good deal of research has been done on trichome structure in relation to the secretory process, in order to elucidate the secretory mechanism (Bosabalidis and Tsekos, 1982).

Most of this research has focused on only a few species, such as *Origanum dictamnus* (Bosabalidis and Tsekos, 1982), *Perilla ocymoides* (Danilova & Kashina, 1987), *Leonotis leonurus* (Ascensao et al. 1995), *Salvia officinalis* (Corsil and Bottega, 1999), *Mentha piperita* (Turner et al. 2000), and *Prostanthera ovalifolia* (Gersbach, 2002). The research has shown that most organelles participate in the production or storage of the essential oil. This includes the plastids, endoplasmic reticulum (ER), and vacuoles. Exocytosis is the most probable mechanism for movement of the essential oil from the cell to the subcuticular space (Huang et al., 2008).

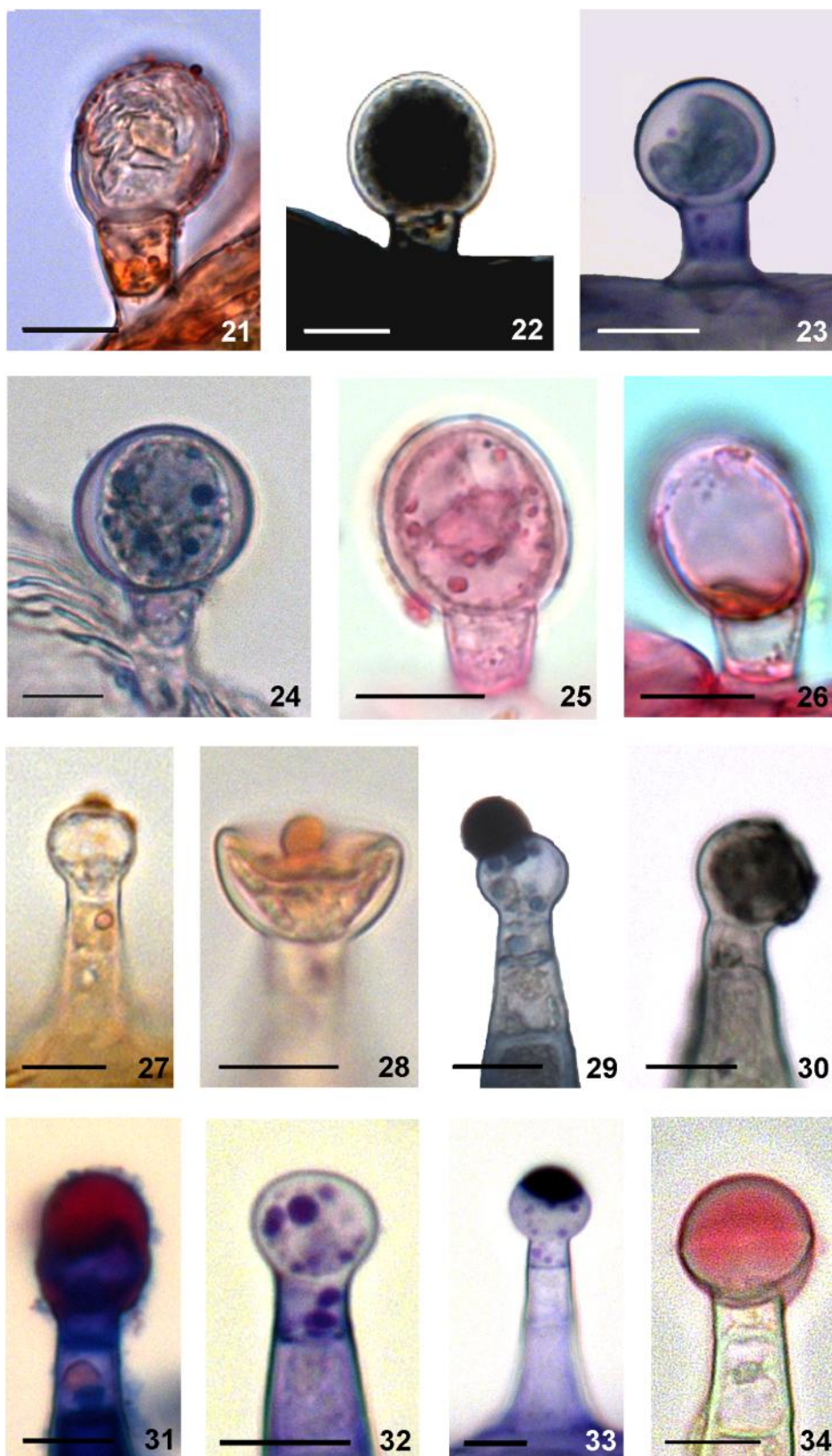
The results of the histochemical tests carried out to characterize the main classes of compounds contained in the glandular trichomes of *S. aegyptiaca* are summarized in Table 1.

Capitate trichomes type I have a small subcuticular space and secretory material mainly accumulates inside the glandular cell (Figs. 21-26), whereas capitate trichomes type II accumulate secretion inside head cell and in the subcuticular space (Figs. 27-34). Peltate trichomes store in their large subcuticular saces abundant secretory product with an emulsion-like appearance (Fig. 35). The secretion stored in the subcuticular space of mature capitate trichomes type II (Figs. 27-33) and peltate trichomes (Figs. 36-43) contained copious amount of lipophilic substances. Reaction with OsO₄ showing black color confirmed the presence of bulk of unsaturated lipid substances in the subcuticular space of capitate trichomes type II (Fig. 30) and peltate trichomes (Fig. 38). In the capitate trichomes type I strong osmiophilic reaction was observed in the glandular head (Fig. 22). The presence of essential oil was identified by Nile blue A in secreted material of capitate trichomes type II (Fig. 31) and peltate trichomes (Figs. 41 and 42). The secretion of capitate trichomes type I contained only a small amount of terpenoids (Fig. 23), while capitate trichomes type II (Figs. 32 and 33) and peltate trichomes (Figs. 39 and 40) produce large amount of terpenoids.

Table 1. Results of the histochemical characterization of the main secretory products of the *S. aegyptiaca* glandular trichomes.

Staining procedure	Target compounds	Capitate trichomes type I		Capitate trichomes type II		Peltate trichomes	
		Secretory cell	Subcuticular space	Secretory cell	Subcuticular space	Secretory cell	Subcuticular space
Sudan IV	Total lipids	+	+	+	++	-	+++
Sudan Black B	Total lipids	+	+	+	++	-	+++
OsO₄	Unsaturated lipids	+	-	++	++	++	+++
Nile blue A	Essential oils	-	-	+	+++	-	+++
Nadi reagent	Terpenes	+	+	+++	+++	++	+++
FeCl₃	Phenolic compounds	-	-	-	-	-	-
Toluidine blue O	Tannins	++	-	nt	nt	-	++
AlCl₃ (UV)	Flavonoids	-	-	-	-	-	-
PAS	Polysaccharides	+	-	nt	nt	+	-
Ruthenium red	Pectins	+	+	+	++	+	-
UV (autofluorescence)	Phenolic compounds	-	-	-	-	-	-

+++ very strong reaction; ++ strong reaction; + moderate reaction; - negative reaction, nt = not tested



Figs. 21–34. Histochemical characterization of the secretions of capitulate trichomes of *S. aegyptiaca*.

21–26. Capitate glandular trichomes type I.

21. Small droplets in the subcuticular space and lipophilic content of the head cell stained orange-brown with Sudan IV; lipophilic, orange, droplets of various sizes are also noted in the neck cell.

22. OsO₄ test showing black staining in the head cell and neck cell.

23. Violet-blue droplets of essential oils in the subcuticular spaces, head and neck cell can be seen after staining with Nadi reagent.

24. Deep blue droplets in the secretory head indicate the presence of tannins and pink colored droplets at the periphery of cytoplasm signify the presence of pectins after staining with Toluidin blue O.

25. The presence of pink colored droplets in head cell after PAS reaction indicates polysaccharides.

26. Pectins stained pink with Ruthenium red.

27–31. Capitate glandular trichomes type II.

27. Secretory material positively stained with Sudan IV in the subcuticular space, head cell and neck cell.

28. Lipophilic droplet on the deflated cup-shaped head cell.

29. Black-blue colored secretion of lipophilic substances after staining with Sudan Black B.

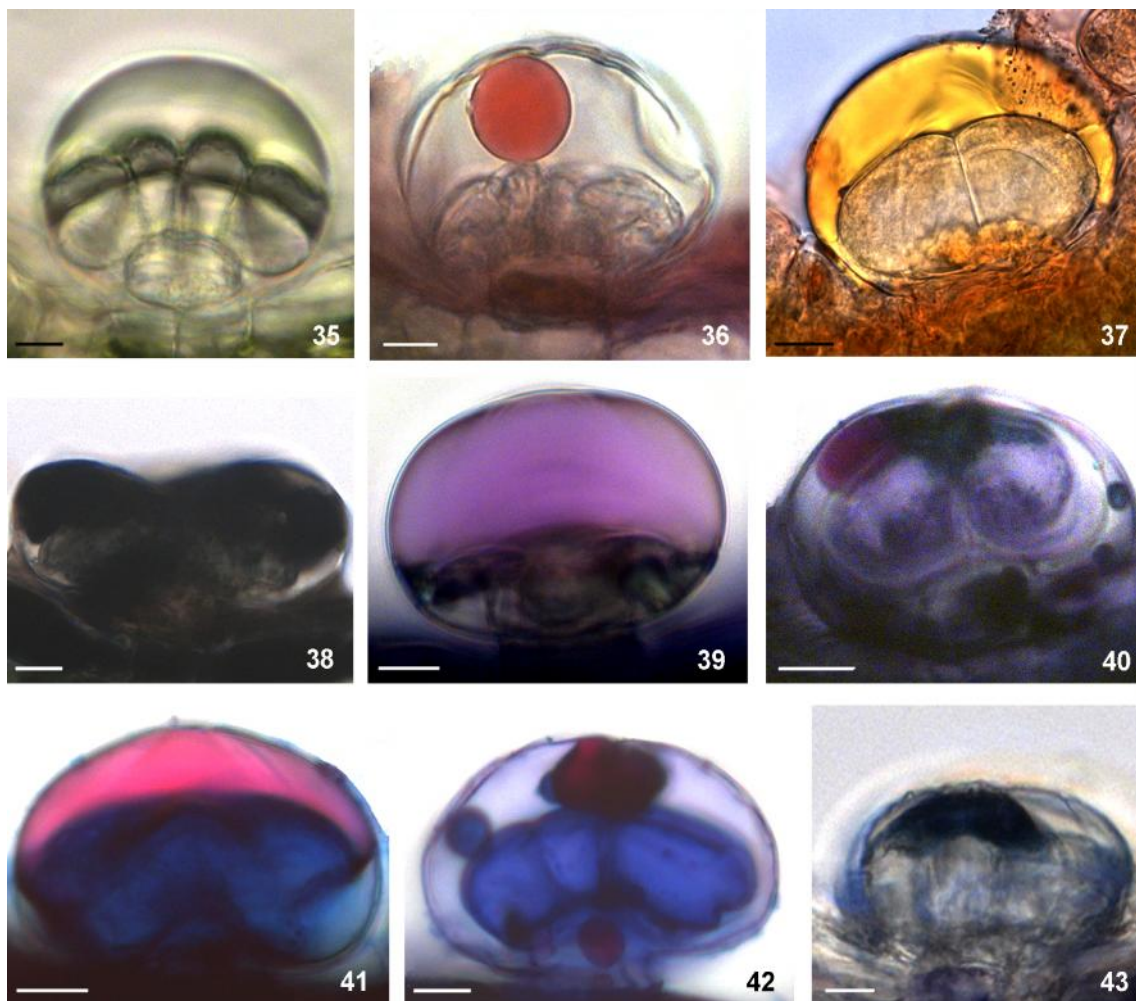
30. OsO₄ test showing black staining of secretory material in the subcuticular space and in the head cell.

31. Neutral lipids/essential oils (red color) and acidic lipids/resins (blue color) are evident after staining with Nile blue A.

32–33. Successive stages of terpene secretion; droplets of various sizes in the subcuticular space, head cell and neck cell stained violet-blue after staining with the Nadi reagent.

34. Pectins stained pink with Ruthenium red in the subcuticular space and head cell.

Bar = 10 µm (21-34).



Figures 35–43. Histochemical characterization of the secretions of peltate trichomes of *S. aegyptiaca*.

35. Full-developed peltate trichome *in vivo*.

36–38. Peltate trichomes showing lipophilic secretion (Sudan red 7B/hematoxylin, Sudan IV and OsO_4 , respectively).

39–40. The terpenes within the subcuticular space has reacted positively with Nadi reagent (violet-blue secretion).

41–42. Secreted material within the subcuticular space; essential oils stained pink-red, while resins stained blue with Nile blue A.

43. Peltate trichomes showing positive reaction, in the subcuticular space, for tannins with Toluidine blue O (deep blue color).

Bar = 10 μm (35-43)

Histochemical tests carried out to detect tannins gave a positive results in capitate trichomes type I (Fig. 24), and peltate trichomes (Fig. 43). The abundant polysaccharidic compounds other than cellulose were accumulated in the subcuticular space of capitate trichomes type II (Fig. 34) and in the cytoplasm of head cell of capitate trichomes type I (Figs. 25 and 26) and type II. The polysaccharides were not detected in the secretion of peltate trichomes (Table 1).

The Sudan IV reaction showed that in all trichome types the anticlinal cell walls of stalk cells were impregnated with lipid substances. The reaction with Nile blue and Nadi reagent confirmed the presence of essential oils in the stalk cells of capitate type I (Fig. 23), capitate type II (Figs. 31-33) and peltate trichomes (Figs. 39-42).

Data obtained from histochemical tests revealed that the secreted material in all types of *S. aegyptiaca* glandular trichomes was of a complex nature. Most of the material was lipophilic, as shown by positive reactions with Sudan dyes. The secreted product also included non-cellulosic polysaccharides in the capitate trichomes as shown using PAS and Ruthenium red tests. Polysaccharides were detected in both types of capitate trichomes, but were not detected in peltate trichomes. According to Werker (1993) capitate trichomes are assumed to secrete varying amounts of polysaccharides along with essential oils. Peltate trichomes secrete only lipophilic substances, while capitate trichomes mainly secrete polysaccharide products (Huang et al., 2008).

Modenesi et al. (1984) suggested that the presence of a viscous secretion might act as lubricant to facilitate leaf growth, from the origin of the bud to full laminar expansion. The presence of viscous, adhesive polysaccharides, especially in the calyces of blooming flowers and dry calyces provide good mechanical defense from aphids during flowering and seed development (Corsi & Bottega, 1999).

Comparing the results of histochemical analysis, it can be observed that lipids and terpenes were detected in all types of trichomes. Pectins were detected as secretory product in both types of capitate trichomes, while in peltate trichomes the reaction was negative in subcuticular space where secretion is accumulated. The peltate trichomes and capitate trichomes type II are the main structures that secrete the essential oil in *S. aegyptiaca*. Previous findings showed that the peltate trichomes produce most of the essential oils in the Lamiaceae (Werker et al. 1985; Werker, 1993; Bourett et al. 1994; Clark et al. 1997; Huang et al., 2008), although the capitate trichomes also produce a

few lipophilic compounds (Ascensao & Pais, 1998; Ascensao et al. 1999; Corsi & Bottega, 1999). Comparing to other Lamiaceae species, the differences in the amounts of secretory products and production sites can be observed (Mota et al. 2013).

The histochemical study indicate that the secretion of *S. aegyptiaca* peltate trichomes is an oleoresin containing terpenoids (essential oils and resiniferous acids), which was reported in other plants (Ascensao et al. 1999; Gersbach, 2002).

The capitate trichomes contain lipids and polysaccharides. This result is consistent with the phytochemical data available for other species of the Lamiaceae (Werker et al. 1985, Dudai et al. 1988; Ascensao & Pais 1998; Ascensao et al. 1999). Positive reactions confirmed that the stalk cells of both trichome types contained lipophilic compounds – essential oils. The synthesis of essential oil in stalk cells was confirmed in our investigation for all trichome types. In most glandular trichomes the stalk cells contain lipophilic substances (Ascensao & Pais, 1998; Ascensao et al. 1999; Corsi & Bottega, 1999) but the thick cuticle or cuticular thickenings on the lateral walls of the stalk cells, as well as presence of barrier cells with suberized lateral walls, provides structural support and probably prevent the flow of secreted products into mesophyll tissue and regulate directional transport of metabolites to the glandular cells above (Fahn, 1988; Werker, 2000; Gersbach, 2002).

The presence of terpenes in the essential oils of the glandular trichomes on *S. aegyptiaca* leaves is probably responsible for the medicinal properties, and suggestive of a protective function as demonstrated for other members of the Lamiaceae. As the largest class of natural products, terpenes have a variety of roles in mediating antagonistic and beneficial interactions among organisms. They defend many species of plants, animals and microorganisms against predators, pathogens and competitors having potent antibacterial and antifungal activity, and they are toxic to insects, nematodes, mollusks, etc. (Nishida, 2002; Gershenzon & Dudareva, 2007).

Phenolic compounds were not detected by applied tests, but in the subsequent research of *S. aegyptiaca* extracts, phenols and flavonoids were detected using spectrophotometric method.

The composition of secreted material of the glandular hairs suggest that these trichomes are involved in chemical defense, because essential oils are poisonous to most insects, terpenoids are olfactory deterrents for insects, and steroids, flavonoids and

especially tannins are very strong insect deterrents (Corsi & Bottega, 1999; Simmonds 2003; Barbehenn & Constabel, 2011).

The secreted material is mostly accumulated in subcuticular spaces of trichomes, as it was described for other aromatic Lamiaceae (Bosabalidis & Tsekos, 1982; Werker et al. 1985; Bruni & Modenesi, 1983; Bourett et al. 1994; Serrato-Valenti et al. 1997). Heterogeneous secreted material was temporarily stored in the subcuticular space in mature peltate trichomes and released by rupture of the cuticle, while in capitate trichomes it was probably released through micropores. It is likely, as proposed by Ascensao et al. (1995) that, under natural conditions, factors such as high temperature and low air humidity can cause the cuticle to burst, releasing the essential oil. Other factors, such as contact with predators can be involved in the rupture mechanism (Werker, 1993).

When the cuticular sac ruptures due to external pressures these substances are released to the outside of the cuticular sac and kept on the surface layer of the plant. These substances may provide chemical protection to the plant against various types of herbivores and pathogens by entrapping, deterring and poisoning (Wagner, 1991). The fact that various compounds (terpenoids, phenolics, flavonoids, etc.) are localized in the glandular trichomes that are distributed over much of the plant's exterior suggests that they have a protective function. Essential oils could contain repellent substances which are effective against herbivores and pathogens (Werker, 1993; Corsi & Bottega, 1999).

4.1.3. Total phenolic and flavonoid content and antioxidant effects of different extracts

The total phenolic and flavonoid content, as well as the antioxidant activity of dichloromethane, ethyl acetate, chloroform and ethanol extracts prepared from the aerial parts of Libyan *S. aegyptiaca* were analysed for the first time.

4.1.3.1. The yield of extracts, total phenolic and flavonoid content

As presented in Table 2, yields of extracts ranged from 0.42% for ethyl acetate to 12.78% for ethanol extract. The highest and the lowest contents of total phenolics were measured for ethanol and ethyl acetate extract (150.71 and 30.82 mg GAE/g,

respectively). Conversely, ethyl acetate extract showed the highest flavonoid content (43.26 mg QE/g) and ethanol extract the lowest (14.64 mg QE/g).

Table 2. The yield of extracts, total phenolic content (TPC) and total flavonoid content (TFC) of *S. aegyptiaca* extracts.

Extracts	Yield	TPC	TFC
Dichloromethane	0.70	30.82 ± 1.100	40.72 ± 0.940
Chloroform	11.12	33.63 ± 0.901	29.38 ± 0.335
Ethyl acetate	0.42	146.77 ± 0.497	43.26 ± 1.195
Ethanol	12.78	150.71 ± 0.300	14.64 ± 0.577

^a percentage of yield (%). ^b mg GAE/g dry extract. ^c mg QE/g dry extract

The largest amounts of yield were obtained for ethanol and chloroform extracts, 12.78% (w/w) for ethanol and 11.12% for chloroform extract. Similar result was obtained in our research of *S. amplexicaulis*, 13.18% for ethanol extract (Alimpi et al., 2014). enol et al. (2010) obtained very low yield for ethyl acetate extract of *S. trichoclada* (0.14%). The variations in extract yields depend on seasons, growth phase, localities, etc. (Perry et al., 1999; Kamatou et al., 2008; enol et al., 2010; Orhan et al., 2012; Ben Farhat et al., 2013a).

Extraction into an appropriate solvent is the first step for the recovery of bioactive phytochemicals from plant materials (Guo et al., 2001; Gao et al., 2006). Ethanol and methanol are the most used solvents for the extraction of antioxidants from herbs. Ethanol is often the final choice since it is non-toxic, compatible with food industry, providing good yield, and can be mixed with water in different ratios (Pan et al. 2003; Pasias et al., 2010).

4.1.3.2. Antioxidant effects of different extracts

Antioxidant activity of extracts was measured by four parallel assays: DPPH, ABTS, FRAP and β-carotene bleaching (β-CB) assay and results are presented in Table 3. Ethanol extract showed the strongest activity in DPPH, ABTS and FRAP assays, whereas dichloromethane and chloroform extracts performed the weakest antioxidant

effect. DPPH activity of ethanol and ethyl acetate extracts was close to positive controls (IC₅₀ values about 20 µg/ml), while dichloromethane and chloroform extracts showed IC₅₀ values above 100 µg/ml. Similarly, ABTS activity of ethanol and ethyl acetate extracts was the highest (1.98 and 1.64 mg AAE/g) and close to synthetic antioxidants BHA and BHT, unlike dichloromethane and chloroform extracts with activity about 0.1 mg AAE/g. Ethanol extract showed the highest and dichloromethane the lowest reducing activity measured using FRAP assay (1500.38 and 130.73 µmol Fe(II)/g, respectively). In the β-CB assay, ethanol extract showed low activity (9.04%) comparing to standards (17.82-57.71%).

Table 3. Antioxidant activity of *S. aegyptiaca* extracts.

Samples	DPPH assay (IC ₅₀ , µg/ml)	ABTS assay (mg AEE/g)	FRAP assay (µmol Fe(II)/g)	β-CB assay (% inhibition)
Extracts				
DCM	247.41 ± 19.897	0.14 ± 0.010 ^a	130.73 ± 1.147 ^b	nt
CHL	149.13 ± 5.439	0.11 ± 0.013 ^a	197.63 ± 1.324 ^b	nt
ETAC	24.92 ± 0.553	1.64 ± 0.027 ^a	556.77 ± 8.920 ^b	nt
ETOH	20.74 ± 0.473	1.98 ± 0.013 ^a	1500.38 ± 6.521 ^b	9.04 ± 1.596 ^b
Standards				
BHT	17.94 ± 0.168	2.75 ± 0.021 ^c	445.34 ± 5.772 ^c	57.71 ± 3.385 ^b
BHA	13.37 ± 0.430	2.82 ± 0.011 ^c	583.72 ± 5.255 ^c	53.72 ± 2.257 ^b
Ascorbic acid	5.11 ± 0.143	nt	180.81 ± 8.607 ^c	17.82 ± 1.128 ^b

^aat concentration of 1 mg/ml. ^bat concentration of 0.5 mg/ml. ^cat concentration of 0.1 mg/ml; nt –not tested; DCM-dichloromethane; CHL-chloroform; ETAC-ethyl acetate; ETOH-ethanol;

4.1.3.3. Correlation between antioxidant assays, total phenolic and flavonoid contents

Antioxidant activity measured by DPPH, ABTS and FRAP assay and total phenolic (TPC) and total flavonoid content (TFC) were correlated in different ways, as presented in Table 4. All of assays were strongly correlated to total phenolic content (r value from ± 0.806 to ± 0.993 , respectively). Flavonoid content of extracts was in moderate to strong negative correlation with their antioxidant activity. All of antioxidant assays were strongly ($r > 0.67$) correlated to each other, particularly DPPH and ABTS free radical scavenging assays ($r = -0.918$). Total phenolic and flavonoid contents were poorly correlated ($r = 0.297$).

Table 4. Pearson's correlation coefficients between antioxidant activity and content of phenolic components.

	DPPH assay	ABTS assay	FRAP assay	TPC	TFC
DPPH assay	1				
ABTS assay	-0,918 ^c	1			
FRAP assay	-0,761 ^c	0,869 ^c	1		
TPC	-0,936 ^c	0,993 ^c	0,806 ^c	1	
TFC	0,395 ^b	-0,389 ^b	-0,775 ^c	-0,297 ^a	1

^a $r < 0.35$ weak correlation, ^b $0.36 < r < 0.67$ moderate correlation, ^c $0.68 < r < 1$ strong correlation (According to Taylor, 1990).

Recent development in biomedical science emphasis the role of free radicals in many diseases, such as brain disfunction, cancer, heart disease and immune system (Halliwell, 1999, 2009; Griendling and FitzGerald, 2003, Valko et al., 2006). Antioxidants have great importance in reducing oxidative stress which could cause damage to biological molecules, especially antioxidants of natural origin, because the safety of widely used synthetic antioxidants has been questioned (Roby et al., 2013). Concern about the safety of synthetic antioxidants together with consumer preference for natural products has resulted in increased research on natural antioxidants. Phenolic compounds such as flavonoids, phenolic acids, diterpenes and tannins have received

attention for their high antioxidant activity. The antioxidant activity of *Salvia* species extracts has been attributed to their total phenolic content, hence plant extracts rich in polyphenols usually have a higher antioxidant capacity (Lu & Foo, 2002; Tosun et al., 2009; Roby et al., 2013; Ben Farhat et al., 2013a).

The interest in polyphenols has grown considerably because of their high capacity to trap free radicals associated with different diseases. Phenols and flavonoids are very important plant constituents because of their antioxidant activity. The plant phenolics are the most widespread secondary metabolite in plant kingdom, commonly present in fruits, vegetables, leaves, nuts, seeds, barks, roots and in other plant parts. These diverse groups of compounds have received much attention as potential natural antioxidant in terms of their ability to act as radical scavengers (Sahoo et al., 2012). The antioxidant activity of phenolic compounds is mainly due to their redox properties which play an important role as free radical scavengers, reducing agents, quenchers of singlet oxygen and complexes of pro-oxidant metals. The total phenolic compounds may contribute directly to the antioxidant action, therefore, it is necessary to investigate total phenolic content (Tohamy et al., 2012). Cuvelier et al. (1994) and Kamatou et al. (2010) highlighted the fact that *Salvia* antioxidant properties are essentially attributed to abietane diterpenoids (carnosic acid and carnosol) and caffeic acid derivatives (rosmarinic acid, caffeic acid, ferulic acid, chlorogenic acid, etc.).

Several reports have shown close relationship between total phenolic contents and antioxidant activity of the fruits, plants and vegetables (Vinson et al., 1998; Deighton et al., 2000; Abdille et al., 2005). The chemical composition and chemical structures of active extract components are important factors governing the efficacy of natural antioxidants, and the antioxidant activity of an extract could not be explained on the basis of their phenolic content, which also needs their characterization (Heinonen et al., 1998).

S. aegyptiaca was subjected to the chemical analyses, but studies of antioxidant properties of different extracts are still rare (Salah et al., 2006; Akbar & Al-Yahya, 2011; Ben Farhat et al., 2013a; Firuzi et al., 2013), as well as lacking for the plants growing in Libya. The phytochemical analysis of *Salvia* species show the presence of many compounds belonging mainly to the group of phenolic acids, phenolic glycosides,

flavonoids, anthocyanins, coumarins, polysaccharides, sterols, terpenoids and essential oils (Lu & Foo, 2002).

The antioxidant capacity of *S. aegyptiaca* was evaluated applying four common antioxidant tests used for the characterization of plant extracts, DPPH, ABTS, FRAP and β -carotene bleaching assays. The antioxidant capacity of the plant extract largely depends on both the phenolic and/or flavonoid content of the extract and the test system. It can be influenced by a large number of factors, and cannot be fully evaluated by one single method. It is necessary to perform more than one type of antioxidant capacity measurement to take into account the various mechanisms of antioxidant action (Song et al., 2010). Applying of multiple assays in screening work is recommended by several authors (Tepe et al., 2007; Tepe, 2008; Ben Farhat et al., 2013a), because the results of a single assay can give only a reductive suggestion of the antioxidant properties of extracts towards food matrices and must be interpreted with some caution. The chemical complexity of extracts, often a mixture of numerous compounds with different functional groups, polarity and chemical behaviour, could lead to scattered results, depending on the test employed (Tepe et al., 2007).

S. aegyptiaca in our study showed the best antioxidant activity for ethanol extract, which was correlated to the phenolic content.

In the research of cytotoxic, antioxidant, total phenol contents and antimicrobial activities of extracts of eleven Iranian *Salvia* species, Firuzi et al. (2013) obtained low radical scavenging potential for *S. aegyptiaca* 80% methanol extract in DPPH test, correlated with low phenolic content. The antimicrobial activity was weak, but cytotoxic effect of dichloromethane extract on human cancer cell lines was significant. In our study DPPH activity of ethanol (IC_{50} value 20.74 μ g/ml), and ethyl acetate (24.92 μ g/ml) extracts were close to positive controls (BHT, 17.94 μ g/ml and BHA, 13.37 μ g/ml), which is significantly higher activity comparing to Firuzi et al. (2013).

Ben Farhat et al. (2013a) studied phenolic contents and compositions and antioxidant activities of methanolic extracts of four Tunisian *Salvia* species, among them *S. aegyptiaca*. In the DPPH, ABTS and FRAP tests *S. aegyptiaca* showed low variation between collection sites (IC_{50} 22.62 μ g/ml, and 21.00 μ g/ml) which is close to our results for DPPH assay. The authors applied the classification of Kamatou et al. (2010) who placed analyzed *Salvia* species into plants with good (IC_{50} <30 μ g/ml),

moderate ($30 < IC_{50} < 80 \mu\text{g/ml}$) and poor ($IC_{50} > 80 \mu\text{g/ml}$) antioxidant activity using the DPPH assay, and *S. aegyptiaca* samples were marked by a good free radical-scavenging activity. The similar results were obtained when our results for ethanol and ethyl acetate extracts were compared with results of methanolic extracts of *S. aegyptiaca* growing wild in Tunisia (Ben Farhat et al., 2013a).

The study of Firuzi et al. (2013) showed lower values of IC_{50} and phenolic content for *S. aegyptiaca* than those obtained for different extracts in the present study. The methanolic extracts of three Tunisian *Salvia* species were screened for their antioxidant activity and gave positive results in applied tests. The extracts of *S. aegyptiaca* were the most active in both tests (Salah et al., 2006). Our results confirmed the high antioxidant capacity of *S. aegyptiaca*.

A positive linear correlation was obtained between total phenolic content and antioxidant activity of the extracts, which indicated that the phenolic compounds could be main contributor of the antioxidant activity of these extracts. This result was in agreement with previous studies of different plants (Cai et al., 2004; Tosun et al., 2009; Li et al. 2013; Alimpi et al., 2014). Weak and negative correlation was obtained for TPC and TFC ($r = -0.297$), which could be explained by variety of phenolic constituents and their concentrations.

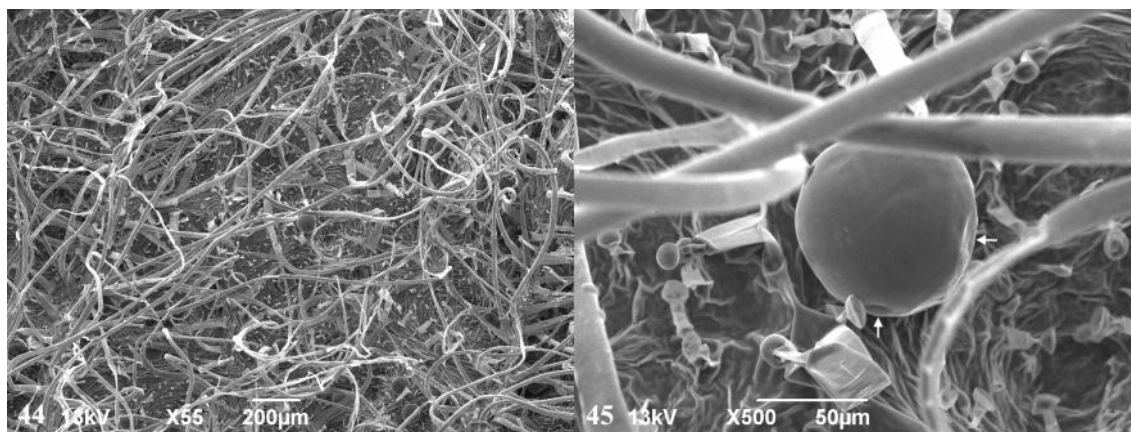
The current investigation confirms the importance of *S. aegyptiaca* for its traditional use as food condiment and medicinal plants and emphasizes the potential further use as functional food ingredient and therapeutic agent.

4.2. *Salvia fruticosa*

S. fruticosa, endemic species of the Mediterranean region, is one of the most commercially important *Salvia* species, appreciated for its beauty, medicinal and culinary properties. Micromorphological and ultrastructural analyses of the leaf trichomes of *S. fruticosa* were done using light and electron microscopy. Since the plant possess medicinal value, and there is no data about biological activities of *S. fruticosa* originated from Libya, various extracts of plant material was subjected to the analysis of several biological activities.

4.2.1. Micromorphological, histological and ultrastructural analyses of leaf trichomes of *S. fruticosa*

The densely-pubescent leaves bear numerous non-glandular and glandular trichomes on both surfaces. Non-glandular trichomes densely covered the whole leaf surface, but were more abundant on the abaxial leaf side (Fig. 44). Especially abundant were noticed to be on margins. They are single, uniseriate, multicellular, pointed, and erect. Numerous trichomes are very elongated, flagelliform, vary in length, consist of five or more cells and are supported by epidermal cells. During the development of the leaf, the density of non glandular trichomes decreases, although they remain abundant on both surfaces of mature leaves, predominating on the margin and veins of the abaxial surface.



Figures 44-45. SEM micrographs of *S. fruticosa* leaf trichomes

44. SEM micrograph showing the morphology of non-glandular and glandular trichomes on the leaf surface of *S. fruticosa*.

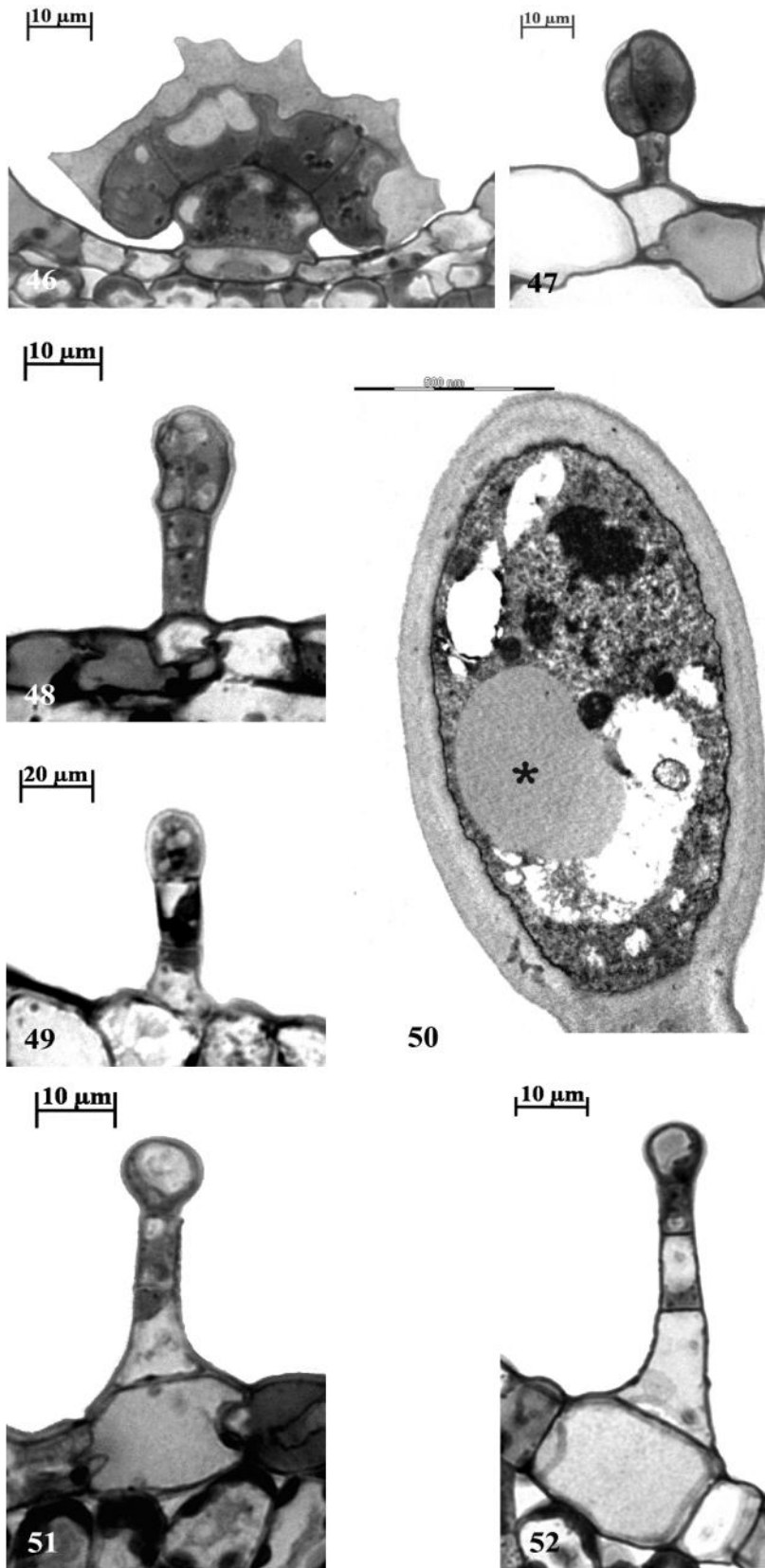
45. SEM micrograph of peltate trichome of *S. fruticosa*. Note equatorial line ().

The glandular trichomes are of three main types – peltate, capitate and digitiform. *In vivo*, peltate trichomes have green to brownish colour and balloon shape (Fig. 45). They consist of basal epidermal cell, very short stalk cell and large round head of eight secretory cells arranged in a circle (Fig. 46). Peltate glandular hairs show a smooth surface indicating the close attachment of the cuticle to the secretory upper cell walls (Fig. 45). Large space in which the secreted material accumulated developed at the time of secretion by the elevation of the cuticle together with outermost layer of the

secretory cell walls (Fig. 46). An equatorial line of weakness became visible round head (Fig. 45); the rupture of the cuticle along this line and subsequent rise of the cuticular cap led to release of exudate.

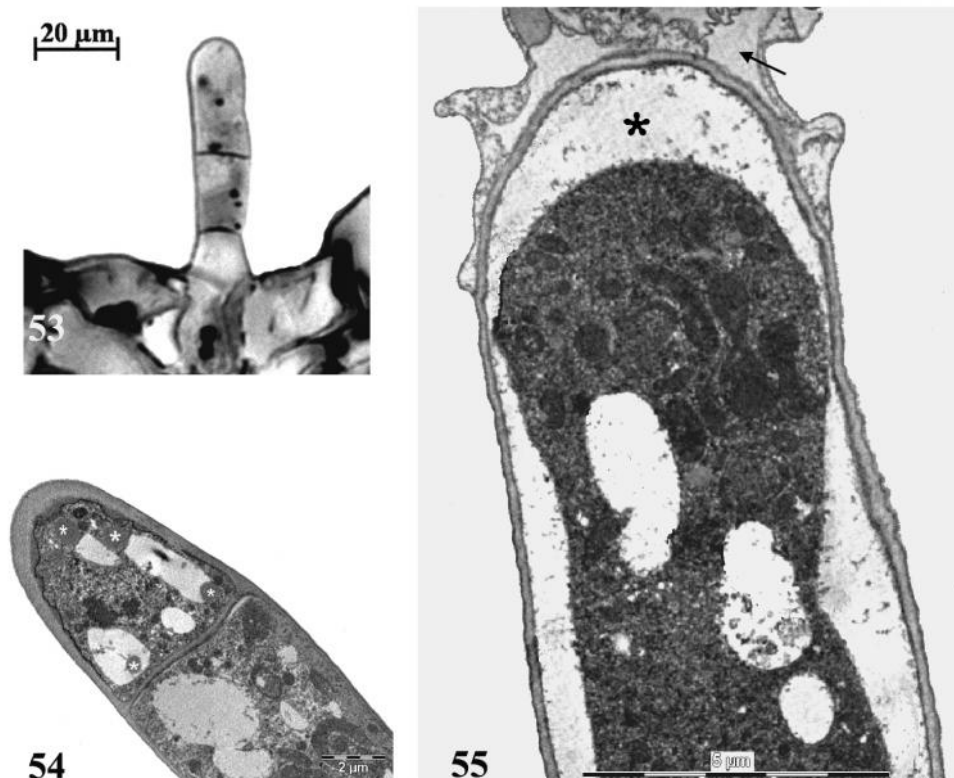
Capitate trichomes can be divided into two main types, short stalked and long stalked, and further into five subtypes according to the number of stalk cells, the morphology and the number of the glandular head cells and the secretion process. Short stalked capitate trichomes subtype I possess one basal cell, one stalk cell with thick cutinized lateral walls and a bicellular ovoid glandular head (Fig. 47). Subtype II have one basal cell, two stalk cells and globoid secretory head of two cells (Fig. 48), while subtype III capitate trichomes have one basal cell, one stalk cell and one head cell (Fig. 49). The secretory product accumulates inside the apical cells (Fig. 50). Long-stalked capitate trichomes subtype IV possess one basal cell, long two-celled stalk and an unicellular spherical head (Fig. 51), subtype V have one basal cell, three celled stalk and also one cell in the spherical glandular head (Fig. 52).

Digitiform trichomes consist of one basal cell, one or two stalk cells and one apical secretory cell. The cells are of similar diameter and approximately equal length (Fig. 53). There is no clear distinction between head and stalk cells. The glandular apical cells have rounded tips, thin walls and are rich in cytoplasm (Fig. 54). Apical cell of few digitiform trichomes posses a very small subcuticular spaces, but numerous of them have not developed subcuticular space (Fig. 54, 55). In the secretory phase, digitiform trichomes cells contained dense cytoplasm, numerous dark organelles, osmiophilic drops, and translucent vacuoles and the outer cell wall was covered with a thick cuticle (Fig. 54). During further development as a result of accumulation of secretion the periplasmatic space gradually enlarged, leading to a drastic retraction of the plasma membrane from the cell wall (Fig. 55).



Figures 46-52. LM and SEM micrographs of *S. fruticosa* leaf trichomes.

46. LM micrograph of peltate trichome of *S. fruticosa* showing basal epidermal cell, very short stalk cell and large head of eight secretory cells.
47. LM micrograph of short stalked capitate trichomes subtype I.
48. LM micrograph of short stalked capitate trichomes subtype II.
49. LM micrograph of short stalked capitate trichomes subtype III.
50. TEM micrograph of capitate trichome subtype III showing the secretory product-lipid droplet (*) inside the apical cell.
51. LM micrograph of long-stalked capitate trichomes subtype IV.
52. LM micrograph of long-stalked capitate trichomes subtype V.



Figures 53-55. LM and TEM micrographs showing digitiform trichomes of *S. fruticosa*.

53. LM micrograph of digitiform trichome of *S. fruticosa*.
54. TEM micrograph of the apical cell of digitiform trichome. Note lipid droplets (*).
55. TEM micrograph of the apical cell of digitiform trichome. Note periplasmic space (*) and subcuticle space () filled with secreted material.

The presence of non-glandular and glandular hairs is characteristic for Lamiaceae species (Werker et al., 1985). Distribution of non-glandular trichomes on epidermis is a natural phenomenon in most angiosperms and could be associated with protection of foraging insects and airborne propagules of fungi (Fahn, 1967). These non-glandular trichomes are considered to shield the stoma and oil glands from intensive heat during the dry and hot season as they had shown high distribution and surround the stoma. Such basic phenomena involved in characterization of the boundary layer resistance have also been shown in xeromorphic species. At maturity, some of the organs of the plants that have undergone secondary development are more likely to have less number of the non-glandular trichome than glandular trichome (Mayekiso et al., 2009).

Salvia dominica, *S. fruticosa*, *S. sclarea* and *S. officinalis* were some of the species of these genera that composed of different types of glandular hairs than any other genera in the Lamiaceae family (Werker et al., 1985; Serrato-Valenti et al., 1997).

Glandular trichomes vary in morphology, structure and in number per unit area of epidermis, among species and organs (Serrato-Valenti et al., 1997; Ascensao et al., 1999). The researchers mostly have found two main types, peltate with large secretory head, and capitate trichomes with stalk and small head, on studied leaves and flowers of different Lamiaceae species. Different variations have been observed in studied species, in stalk length, head shape and number of cells, release of secretion products, etc.

Several *Salvia* species were studied for their glandular hairs characteristics and essential oils composition. Different types of glandular trichomes were found in different species, while results obtained in this research showed that peltate, capitate and digitiform glandular hairs are present on leaves of *S. fruticosa*.

The present study showed that *S. fruticosa* possess the peltate glandular trichomes consisted of the basal epidermal cell, very short stalk cell with cutinized lateral walls and large round head of eight secretory cells arranged in a circle, as it was observed in some studied *Salvia* species. Werker et al. (1985) also found that head of *S. fruticosa* peltate trichomes composed of eight cells was common. The number of secretory cells composing the head could vary in number among *Salvia* species, from four to eight, or even 12-18 cells (Bisio et al., 1999; Serrato-Valenti et al., 1997; Corsi

& Bottega, 1999; Kamatou et al., 2006; Özkan, 2008; Baran et al., 2010; Kahraman et al., 2010).

Capitate trichomes are more variable in stalk length and head shape. Several researchers divided capitate trichomes into two types according to the dimensions of the stalk, the morphology of glandular head and the secretion mode (Ascensao et al., 1999; Serrato-Valenti et al., 1997, Bisio et al., 1999), but Werker et al. (1985) classified capitate hairs in type I, II and III, and Corsi & Bottega (1999) described type IV in *S. officinalis*. In our research we have noticed two main types, short stalked and long stalked capitate trichomes, but further divided into five subtypes according to the number of stalk cells, the morphology and the number of the glandular secretory head cells and the secretion process. Short stalked capitate trichomes subtype I, have one stalk cell and bicellular ovoid head, while subtype II have two stalk cells and globoid glandular head, which are consisted of two cells. Subtype III of capitate trichomes has one stalk cell and one head cell. Long stalked capitate trichomes subtype IV and subtype V have unicellular spherical glandular cell, but these type of trichomes have a different number of stalk cells. Some other *Salvia* species also showed variations in morphology of capitate trichomes, and divided in more subtypes, such as *S. chrysophylla* with 4 subtypes (Kahraman et al., 2010), *S. officinalis* (Corsi & Bottega, 1999), or 3 types in *S. smyrnea* (Baran et al., 2010), *S. verticillata* (Krstić et al., 2006), *S. argentea* (Baran et al., 2010). Because of great variation in structure and size of capitate trichomes, and since they form part of the floral specialized properties for pollination, some authors emphasize their significance as taxonomic character of the Lamiaceae (Navarro & El Oulalidi, 2000).

In the analysed leaves of *S. fruticosa* one specific type of glandular trichomes was observed – digitiform trichomes. These trichomes are less abundant than peltate and capitate trichomes. A digitiform type of glandular trichomes was reported for some Lamiaceae species, such as *Plectranthus ornatus* (Ascensao et al., 1999). These trichomes consist of three to four cells, in line, of similar diameter and approximately equal length (one basal, one to two stalk cells and one apical, head-like cell which do not develop subcuticular space). There is no clear distinction between head and stalk cells. Talebi et al. (2012) found four types of digitiform trichomes in *Ziziphora tenior* (one- to four-celled).

Ultrastructural analyses are important in the trichome research because the relations among morphology, cytology, and secretion processes could be established. Ultrastructural changes during secretory phase of glandular trichomes were characterized by proliferation of endomembranous system. Numerous mitochondria are connected with high metabolic activity of the cell (Dunki et al., 2007). Smooth ER, leucoplasts without thylakoids containing osmophilic drops, are characteristic organelles involved in terpene production, while dictyosomes are responsible for polysaccharide production (Cheniclet & Carde, 1985; Dunki et al., 2007; Huang et al., 2008).

The results of performed analysis showed that *S. fruticosa* is very rich from the point of glandular trichome diversity and quantity. It is suggested that the secretions of the glandular trichomes may be involved in the chemical defense of plants, or may guide insects, or act as floral rewards to pollinators, but the specific function of each trichome type is not known (Ascensao et al., 1999).

Detailed studies of morphology, anatomy and ultrastructure may be useful in interpretation of their functions. The abundant non-glandular hairs are involved in mechanical defense, and protection of the plant from excessive transpiration and insolation (Corsi & Bottega, 1999). Trichomes may thus have multiple functions and trichome density may evolve in response to variation in several environmental factors. In general, plants with high density of leaf trichomes can be expected in environments that are dry or cold, where UV-radiation is intense, and in areas where the risk of being damaged by herbivorous insects is high (Ehrlinger, 1984; Løe et al. 2007). Some authors (Baran et al., 2010) assumed that the features like abundance and diversity of glandular trichomes on plant organs, presence or absence of neck cells, the thickness of their side walls, and the stalk length, could show variation according to xeromorphic character of plants.

The abundant and diverse glandular trichomes and long-length stalks of capitate trichomes of *S. fruticosa* show the xeromorphic character of the investigated plant species which in its native environment grows as part of the Maquis shrubland and it is very drought resistant.

4.2.2. Composition and biological effects of different extracts

In the present study, dichloromethane, ethyl acetate, methanol, ethanol and water extracts obtained from wild growing Libyan *Salvia fruticosa* were analyzed for their total phenolic and flavonoid content, phenolic composition of extracts, and antioxidant activities using DPPH, ABTS, FRAP and β -carotene bleaching (β -CB) assays. For the further investigation of antimicrobial, cytotoxic and enzyme inhibition activities, the ethanol and water extracts were chosen, because of their wide usage in phytotherapy.

4.2.2.1. The yield of extracts, total phenolic and flavonoid content

In the present study, the yields of extracts were measured from 3.43% for ethyl acetate to 8.92% for ethanol extract (Table 5). It can be observed that alcoholic and water extracts yielded more abundantly than extracts obtained by non-polar and medium polar organic solvents such as dichloromethane, ethyl acetate).

Table 5. The yield of extracts, total phenolic content (TPC) and total flavonoid content (TFC) of *S. fruticosa* extracts.

Extract	Yield ^a	TPC ^b	TFC ^c
Ethanol	8.92	154.18 \pm 2.789	29.08 \pm 0.544
Water	7.62	67.68 \pm 0.617	21.73 \pm 0.163
Dichloromethane	3.60	164.71 \pm 0.727	38.38 \pm 0.538
Ethyl acetate	3.43	105.10 \pm 0.910	17.00 \pm 0.769
Methanol	6.90	132.00 \pm 3.509	22.69 \pm 0.705

^a percentage of yield (%). ^b mg GAE/g dry extract. ^c mg QE/g dry extract

Water extract and methanol extract yields from dried leaves of *S. fruticosa* showed higher yield (9.8 and 22.4%, respectively) (Ibrahim & Aqel., 2010), than the same extracts in our study. The results obtained from extracts of the leaves of *S. fruticosa* grown in Greece, showed that ethanol and ethyl acetate produced similar yields, higher than methanol extracts (Pasias et al., 2010). The results of extract yields of *S. fruticosa* grown in Turkey were 7.48% for dichloromethane extracts, 2.58% for

ethyl acetate extracts and 9.87% for methanol extracts (Enol et al., 2010). The extract yields of wild and cultivated *Salvia fruticosa* changed in 17.98–20.32 g/100 g dw and the highest yield was obtained with the wild *S. fruticosa* (Dincer et al., 2012). Papageorgiou et al. (2008) have reported that extract yield of the leaves of *S. fruticosa* harvested in different year and season ranged between 19.40% and 29.20%. Pizzale et al. (2002) have reported the extraction yield of *S. fruticosa* methanolic extract (25.5%). In addition, Bozan et al. (2002) determined that methanol extracts yields of aerial part of eight *Salvia* species varied in the range of 12.8–26.3% and they found the highest extract yield in *S. fruticosa*. The differences can be related with tested sample parts (Bozan et al., 2002; Pizzale et al., 2002), as well as extraction conditions, geographical region, collecting season, or ecological conditions. (Papageorgiou et al., 2008).

The current study showed that dichloromethane extracts had the highest contents of phenolics and flavonoids (164.71 mg GAE/g and 38.38 mg QE/g, respectively). Ethyl acetate and water had the lowest contents of phenolics (105.10 and 67.68 mg GAE/g, respectively) and flavonoids (17.00 and 21.73 mg QE/g, respectively).

S. fruticosa contains considerable amounts of secondary metabolites such as phenolics and terpenoids which have anti-inflammatory, antimicrobial and antioxidant properties (Dincer et al., 2012). Tawaha et al. (2007) studied the total phenolic content of *S. fruticosa* and reported as 24.1 mg GAE/g dry weight. Papageorgiou et al. (2008) found the total phenolic content of the same plant, in different seasons of the year, ranged between 63.7 and 144 mg GAE/g dry weight. According to the authors, these differences are because of different extraction methods, geographical coordinates, climate and ecological conditions. Our results showed different findings of Tawaha et al. (2007) and Papageorgiou et al. (2008), these differences can be related with tested sample parts and storage conditions before analysis. As a matter of fact, Papageorgiou et al. (2008) used only leaves like our samples; however, they stored their samples under nitrogen. On the other hand Tawaha et al. (2007) used all aerial parts of the plants.

Our findings for total phenolics and total flavonoid dichloromethane extract were 164.71 mg GAE/g and 38.38 mg QE/g, respectively. These findings are higher than total phenol and total flavonoid dichloromethane extract which were found in the dichloromethane extract of Turkish *S. fruticosa* (87.86 mg GAE/g extract and 3.70 mg

rutin equivalent/g extract, respectively). It can be also speculated that the activity might be depending on a possible synergistic interaction between its terpenoids as its total phenol content (Enol et al., 2010).

Among examined species, leaves of Libyan *S. fruticosa* showed the moderately high quantity of phenols (Gonaid et al., 2012). Pizzale et al. (2002) found the total phenolic compound content of 61.3-113.4 g/kg in the extracts of Cretan *S. fruticosa* samples. The total flavonoid contents of wild and cultivated *Salvia fruticosa* from Turkey were found between 25.79 and 38.61 mg of CE g/dw depending on the harvesting year, growing conditions and storage period. It may also be related with stressing factors such as drought, salinity, cold, soil characteristics etc. which may increase secondary metabolites synthesis in wild samples (Mittler, 2006; Rebey et al., 2012). The total phenolic contents of wild and cultivated *Salvia fruticosa* were found in range of 41.58-44.60 mg GAE g/dw (Dincer et al., 2012). The total phenol content of ethanol extract for 14 Turkish *Salvia* species ranged from 57.10-218.09 mg GAE/g and 8.29-108.78 mg QE/g for flavonoids (Orhan et al., 2013). Our results of ethanol extracts are in agreement with the range of this values for total phenol (154.18 mg GAE/g) and (29.08 mg QE/g) for flavonoids.

4.2.2.2. Composition of different extracts

Qualitative and quantitative composition of five *S. fruticosa* extracts was investigated using HPLC analysis and results are presented in Table 6. The most of the components were identified in water and methanol, followed by ethanol extract (88.65%, 80.62% and 67.07%, respectively). Polyphenolic components were classified according to Bolton et al. (2008) and Neveu et al. (2010) in the phenolic acids, flavonoids, including flavones and flavonols and other polyphenols.

Among phenolic acids, caffeic acid was present in methanol, ethanol and hot water extracts (1.39-4.02%). Rosmarinic acid was found only in methanol extract (1.41%), although many researchers reported on rosmarinic acid as the most abundant phenolic acid in *Salvia* species (Skoula et al., 2000; Lu & Foo, 1999; 2002; Pizzale et al., 2002; Dincer et al., 2012; Orhan et al., 2012; Ben Farhat et al., 2013a,b, 2014). Askun et al. (2009) also found higher level of caffeic acid in methanol extract of *S.*

fruticosa, while Papageorgiou et al. (2008) reported hydroxibenzoic acid as the major phenolic acid.

Table 6. Phenolic components of *S. fruticosa* extracts presented as percentage (%).

Extracts ^a	DCM	ETAC	MEOH	ETOH	W
Constituents					
PHENOLIC ACIDS					
Caffeic acid	-	-	0.24	1.39	4.02
Rosmarinic acid	-	-	1.41	-	-
FLAVONOIDS					
<i>Flavones</i>					
Apigenin	0.96	8.34	5.57	5.68	-
Apigenin glycosides	-	-	5.98	-	5.37
Luteolin	-	6.79	6.22	7.93	1.51
Luteolin glycoside	-	-	6.10	-	9.70
Genkwanin glycosides	-	-	3.60	9.51	7.99
<i>Flavonols</i>					
Kaempferol glycosides	-	-	43.93	42.56	58.39
Rutin	-	-	4.21	-	1.67
Hyperoside	18.06	11.24	1.40	-	-
OTHER POLYPHENOLS					
Coumarin	-	4.21	1.96	-	-
Total (%)	19.02	30.58	80.62	67.07	88.65

^aDCM-dichloromethane; ETAC-ethyl acetate; MEOH-methanol; ETOH-ethanol; W-water

The study of Cvetkovikj et al. (2013) showed differences among populations of *S. fruticosa* originated from Albania, with higher content of rosmarinic acid, and Greece, with higher content of caffeic acid. Differences could be attributed to developmental stage of plants and also to genetic variation in populations (Skoula et al., 2000; Dincer et al., 2012).

Flavones were present in range from 0.96% to 9.70%, Aglycones, apigenin and luteolin were abundant in ethyl acetate and ethanol extracts, while apigenin, luteolin and genkwanin glycosides were mostly present in methanol, water and ethanol extract, respectively. Kaempferol glycosides were predominant among flavonols (42.56-58.39%), in hot water, methanol and ethanol extracts.

Dincer et al. (2012) also identified kaempferol in the 80% methanolic extract of *S. fruticosa* from Turkey. Among other polyphenols, coumarin was present only in the ethyl acetate and ethanol extracts (4.21% and 1.96%, respectively). There are different results in other studies of *S. fruticosa* samples originated from other Mediterranean countries, with myricetin and morin (Dincer et al., 2012), quercetin (Papageorgiou et al., 2008), apigenin and luteolin (Askun et al., 2009), apigenin and luteolin glycosides (Cvetkovikj et al., 2013) as the most abundant flavonoids, which could be affected by geographic conditions and seasonal variations.

Our study revealed that Libyan *S. fruticosa* have significant amount of phenolic compounds, particularly kaempferol glycosides, which are reported before as powerful antioxidant and antimicrobial agents (Tatsimo et al., 2012).

Previous study on *S. fruticosa* showed seventeen different phenolic compounds consisting of 7 phenolic acids and 10 flavonoids. The flavonoids were composed of flavonols (rutin, myricetin, morin, quercetin and kaempferol), flavones (luteolin and apigenin), flavanones (hesperetin) and catechins ((+)-catechin and (-)-epicatechin) (Dincer et al., 2012).

Extracts of *Salvia fruticosa* were analyzed using HPLC-SPE-NMR hyphenation and the results showed that diterpenes (carnosol, carnosic acid, 12-methoxycarnosic acid) and flavonoids (hispidulin, cirsimaritin, salvigenin) are the main constituents of the ethyl acetate extract (Exarchou et al., 2015).

4.2.2.3. Antioxidant effects of different extracts

Antioxidant activity of *S. fruticosa* extracts was evaluated using four test-systems: DPPH, ABTS, FRAP and β -carotene bleaching assays and results are presented in Table 7. Ethanol extract showed the strongest activity in all applied assays, whereas ethyl acetate extracts performed the weakest antioxidant effect, except in the β -CB assay.

All of the extracts performed DPPH activity (ranged as 29.55-62.68 $\mu\text{g/ml}$), but weaker than positive controls. Regarding applied sample concentration, ethanol extracts showed ABTS activity (2.31 mg AAE/g) close to synthetic antioxidants BHA and BHT. Ethanol and dichloromethane extracts showed high reducing activity measured using FRAP assay (834.10 and 653.67 $\mu\text{mol Fe(II)/g}$, respectively), but water extract showed the best result in FRAP assay, 1191.51 $\mu\text{mol Fe(II)/g}$. In β -carotene-linoleic acid system, the ethanol (85.11%) and water (69.68%) extracts exhibited stronger inhibition comparing to standards (17.82-57.71%).

Many diseases such as atherosclerosis, arthritis, cardiovascular disorders, Alzheimer disease, cancer and many others may be caused by oxidative cell damages (Halliwell, 1999, 2001a,b). Antioxidants can reduce or prevent oxidation and protect against the damaging effects of free radicals which are produced in the human body from normal metabolism or induced by physical or chemical factors of the environment. The major action of antioxidants is to prevent damage caused by the action of reactive oxygen species (ROS) such as superoxide radicals, hydroxyl, and peroxides by donating an electron to a molecule that has been compromised by oxidation, bringing it back into a state of proper function (Lu & Foo, 2002). Synthetic antioxidants, such as BHA and BHT are very effective, but their use is now restricted due to adverse side effects and possible potential health risks and toxicity (Kahl & Kappus, 1993). Thus, the search for naturally occurring antioxidants has attracted increasing interest among scientists in searching for natural products that can be used in food, cosmetic and pharmaceutical products instead of synthetic antioxidants. Numerous studies have demonstrated that medicinal plants contain various components possessing antioxidant properties causing beneficial health effects.

Table 7. Antioxidant activity of *S. fruticosa* extracts.

Samples	DPPH assay (IC ₅₀ , µg/ml)	ABTS assay (mg AEE/g)	FRAP assay (µmol Fe(II)/g)	β-CB assay (% of inhibition)
Extracts				
Ethanol	29.55 ± 0.356	2.31 ± 0.025 ^a	834.10 ± 7.636 ^b	85.11 ± 1.843 ^b
Water	48.11 ± 1.495	1.98 ± 0.005	1191.51 ± 8.109	69.68 ± 2.764 ^b
Dichloromethane	33.23 ± 0.171	0.64 ± 0.020 ^a	653.67 ± 4.865 ^b	50.53 ± 4.222 ^b
Ethyl acetate	62.68 ± 2.587	0.55 ± 0.020 ^a	342.32 ± 10.542 ^b	32.98 ± 1.596 ^b
Methanol	36.37 ± 1.179	1.83 ± 0.088 ^a	332.19 ± 5.772 ^b	12.23 ± 4.787 ^b
Standards				
BHT	17.94 ± 0.168	2.75 ± 0.021 ^c	445.34 ± 5.772 ^c	57.71 ± 3.385 ^b
BHA	13.37 ± 0.430	2.82 ± 0.011 ^c	583.72 ± 5.255 ^c	53.72 ± 2.257 ^b
Ascorbic acid	5.11 ± 0.143	nt	180.81 ± 8.607 ^c	17.82 ± 1.128 ^b

^a at concentration of 1 mg/ml, ^b at concentration of 0.5 mg/ml, ^c at concentration of 0.1 mg/ml; nt -not tested.

Among numerous studies on the antioxidant potential of various *Salvia* species, *S. fruticosa* was analysed mostly by Turkish (Bozan et al., 2002; enol et al., 2010; 2011; Dincer et al., 2012; Topcu et al., 2013), Greek (Exarchou et al., 2002; Papageorgiou et al., 2008; Pasiás et al., 2010; Stagos et al., 2012) and Jordanian (Tawaha et al., 2007; Al-Mustafa & Al-Thunibat, 2008) authors. In the previous study of the antioxidant activity of the essential oil of the Libyan *S. fruticosa* using the DPPH free radical scavenging method (Giweli et al., 2013b), low antioxidant activity was found ($IC_{50} = 15.53$ mg/ml). The results on DPPH radical scavenging activity of *S. fruticosa* were in consistence with reported data, especially when polar extracts (methanol, ethanol, water) were applied, having rich polyphenol content.

S. fruticosa extracts showed lower DPPH activity than those measured by Stagos et al. (2012) for methanolic and water *S. fruticosa* extracts, 22 and 16 μ g/ml, respectively. In ABTS test *S. fruticosa* showed activity of 13 μ g/ml for methanolic and 29 μ g/ml for water extract (Stagos et al., 2012). Bozan et al. (2002) in analyzing of antioxidant properties of eight Turkish *Salvia* species found that *S. fruticosa* has moderate antioxidant activity in comparison to the other species. In the study which comprised 165 extracts of 55 Turkish *Salvia* taxa (enol et al., 2010), the methanol extract of *S. fruticosa* showed 93.06%, dichloromethane extract 89.23%, and ethyl acetate extract 63.86% at 100 μ g/ml concentration. enol et al. (2011) determined the antioxidant activities of the dichloromethane, ethyl acetate, and ethanol extracts of three wild-growing and one cultivated sample of *S. fruticosa*. All extracts of cultivated *S. fruticosa* showed better scavenging activity against DPPH than the wild samples. The ethanol extract of cultivated *S. fruticosa* also showed greater activity and dose-dependent in the FRAP test (absorbances of 0.699-1.755) comparing to the ethanol extract of wild *S. fruticosa*.

Dincer et al. (2012) compared wild and cultivated *S. fruticosa* in two seasons using different methods, and obtained higher activity for samples in the second harvesting year. Topcu et al. (2013) examined the Anatolian *S. fruticosa* for the antioxidant activity and have found high DPPH free radical scavenging activity (93.46%) at concentration of 100 μ g/ml, and also high radical scavenging activity in the other tests. Pizzale et al. (2002) also have found high antioxidant activity for Cretan *S. fruticosa*. Pasiás et al. (2010) determined the IC_{50} values for ethanolic extract of Cretan

S. fruticosa leaves ranging from 14.1 to 27.4 mg/l. Papageorgiu et al. (2008) reported the IC₅₀ values for the methanolic extract of *S. fruticosa* collected in Zakynthos island ranging from 21.30 to 46.15 mg/l. Matsingou et al. (2003) analyzed the antioxidant activity of four organic solvent extracts from aqueous infusions of *S. fruticosa* and found antioxidant activity, reflecting 77% inhibition of oxidation in comparison to the control. The less polar solvent extracts of *n*-hexane, diethyl ether, and ethyl acetate exhibited high antioxidant activity, reflecting 76%, 68% and 52% inhibition of oxidation, respectively, in comparison to the control. Tawaha et al. (2007) obtained 175 µmol TE/g dry weight for methanolic and 53.4 µmol TE/g dry weight for water extract of Jordanian *S. fruticosa*. Al-Mustafa & Al-Thunibat (2008) examined the antioxidant activity of some Jordanian medicinal plants used traditionally for treatment of diabetes, and found moderate (DPPH-TEAC range 20-80 mg/g activity of *S. fruticosa* shoot.

The infusions of the seven plant species sold in Turkey, including *S. fruticosa*, were preliminarily tested for their antioxidant activity and the *S. fruticosa* infusion exerted the best activity in the DPPH radical scavenging and FRAP assays. Several studies also showed significant antioxidant activity of the polar (water, ethanol, methanol, etc.) extracts of *S. fruticosa*, which was attributed to the existence of phenolic compounds, including rosmarinic acid, caffeic acid, carnosol, apigenin, and luteolin. Accordingly, it could be speculated that the high antioxidant activity of this species could result from the presence of similar phenolic compounds in the infusion. However, not all the *S. fruticosa* samples purchased for this study displayed high radical scavenging activity (Orhan et al., 2010).

4.2.2.4. Correlation between antioxidant assays, total phenolic and flavonoid contents

Pearson's correlation coefficients (*r*) were calculated between content of polyphenols and antioxidant activity of extracts measured by four different assays, and presented in Table 8.

Table 8. Pearson's correlation coefficients between antioxidant activity and content of phenolic components.

	DPPH assay	ABTS assay	FRAP assay	β-CB assay
PHENOLIC ACIDS	-0.001 ^a	0.724 ^c	0.741 ^c	0.341 ^a
Caffeic acid	0.084 ^a	0.602 ^b	0.905 ^c	0.597 ^b
<i>Flavones</i>	0.030 ^a	0.803 ^c	0.148 ^a	-0.007 ^a
Apigenin and its glycosides	0.287 ^a	0.272 ^a	-0.520 ^b	-0.609 ^b
Luteolin and its glycosides	0.167 ^a	0.692 ^c	0.105 ^a	-0.154 ^a
Genkwanin glycosides	-0.366 ^b	0.943 ^c	0.705 ^c	0.691 ^c
<i>Flanonols</i>	-0.308 ^a	0.892 ^c	0.580 ^b	0.251 ^a
Kaempferol glycosides	-0.270 ^a	0.936 ^c	0.573 ^b	0.309 ^a
Quercetin glycosides	0.126 ^a	-0.923 ^c	-0.471 ^b	-0.426 ^b
TPC (HPLC)	-0.153 ^a	0.889 ^c	0.437 ^b	0.144 ^a
TFC (HPLC)	-0.204 ^a	0.908 ^c	0.457 ^b	0.172 ^a
TPC (spec)	-0.729 ^c	-0.133 ^a	-0.381 ^b	-0.002 ^a
TFC (spec)	-0.765 ^c	-0.104 ^a	0.180 ^a	0.324 ^a
DPPH assay	1	-0.473 ^b	-0.168 ^a	-0.291 ^a
ABTS assay	-	1	0.510 ^b	0.439 ^b
FRAP assay	-	-	1	0.824 ^c
β-CB assay	-	-	-	1

^a $r < 0.35$ weak correlation, ^b $0.36 < r < 0.67$ moderate correlation, ^c $0.68 < r < 1$ strong correlation (According to Taylor, 1990).

DPPH assay was weakly correlated to content of identified phenolic components. Regarding the fact that this assay showed strong correlation to spectrophotometrically measured total phenolic and flavonoid content, it might be assumed that some of non-identified component could be responsible for DPPH scavenging activity. The other assays were more strongly correlated to total flavonoid contents determined by HPLC than to those measured by spectrophotometer. Content of the genkwanin, and especially, kaempferol glycosides were the most strongly correlated to ABTS activity ($r > 0.900$). Presence of caffeic acid ($r = 0.905$) and genkwanin glycosides ($r = 0.705$) could contribute to FRAP activity of extracts. Also, genkwanin glycosides could be responsible for inhibition in the β -CB assay ($r = 0.691$). Antioxidant tests were weakly to strongly correlated (correlation coefficients from 0.169 to 0.824).

The other researchers also have found the correlation of antioxidant activity to the amount of total phenolic content in *S. fruticosa* (Pizzale et al., 2002; Tawaha et al., 2007; Al-Mustafa and Al-Thunibat, 2008; Papageorgiou et al., 2008; Stagos et al., 2012).

4.2.2.4. Cytotoxic effects of different extracts

Cytotoxic activity of *S. fruticosa* extracts was determined using MTT cell viability assay against HCT-116 and K562 cell lines. The ethanol and water extract were tested on human carcinoma cell line HCT-116 after 24 and 72 h of treatments. As presented in Table 9 and Figure 56, only *S. fruticosa* ethanol extracts showed cytotoxic activity with IC_{50} values (concentration which kill 50% of cells) of 375.96 μ g/ml after 72 h of treatment.

Water extract was not active against HCT-116 cells at tested concentrations. Only the highest concentrations (250 and 500 μ g/ml) of *S. fructiosa* ethanolic extracts possessed cytotoxic properties (Figure 56). Water extract was tested using human immortalized myelogenous leukemia K562 cell line, and results were monitored after 48h of treatment (Table 9). Obtained results showed that *S. fruticosa* water extract performed low cytotoxic activity (IC_{50} value 386 μ g/ml).

Table 9. Cytotoxic effects of *S. fruticosa* extracts presented as IC₅₀ values (µg/ml) on HCT-116 and K562 cell lines.

Extract	24 h	48 h	72 h
Ethanol	>500 ^a	nt	375.96 ± 2.550 ^a
Water	>500 ^a	386.00 ± 31.607 ^b	>500 ^a

^a HCT-116 cell line, ^b K562 cell line.

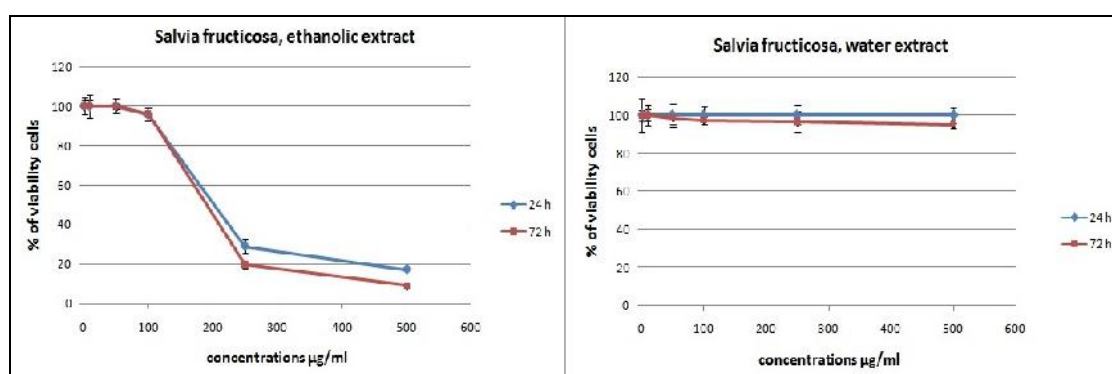


Figure 56. Cytotoxic effects of *S. fruticosa* ethanol and water extracts on HCT-116 cell line after 24 and 72 h of treatment.

Compounds from plants are important source of anti-cancer agents and although plant natural products have long history of use in the treatment of cancer, there is continuing need for development of new anticancer drugs (Hartwell, 1982; Mukherjee et al., 2001; Cragg & Newman, 2005a,b). Although in our study *S. fruticosa* extracts at tested concentrations did not show significant cytotoxic activity on investigated cell lines, *Salvia* species are used in folk medicine worldwide for centuries and several studies noticed potential of *Salvia* species in cancer treatment. among them *S. fruticosa* (Badisa et al., 2005; Kaileh et al., 2007; Xavier et al., 2009; Abu-Dahab et al., 2012).

Xavier et al. (2009) examined the antiproliferative and proapoptotic effect of water extracts of *S. fruticosa*, *S. officinalis* and rosmarinic acid on human colorectal carcinoma cell lines, HCT15 and CO115, which have different mutations in the MAPK/ERK and PI3K/Akt signalling pathways. Their results showed that the *S. fruticosa* water extract induced apoptosis in both cell lines, whereas cell proliferation

was inhibited only in HCT15, and concluded that the activity of sage extract seems to be mostly due to the inhibition of MAPK/ERK pathway.

Crude aqueous extracts of eleven *Salvia* spp., grown and sold in Jordan including *S. fruticosa* were tested for general cytotoxicity against a panel of colorectal cancer cell lines (HT29, HCT116, SW620 and Caco2). None of the tested extracts were found to possess cisplatin- or doxorubicin-like antiproliferative capacities in comparison to non induced basal incubations (Kasabri et al., 2014).

Kaileh et al. (2007) tested the organic extracts of 24 selected Palestinian medicinal plant species for their potential cytotoxic effect on the murine fibrosarcoma L929sA cells, and on the human breast cancer cells MDA-MB231 and MCF7. Among examined plant extracts using MTT colorimetric assay, *S. fruticosa* displayed a remarkable activity. The essential oil was found to be cytotoxic at concentrations that range between 180 µg/ml in L929sA mouse fibrosarcoma cells to 290 µg/ml in MDA-MB 231 metastatic human breast carcinoma cells, indicating differential sensitivities of various cancer cell lines to the oil.

Abu-Dahab et al. (2012) investigated ethanol extracts from nine *Salvia* species grown in Jordan for their antiproliferative activity using a panel of breast cancer cell. The results indicated that the extracts of three *Salvia* species, among them *S. fruticosa*, exhibit selective antiproliferative activity against estrogen positive cell lines and with minimum toxicity against normal human periodontal fibroblasts.

Against four cell lines *S. fruticosa* showed IC₅₀ value bellow 30 µg/ml. Jordanian *S. fruticosa* was investigated also by Ibrahim and Aqel (2010). Water and methanolic extracts applied to different cell lines of human carcinoma exhibited time-dependent, cell specific inhibitory effects on HEP-2, RD and AMN3 malignant cell lines. AMGM5 cell line was resistant to the effects of both extract as inhibition could only be recorded after 72 h of exposure to the highest extracts concentrations, 625 and 1250 µg/ml, which were much higher comparing to concentrations used in our study.

In the study of Al-Kalaldehy et al. (2010) the crude ethanol extract of Jordanian *S. fruticosa* showed antiproliferative activity to the adenocarcinoma of breast cell line (MCF7) with IC₅₀ values of 25.25 µg/ml, but the aqueous extract and hydrodistilled essential oil did not demonstrate cytotoxic activity.

Five *Salvia* species, including *S. fruticosa*, were collected from different Greek islands and evaluated for cytotoxic activities against brine shrimps and four human cancer cell lines (human colon adenocarcinoma (HCA), HepG2, MCF-7 and human pancreatic carcinoma (HPC) (Badisa et al., 2005). The obtained results showed high activity of *S. fruticosa* against human cancer cell lines, especially the samples from Kalymnos and Crete. Among tested samples, only *S. fruticosa* collected from Kalymnos was active against HepG2 cells with $IC_{50}=68.1 \mu\text{g/ml}$.

The cytotoxic effects of investigated extracts varied depending on the localities and seasons, which was already concluded in the studies of *S. fruticosa* essential oils (Skoula et al., 2000). Further analysis of essential oil and extracts from different seasons and localities in Libya should be done in order to clarify the cytotoxic potential of Libyan *S. fruticosa*.

4.2.2.5. Antimicrobial effects of different extracts

4.2.2.5.1. Antibacterial activity

Antibacterial activity of *S. fruticosa* ethanol and water extracts was tested using microdilution method against six Gram-negative and five Gram-positive pathogenic bacteria. All of examined extracts inhibited bacterial growth with MIC values ranged as 10-35 mg/ml. Ethanol extract showed stronger activity (MICs 10-25 mg/ml) than water extract (MICs from 20 to 35 mg/ml). The inhibitory effect of sage both extracts were more pronounced against Gram positive bacteria (10-25 mg/ml). Inhibitory effect of extracts was weaker comparing to streptomycin (MICs ranged as 0.005-0.016 mg/ml). The most resistant bacteria were *Pseudomonas tolasii* and *P. aeruginosa*, while the most sensitive bacteria were *S. aureus*, *B. cereus* and *L. monocytogenes* (Table 10).

The ethanol and water extracts were chosen for testing of the antimicrobial activity since the obtained results in the present study of antioxidant activities showed significant activity for that extracts. Moreover, mixtures of ethanol/water and water extracts are recommended for human usage because of their non-toxicity (Durling et al., 2007; Stagos et al., 2012).

Table 10. Antibacterial activity of *Salvia fruticosa* extracts and standard antibiotic streptomycin expressed as minimal inhibitory concentration (MIC, mg/ml).

Bacteria	Extracts		Streptomycin
	Ethanol	Water	
Gram-negative bacteria			
<i>Esherishia coli</i> ATCC 25922	15	25	0.012
<i>Salmonella typhimurium</i> ATCC 14028	15	30	0.010
<i>Salmonella enteritidis</i> ATCC 13076	15	25	0.010
<i>Pseudomonas tolasii</i> NCTC 387	20	35	0.016
<i>Pseudomonas aeruginosa</i> ATCC 27853	25	35	0.016
<i>Proteus mirabilis</i> ATCC 14273	15	30	0.005
Gram-positive bacteria			
<i>Staphylococcus aureus</i> ATCC 25932	10	25	0.016
<i>Bacillus cereus</i> ATCC 10876	10	25	0.005
<i>Micrococcus flavus</i> ATCC 14452	12	20	0.010
<i>Sarcina lutea</i> ATCC 10054	15	30	0.012
<i>Listeria monocytogenes</i> ATCC 15313	10	25	0.010

In the present study, data indicates that both Gram-positive and Gram-negative bacteria were affected by the sage extracts tested. However, Gram positive strains were more sensitive. This finding is consistent with previous studies on a range of herbs and spices (Shan et al., 2007). This could be due to the fact that the cell wall of Gram positive bacteria is less complex and lack the natural sieve effect against large molecules due to the small pores in their cell envelope. There is also a difference in the outer membrane arrangement which, in Gram-negative strains, serves as penetration barrier towards macromolecules (Nikaido, 1996, 2003).

Gonaid et al. (2012) analyzed the ethanol extract obtained from Libyan *Salvia fruticosa* and their results showed a moderately high activity against *E. coli* and *Pseudomonas aeruginosa*. *Salvia fruticosa* possess higher inhibitory effects against *Bacillus subtilis* and a moderate effect on *Staphylococcus aureus*. In the analysis of antimicrobial activities of methanol extracts of Turkish medicinal plants, Askun et al. (2009) found that *S. fruticosa* displayed high activity on Gram-negative bacteria, with MIC 640 µg/ml against *S. typhimurium* and *E. aerogenes*, which could be probably attributed to the significant amount of carvacrol in the extract.

Several papers on *S. fruticosa* antibacterial or antifungal activities were dealing with essential oil (Shimoni et al., 1993; Muller-Riebau et al., 1995; Sivropolou et al., 1997; Adam et al., 1998; Pitarokili et al., 2003; Delamare et al., 2007), which has shown effectiveness varying from slight to high, against tested microorganisms. In the previous study of the antimicrobial activity of the essential oil of the libiyan *S. fruticosa* against eight bacteria and eight fungi, the essential oil showed minimal inhibitory activity (MIC) at 0.125-1.5 mg/ml and bactericidal (MBC) at 0.5-2.0 mg/ml, and fungistatic (MIC) at 0.125-1.0 mg/ml and fungicidal effect (MFC) at 0.125-1.5 mg/ml (Giweli et al., 2013b).

4.2.2.5.2. Antifungal activity

Antifungal activity of *Salvia fruticosa* ethanolic and water extracts was investigated using microdilution assay and results were shown in Table 11 along with those for the ketoconazole. *Salvia fruticosa* extracts did not show fungistatic and fungicidal effects on tested *Candida* species at all tested concentrations. *A. glaucus* was the only sensitive *Aspergillus* species, particularly to the water extract of *S. fruticosa* (MIC 16 mg/ml, MFC 32 mg/ml).

Water extract in concentration of 32 mg/ml showed fungicidal effect on *T. mentagrophytes* while ethanolic extract had no effect on its growth. The activity of *S. fruticosa* extracts and essential oil was estimated as poor comparing to values obtained for ketoconazole.

Table 11. Antifungal activity of *S. fruticosa* extracts and standard antimycotic ketoconazole against selected micromycetes presented as minimal inhibitory concentration (MIC - mg/ml) and minimum fungicidal concentration (MFC - mg/ml).

Micromycetes	Extracts				Ketoconazole	
	Ethanol		Water		MIC	MFC
	MIC	MFC	MIC	MFC		
<i>Candida krusei</i>	-	-	-	-	0.0078	0.0156
<i>Candida albicans</i>	-	-	-	-	0.0078	0.0156
<i>Candida parapsilosis</i>	-	-	-	-	0.0078	0.0156
<i>Aspergillus glaucus</i>	64	64	16	32	0.0078	0.0078
<i>Aspergillus fumigatus</i>	-	-	-	-	0.0078	0.0156
<i>Aspergillus flavus</i>	-	-	-	-	0.0078	0.0078
<i>Trichophyton mentagrophytes</i>	-	-	32	32	0.0019	0.0039

In the research of Gonaïd et al. (2012) the ethanol extract of Libyan *S. fruticosa* demonstrated a moderate inhibitory activity against *Candida albicans*, with no effect on *Aspergillus flavus*.

Exarchou et al. (2015) studied the antifungal activity of the crude ethyl acetate extract and selected phytochemicals was estimated against the plant pathogenic fungal species *Aspergillus tubingensis*, *Botrytis cinerea*, and *Penicillium digitatum*. The estimated MIC and MFC values of the ethyl acetate extract of *S. fruticosa*, as well as three of its major constituents (carnosic acid, carnosol, and hispidulin), demonstrated antifungal activity, especially against *B. cinerea* and *P. digitatum*, suggesting their potential use in food and agricultural systems.

The antimycotic activity of petroleum ether and methanolic extracts of nine Lebanese wild plant species against eight phytopathogenic fungi was evaluated. The efficacy of *S. fruticosa* petroleum ether extract against phytopathogenic fungi tested was

higher than that of methanolic extract. *S. fruticosa* PE extract was active on *Botrytis cinerea*, *Verticillium dahlia*, *Fusarium oxysporum* f. sp. *melonis*, and *Alternaria solani* but not on *Penicillium* sp. and *Cladosporium* sp. (Abou-Jawdah et al., 2002).

4.1.2.6. Acetylcholinesterase and tyrosinase inhibitory activities

Alzheimer's disease (AD) is a chronic, progressive degenerative neurological disorder characterized by senile plaques containing amyloid β protein and loss of cholinergic neuromediators in the brain, which cause learning and memory impairment, cognitive dysfunction, and behavioral disturbances. Alzheimer's disease mainly affects elderly individuals and has become a major threat to the increasing ageing population in developed countries (Perry et al., 1996). The cholinergic hypothesis states that the cognitive decline in AD is consequence of deficit in central cholinergic neurotransmission resulting from a loss of acetylcholine (ACh) (Grossberg, 2003). Therefore, inhibition of acetylcholinesterase (AChE), the enzyme which hydrolysis ACh into choline and acetic acid, is currently the most widely used treatment options against AD (Giacobini et al., 2002). Numerous herbal extracts have been used to treat neurological disorders in folk medicine, among them *Salvia* species are known for their beneficial effects, especially *S. lavandulaefolia*, *S. officinalis*, *S. fruticosa* (Perry et al., 2000b; Howes et al., 2003; enol et al., 2010; Orhan et al., 2012, 2013).

Tyrosinase is a multifunctional, glycosylated, and copper-containing oxidase, which catalyzes the first two steps in mammalian melanogenesis and is responsible for enzymatic browning reactions in damaged fruits during post-harvest handling and processing. Neither hyperpigmentation in human skin nor enzymatic browning in fruits is desirable. These phenomena have encouraged researchers to seek new potent tyrosinase inhibitors for use in foods and cosmetics (Chang, 2009). It might also be involved in neuromelanin production and be associated with Parkinson's disease, by acceleration the induction of catecholamine quinone derivatives by its oxidase activity (Asanuma, 2003; Hasegawa, 2010).

Enzyme inhibition activity of *S. fruticosa* extracts was tested at three concentrations (25, 50 and 100 $\mu\text{g/ml}$) against two enzymes, acetylcholinesterase (AChE) and tyrosinase (TYR), and results were presented in Table 12. In AChE

inhibition assay, extracts exhibited weaker activity (16.28-26.82%) than standard galanthamine (42.38-57.11%). Although galanthamine inhibited AChE in dose-dependent manner, the extracts performed strongest activity at 50 µg/ml, as it was reported before (Enol et al., 2010; Orhan et al., 2013).

Table 12. Acetylcholinesterase and tyrosinase inhibition activity of *S. fruticosa* extracts and standards expressed as percentage (%).

Conc. (µg/ml)	AChE inhibition (%)			TYR inhibition (%)		
	Extracts		Galanthamine	Extracts		Kojic acid
	Ethanol	Water		Ethanol	Water	
25	24.44 ±	23.17 ±	42.38 ± 0.740	62.25 ±	59.38 ±	35.73 ±
	3.717	0.996		0.648	5.749	5.460
50	25.95 ±	26.82 ±	50.56 ± 0.510	62.35 ±	74.66 ±	33.93 ±
	5.463	2.040		1.075	1.576	3.780
100	25.73 ±	16.28 ±	57.11 ± 1.670	62.99 ±	57.89 ±	51.81 ±
	3.701	0.586		0.681	7.380	2.550

As shown in Table 12, *S. fruticosa* extracts performed stronger activity in TYR inhibition assay in comparison with standard kojic acid (33.93-51.81%) at tested concentrations.

Orhan et al. (2012) found no or very low inhibition of 16 *Salvia* species from Turkey towards TYR at 100 µg/ml. In research of Süntar et al. (2011), ethanol extracts of *S. cryptantha* and *S. cyanescens* performed TYR inhibition weaker than those obtained in present study. Caffeic acid (Roseiro et al., 2012), kaempferol, quercetin and its glycosides (Chang, 2009), identified in alcoholic and water extract of *S. fruticosa*, were previously recognized as strong AChE and TYR inhibitors, while literature data on genkwanin are not available till now.

In Anatolian folk medicine, the leaves of *S. fruticosa* are used as infusion (1-5%) for simple disorders, and it is one of the plants used for memory enhancing and neuroprotection (Topcu & Kusman, 2014).

The AChE inhibitory activity of methanol, n-butanol, and water extracts from three different *Salvia* species, which grow in the Mediterranean area were tested, and the methanol extract of *S. trichoclada* showed the highest inhibition on AChE, while methanol extracts of *S. verticillata* and *S. fruticosa* showed lower inhibition (Demirezer et al., 2014).

In the evaluation of cholinesterase inhibitory effects of wild and cultivated samples of *S. fruticosa*, plant extracts showed moderate anti-cholinesterase activity (Senol et al., 2011). However, other studies of a methanol extract of *S. fruticosa* showed strong activity against both AChE and BChE (Topcu & Kusman, 2014).

Orhan et al. (2010) found that infusions of Turkish plants *Salvia fruticosa*, *Sideritis congesta*, *Salvia tomentosa*, *Sideritis pisidica* var. *termessi*, *Sideritis arguta*, *Sideritis perfoliata*, and *Sideritis libanotica* subsp. *linearis* showed no inhibitory effect against AChE. Previous research revealed that the components of sage which are active against AChE were the monoterpenes 1,8-cineol and α -pinene found in major amounts in the essential oils (Savelev et al., 2004). Ineffectiveness of the infusions towards AChE could be attributed to nonsolubility of the essential oils in water.

4.3. *Salvia lanigera*

The aerial parts of Jordanian *S. lanigera* were investigated for the composition of the essential oil (Flamini et al., 2007), but the glandular trichomes, which produces the oil, were not analyzed previously. The leaves of *S. lanigera* are used as an aromatic tea for a variety of abdominal troubles (Al-Hazimi et al., 1984). Since the plant possess potentially medicinal value, and there is no data about biological activities of *S. lanigera* originated from Libya, it was subjected to the analysis of several biological activities.

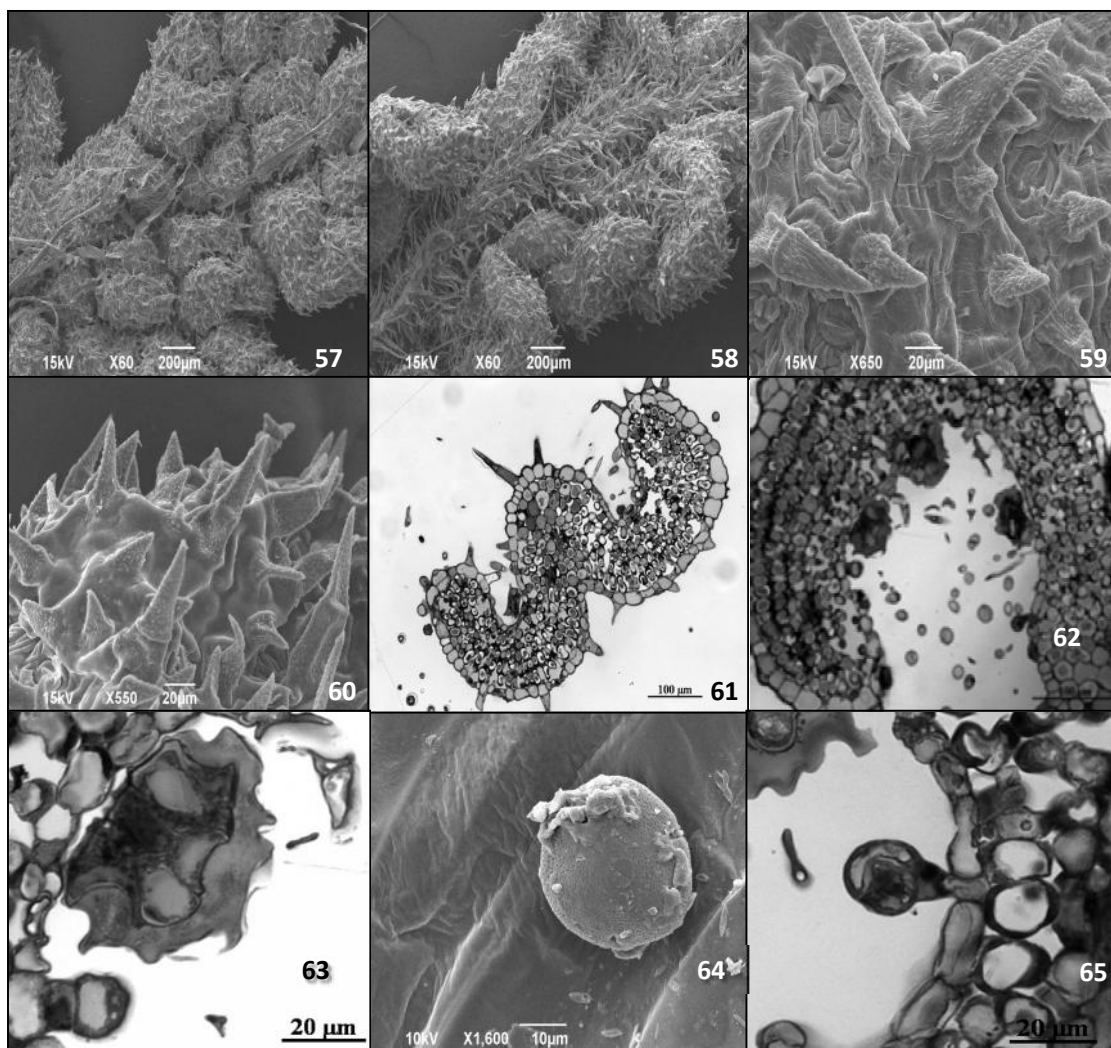
4.3.1. Micromorphological and histological analysis of leaf trichomes of *S. lanigera*

The leaves of *S. lanigera* are deeply pinnatisect, lobes irregular, linear, 10 x 5 cm (Jafri & El-Gadi, 1985) and covered with an indumentum of non-glandular and glandular trichomes on both leaf surfaces. The micromorphological analysis of leaf trichomes of Libyan *S. lanigera* showed the presence of non-glandular and glandular trichomes (Fig. 57 and Fig. 58). The non-glandular trichomes were densely distributed on the adaxial and abaxial side, unbranched, unicellular, and elongated multicellular, uniseriate, erect or slightly leaned toward the epidermis (Fig. 59 and Fig. 60).. The unicellular trichomes are of triangular shape with warty surface (Fig. 59). Unicellular trichomes are epidermal appendages, common in Lamiaceae. The other type of non-glandular trichomes was multicellular, much longer and slimmer than unicellular, variable in length (Fig. 57 and Fig. 58).

The glandular trichomes included peltate and capitate types. The peltate trichomes release the secretion product to the outside when the trichomes is touched («long-term glandular trichomes», as reported in Werker (1993). They were located in concave parts of leaf abaxial side (Fig. 61 and Fig. 62). The peltate trichomes consisted of one basal epidermal cell, embedded in the epidermis, short cylindrical stalk cell with cutinized lateral walls (Fig. 63) and large round or slightly flattened multicellular secretory head (Fig. 64) of eight secretory cells arranged in a single circle.

In capitate trichomes secretory materials are extruded to the outside soon after their production («short term glandular trichomes», as reported in Werker (1993). Capitate trichomes consisted of one-celled glandular head, subtended by a stalk of one cell, so-called short-stalked capitate trichome (Fig. 65).

Trichomes are epidermal appendages of various form, structure and function. They are derived from the various epidermal cells on vegetative organs and floral parts. Trichomes are considered as the physical barriers against external factors, such as animals and pathogens. They also serve to restrict loss of water or to give protection against extreme temperature and ultraviolet radiation (Werker, 2000).



Figures 57 – 65. SEM and LM micrographs of *S. lanigera* leaves.

- 57.** SEM micrograph showing trichome indumentum on adaxial leaf surface.
 - 58.** SEM micrograph showing trichome indumentum on abaxial leaf surface
 - 59.** SEM micrograph showing non-glandular trichomes on adaxial leaf surface.
 - 60.** SEM micrograph showing non-glandular trichomes on abaxial leaf surface.
 - 61.** LM micrograph showing cross-section of whole leaf with non-glandular and glandular trichomes.
 - 62.** LM micrograph of leaf cross-section showing peltate trichomes.
 - 63.** LM micrograph of peltate and short-stalked capitate trichomes.
 - 64.** SEM micrograph showing peltate trichome.
 - 65.** LM micrograph of short-stalked capitate trichome.
- Bar = 200 μ m (57, 58); 100 μ m (61, 62); 20 μ m (59, 60, 63, 65), 10 μ m (64).

Many plants possess glandular trichomes to produce secondary compounds that are used for pollination, defense, and protection. Scientific interest in trichomes is established on their functional and taxonomic importance and on the economic usefulness of some metabolites produced in trichomes. Several Lamiaceae genera have been studied for the essential oils secreted by glandular trichomes and for their potential uses in industry. Many of these plants are cultivated for ornamental and other economical purpose, as in pharmacy and cosmetics (Choi & Kim, 2013).

Considering the large number of *Salvia* species there is a lack of agronomic and morphological comparative information among the different species, which may be relevant for species characterization and industrial applications (Mossi et al., 2011).

The present study was designed to provide useful information on the foliar micromorphology of *S. lanigera*. Like other members of the Lamiaceae, *S. lanigera* carries both peltate and capitate glandular trichomes, as well as unicellular and multicellular non-glandular ones. Unicellular non-glandular trichomes of *S. lanigera* were warty, and these structures are believed to arise either from the cell wall or the cuticle (Werker, 2000), in order to thicken the trichome walls. Their role is protection from water loss, the regulation of temperature through their reflective capacity and mechanical protection from herbivores (Ascensão et al., 1995; 1999). Plant species which contain glandular trichomes generally produce relatively large amounts of bioactive compounds including phytochemicals with biological activity of interest to many industries (Dyubeni & Buwa, 2011).

The density of glandular trichomes decreases with leaf growth (young leaves have a denser pubescence). This might be an adaptive mechanism, whereby the young leaves, most tender and appetizing to herbivores, are given the highest protection (many secretions of glandular trichomes are deterrent or toxic to insects). The density of glandular trichomes is further considered to be associated with transpiration, leaf overheating, UV-B radiation, etc. (Wagner et al., 2004).

On the basis of external morphology and anatomy, the glandular trichomes of *S. lanigera* were similar to the two main types occurring in the Lamiaceae. The current study showed the peltate glandular trichomes consisting of a basal epidermal cell, a very short stalk cell and large round head of eight secretory cells arranged in a circle, which

is in agreement with other studies of the Lamiaceae (Werker et al., 1985; Hallahan, 2000).

In the present research the short-stalked capitate trichomes were noticed. Capitate trichomes are also widespread in Lamiaceae, but they are more variable in stalk length and head shape. They generally consist of one to two stalk cell(s) and one to two cell(s) forming a rounded to pear-shaped secretory head (Werker et al. 1985). Capitate trichomes are assumed to secrete varying amounts of polysaccharides along with essential oils (Werker, 1993). The material within the cuticular sac of capitate trichomes is stored in the form of lipophilic substances such as terpenes, lipids, waxes and flavonoid aglycones. When the cuticular sac ruptures due to external pressures these substances are released to the outside of the cuticular sac and kept on the surface layer of the plant (Dyubeni & Buwa, 2011).

4.3.2. Composition and biological effects of different extracts

This comprehensive research was aimed to investigate phenolic content and composition as well as biological activities of several extracts prepared from the aerial parts of the wild growing Libyan *S. lanigera* for the first time. For testing of cytotoxic, antimicrobial, acetylcholinesterase and tyrosinase inhibitory activities, ethanolic and water extracts, widely used in phytotherapy, were selected.

4.3.2.1. The yield of extracts, total phenolic and flavonoid content

The plant material of *S. lanigera* was extracted using individual and successive extraction procedure and yields of extracts are presented in Table 13. The yields of methanol and ethanol extract were the highest (7.60 and 7.40%, respectively) while successively extracted dichloromethane and ethyl acetate extracts poorly yielded (2.29 and 0.98%, respectively).

Different yields of extracts were obtained in the previous studies of *Salvia* species, which could be influenced by plant development stage, extraction conditions, geographical region, climate, collecting season, or ecological conditions (Papageorgiou et al., 2008). enol et al. (2010) tested 165 extracts prepared with dichloromethane, ethyl acetate, and methanol obtained from 55 Turkish *Salvia* species and found that the

yields of methanolic extracts are the highest (2.88-13.41 %), while the yields for ethyl acetate extracts are the lowest (0.14-1.93 %), which is also obtained in our study. The other authors also confirmed that polar alcoholic extracts, such as ethanol and methanol, provide higher yield than less and/or non-polar solvents extracts (Akkol et al., 2008; Orhan et al., 2012).

Table 13. Yield of the extracts, total phenolic content (TPC) and total flavonoid content (TFC) of *S. lanigera* extracts

Extracts	Yield ^a	TPC ^b	TFC ^c
Ethanol	7.40	43.04 ± 0.849	26.42 ± 0.925
Water	7.32	58.47 ± 0.200	17.18 ± 0.544
Chloroform	5.64	35.54 ± 2.403	36.31 ± 2.502
Acetone	5.88	56.70 ± 0.701	29.27 ± 0.381
Dichloromethane	2.29	67.39 ± 1.596	35.87 ± 0.627
Ethyl acetate	0.98	64.99 ± 1.841	51.59 ± 1.078
Methanol	7.60	51.11 ± 2.203	28.42 ± 1.469

^a percentage of yield (%). ^b mg GAE/g dry extract. ^c mg QE/g dry extract

Total phenolic and flavonoid content were measured using spectrophotometric method and results are presented in Table 13. Among examined extracts, successively extracted dichloromethane and ethyl acetate extracts showed the largest total phenolic content (more than 60 mg GAE/g) whereas chlorophorm extract was the poorest in total phenolics (35.54 mg GAE/g). Flavonoid contents of extracts were measured from 26.42 mg QE/g for ethanol to 51.59 mg QE/g for ethyl acetate extract. The obtained results were in consistent with other studies of *Salvia* species of different origin (Akkol et al., 2008; Kamatou et al., 2010; Asadi et al., 2010; Orhan et al., 2012; Stagos et al., 2012; Alimpi et al., 2015).

4.3.2.2. Composition of different extracts

Five *Salvia lanigera* solvent extracts were investigated for phenolic composition using HPLC and results are presented in Table 14. The majority of identified components was extracted by methanol and water (81.52% and 53.60%), followed by ethanol and less polar ethyl acetate and dichloromethane extracts. Among phenolic acids, it was detected only caffeic acid, mainly in the water extract (16.07%).

Flavonoids, with flavones and flavonols, were predominantly extracted by more polar solvents, such as methanol, ethanol and water. Contents of flavone luteolin glycosides and flavonol kaempferol glycosides, especially in methanol extract (33.25% and 38.91%, respectively), were the highest. Genkwanin glycosides were extracted only by dichloromethane (1.21%), and this extract showed the highest content of hyperoside (7.71%). Among other polyphenols, coumarin was detected (0.82-1.27%), mostly in ethyl acetate extract.

Although numerous researchers have reported the rosmarinic acid as the most abundant phenolic acid in *Salvia* species (Skoula et al., 2000; Akkol et al., 2008; Kamatou et al., 2010; Ko ar et al., 2011; Lu & Foo, 1999; 2002; Dincer et al., 2012; Orhan et al., 2012; Ben Farhat et al., 2013a,b), it was not detected in our samples. Differences could be attributed to the developmental stage of plants and also to genetic variation in populations (Skoula et al., 2000; Dincer et al., 2012).

Table 14. Phenolic components of *S. lanigera* extracts presented as percentage (%).

Extracts	DCM	ETAC	MEOH	ETOH	W
Constituents					
PHENOLIC ACIDS					
Caffeic acid	-	-	0.88	0.30	16.07
FLAVONOIDS					
<i>Flavones</i>					
Apigenin	-	4.36	0.46	-	-
Apigenin glycosides	-	-	4.17	1.07	3.76
Genkwanin glycosides	1.21	-	-	-	-
Luteolin	-	1.35	1.80	0.71	3.89
Luteolin glycosides	-	-	33.25	8.29	6.81
<i>Flavonols</i>					
Kaempferol glycosides	-	-	38.91	9.69	23.07
Hyperoside	7.71	6.63	1.19	5.93	-
OTHER POLIPHENOLS					
Coumarin	0.82	1.27	0.86	-	-
Total (%)	9.74	13.61	81.52	25.99	53.60

^aDCM-dichloromethane; ETAC-ethyl acetate; MEOH-methanol; ETOH-ethanol; W-water

4.3.2.3. Antioxidant effects of different extracts

Reactive oxygen species (ROS) resulting from biochemical reactions or from external factors, are involved in various physiological and pathological processes leading to serious health problems (Haliwell, 1996).

Antioxidants prevent free radical induced tissue damage by acting as free radical-scavengers which may be important in inhibition or decreasing the oxidation and

disease prevention. Since the synthetic antioxidants could be dangerous to human health, the search for effective nontoxic natural compounds possessing antioxidant activity has been intensified in recent years revealing the plant-derived antioxidants as a suitable alternative (Lobo et al., 2010).

The antioxidant activity of *S. lanigera* extracts was performed using four parallel test systems: DPPH, ABTS, FRAP assay and β -carotene (β -CB) assay (Table 15). DPPH scavenging activities of extracts, ranged from 205.45 μ g/ml for water to 1687.45 μ g/ml for chloroform extract, was evaluated as low comparing to standards (5.11-17.94 μ g/ml). Water and methanol extract were the most powerful against ABTS radical (1.77 and 1.06 mg AAE/g, respectively), while chloroform extract showed the poorest activity (0.25 mg AAE/g). Similarly, methanol extract showed the most expressive ability to reduce Fe (III) to Fe (II) ion (200.11 μ mol Fe (II)/g) unlike water extract with 79.13 μ mol Fe(II)/g. Standards BHA and BHT, tested at concentration of 0.1 mg/ml, performed quite stronger activity in ABTS and FRAP assays than *S. lanigera* extracts. In β -CB assay, water and ethanol extracts exhibited activity (77.66% and 76.06%, respectively) stronger than standards BHA and BHT, while other extracts performed inhibition below 20%.

The essential oils and extracts of *S. lanigera* from different countries were previously subjected to research of the antioxidant potential. The antioxidant activity of methanol, acetone and *n*-butanol extracts of Egyptian *S. lanigera* was tested by Shaheen et al (2011). Among examined extracts, acetone extract showed the highest activity in DPPH assay (65.5% decolorisation) while methanol extract (59.8%) and *n*-butanol extract (57.2%) showed slightly more activity than BHT (52.6%). The methanolic extracts also showed high scavenging capacity in previous studies of various *Salvia* species. Ben Farhat et al. (2013a) investigated antioxidant activities of methanolic extracts of *S. officinalis*, *S. verbenaca*, *S. aegyptiaca* and *S. argentea*, growing in various habitats in Tunisia, using DPPH (3.37-77.07 μ g/ml), ABTS (141.23-766.30 μ M TE/mg) and FRAP (81.56-197.33 mM Fe(II)/mg) assay.

Table 15. Antioxidant activity of *S. lanigera* extracts.

Samples	DPPH assay (IC ₅₀ .. µg/ml)	ABTS assay (mg AAE/g)	FRAP assay (µmol Fe(II)/g)	β-CB assay (% inhibition)
Extracts				
Ethanol	448.49 ± 1.555	0.68 ± 0.053 ^a	137.61 ± 2.294 ^b	76.06 ± 2.764 ^b
Water	205.45 ± 5.027	1.77 ± 0.085 ^a	79.13 ± 5.255 ^b	77.66 ± 2.764 ^b
Chloroform	1687.45 ± 88.333	0.25 ± 0.021 ^a	190.75 ± 0.811 ^b	nt
Acetone	1290.63 ± 22.762	0.96 ± 0.038 ^a	83.72 ± 1.662 ^b	nt
Dichloromethane	614.23 ± 7.028	0.64 ± 0.020 ^a	154.43 ± 4.027 ^b	10.64 ± 2.257 ^b
Ethyl acetate	406.86 ± 4.109	0.55 ± 0.020 ^a	80.28 ± 3.244 ^b	18.62 ± 2.257 ^b
Methanol	222.16 ± 5.501	1.06 ± 0.104 ^a	200.11 ± 2.433 ^b	12.23 ± 4.787 ^b
Standards				
BHT	17.94 ± 0.168	2.75 ± 0.021 ^c	445.34 ± 5.772 ^c	57.71 ± 3.385 ^b
BHA	13.37 ± 0.430	2.82 ± 0.011 ^c	583.72 ± 5.255 ^c	53.72 ± 2.257 ^b
Ascorbic acid	5.11 ± 0.143	nt	180.81 ± 8.607 ^c	17.82 ± 1.128 ^b

^a at concentration of 1 mg/ml, ^b at concentration of 0.5 mg/ml, ^c at concentration of 0.1 mg/ml; nt -not tested.

4.3.2.3. Correlation between antioxidant assays, total phenolic and flavonoid contents

Content of phenolic components and DPPH, ABTS, FRAP and β -CB antioxidant activities of extracts were correlated in different ways (Table 16). Antioxidant assays more strongly correlate to TPC and TFC determined by HPLC than to those measured spectrophotometrically. Antioxidant assays were more strongly correlated to total phenolic than to flavonoid content and these findings are in agreement with previous studies (Asadi et al., 2010; Ben Farhat et al., 2009; Li et al., 2008; Stagos et al., 2012).

Antioxidant tests were weakly, moderate to strongly (DPPH and ABTS assays) correlated among them. According to correlation coefficients presented in Table 16, contents of caffeic acid, flavones apigenin, luteolin and its glycosides and flavonol kaempferol glycosides could contribute to antioxidant activity of extracts evaluated by different methods.

A wide range of phytochemicals present in plants are known for the various health-related effects, such as antibacterial, anticarcinogenic, antimutagenic, etc. (Cowan, 1999; Nickavar et al., 2007; Lu & Foo, 2001; Qing et al., 2001; Talib & Mahasneh, 2010; Tenore et al., 2010; Dyubeni & Buwa, 2011; Karaka et al., 2012). Organic solvents such as ethanol, acetone, and methanol are often used to extract bioactive compounds (Eloff, 1998).

Ethanol is the most commonly used organic solvent by herbal medicine manufacturers because the finished products can be safely used internally by consumers of herbal extracts (Low Dog, 2009). The ethanol and water extracts were chosen for further tests of antimicrobial activity, cytotoxicity, and enzyme inhibition.

Table 16. Pearson's correlation coefficients between antioxidant activity and content of phenolic components.

	DPPH	ABTS	FRAP	β-CB
	assay	assay	assay	assay
Caffeic acid	-0.606 ^b	0.940 ^c	-0.521 ^b	0.621 ^b
Total flavones	-0.744 ^c	0.381 ^b	0.595 ^b	-0.136 ^a
Apigenin	0.036 ^a	-0.428 ^b	-0.473 ^b	-0.383 ^b
Apigenin glycosides	-0.896 ^c	0.818 ^c	0.254 ^a	0.260 ^a
Luteolin	-0.872 ^c	0.922 ^c	-0.447 ^b	0.493 ^b
Luteolin glycosides	-0.626 ^b	0.271 ^a	0.718 ^c	-0.169 ^a
Total flavonols	-0.777 ^c	0.543 ^b	0.561 ^b	0.022 ^a
Kaempferol glycosides	-0.834 ^c	0.634 ^b	0.467 ^b	0.089 ^a
Hyperoside	0.941 ^c	-0.904 ^c	-0.027 ^a	-0.348 ^a
Coumarin	0.239 ^a	-0.559 ^b	0.139 ^a	-0.920 ^c
TPC (HPLC)	-0.859 ^c	0.644 ^b	0.431 ^b	0.069 ^a
TFC (HPLC)	-0.761 ^c	0.451 ^b	0.586 ^b	-0.080 ^a
TPC (spec)	0.346 ^a	-0.103 ^a	-0.356 ^b	-0.546 ^b
TFC (spec)	0.480 ^b	-0.775 ^c	-0.158 ^a	-0.080 ^a
DPPH assay	1	-0.767 ^c	0.078 ^a	-0.304 ^a
ABTS assay	-	1	-0.229 ^a	0.512 ^b
FRAP assay	-	-	1	-0.454 ^b
β-CB assay	-	-	-	1

^ar 0.35 weak correlation, ^b0.36<r<0.67 moderate correlation, ^c0.68<r<1 strong correlation (According to Taylor, 1990).

4.3.2.4. Cytotoxic effects of different extracts

Cytotoxic activity of *S. lanigera* extracts was determined using MTT cell viability assay against HCT-116 and K562 cell lines. The ethanol and water extract were tested on human carcinoma cell line HCT-116 after 24 and 72 h of treatments and results were presented in Table 17 and Figure 66. Obtained results showed that *S. lanigera* extracts showed very poor cytotoxic activity with IC₅₀ values (concentration

which kill 50% of cells) higher than the highest investigated concentration (500 µg/ml). Water extract was tested using human immortalized myelogenous leukemia K562 cell line, and results were monitored after 48 h of treatment (Table 17). *S. lanigera* water extract showed IC₅₀ value of 383.29 µg/ml, which is assessed as moderate cytotoxic activity.

Compounds from plants are important source of anti-cancer agents and although plant natural products have long history of use in the treatment of cancer, there is continuing need for development of new anticancer drugs (Hartwell, 1982; Mukherjee et al., 2001; Cragg and Newman, 2005a,b). Although in our study *S. lanigera* extracts at tested concentrations showed low and non-significant cytotoxic activity on investigated cell line, *Salvia* species are used in folk medicine worldwide for centuries and several studies pointed out the potential of *Salvia* species as cytotoxic agents (Fiore et al., 2006; Kamatou et al., 2008; Shaheen et al., 2011; Abu-Dahab et al., 2012).

Table 17. Cytotoxic effects of *S. lanigera* extracts on HCT-116 and K562 cell lines presented as IC₅₀ values (µg/ml).

Extract	24 h	48 h	72 h
Ethanol	>500 ^a	nt	>500 ^a
Water	>500 ^a	383.29 ± 49.812 ^b	>500 ^a

^a HCT-116 cell line, ^b K562 cell line

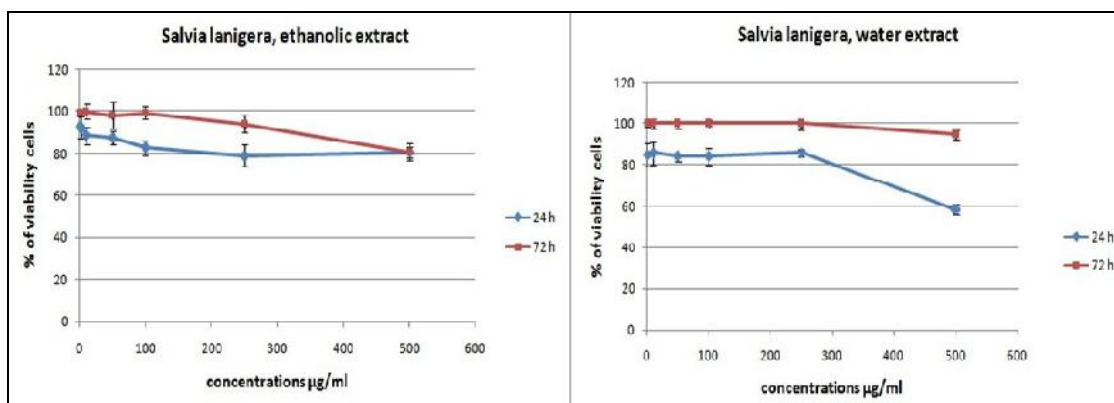


Figure 66. Effects of *S. lanigera* ethanol and water extracts on HCT-116 cell line after 24 and 72 h of treatment.

Abu-Dahab et al. (2012) investigated ethanol extracts of nine *Salvia* species grown in Jordan for their antiproliferative activity using a panel of breast cancer cell lines. Among tested *Salvia* species, four demonstrated substantial antiproliferative effect, inhibiting at least 50% of tumor cell proliferation at 25 µg/ml. In the study of Fiore et al. (2006), methanol extracts of six Jordanian *Salvia* species were screened in human cancer cell lines from different histological types and showed IC₅₀ values ranging from 90 to 400 µg/ml, approximately.

The extract of *S. lanigera* did not show a dose response effect, but displayed toxic activity at all concentrations tested in each cell line. Crude aqueous extracts of eleven *Salvia* spp. grown and sold in Jordan including *S. lanigera*, were tested for general cytotoxicity against a panel of colorectal cancer cell lines (HT29, HCT116, SW620 and Caco2). None of the tested extracts were found to possess cisplatin- or doxorobocin-like antiproliferative capacities in comparison to non induced basal incubations (Kasabri et al., 2014). Shaheen et al. (2011) tested methanol, acetone and *n*-butanol extracts of *S. lanigera* and *S. splendens* from Egypt against selected human cell lines.

The obtained results varied depending on the species, extract type and cell line used in the study. *S. lanigera* extracts in this study were less active than extracts of Egyptian *S. lanigera* (IC₅₀ values from 9.83 to over 100 µg/ml), especially when acetone extract was applied. The best results were obtained for the ovary adenocarcinoma and uterine sarcoma, in all tested extracts.

4.3.2.5. Antimicrobial effects of different extracts

4.3.2.5.1. Antibacterial activity

Antibacterial activity of *Salvia lanigera* extracts was tested by microdilution method against six Gram-negative and five Gram-positive pathogenic bacteria. The most of the examined extracts exhibited inhibitory activity on bacterial growth. Ethanol extract showed stronger activity (MICs 15-35 mg/ml) comparing to water extract (MICs from 30 to over 50 mg/ml). Gram-positive bacteria were more sensitive than those Gram-negative. The most sensitive bacteria were *M. flavus* and *L. monocytogenes* (MICs 15 mg/ml) versus the most resistant *P. mirabilis* and *P. aeruginosa*. Extracts

showed weaker activity compared to streptomycin (MICs ranged as 0.005-0.016 mg/ml) (Table 18).

Table 18. Antibacterial activity of *S. lanigera* extracts and standard antibiotic streptomycin against selected bacteria presented as minimal inhibitory concentration (MIC, mg/ml).

Bacteria	Extracts		Streptomycin
	Ethanol	Water	
Gram-negative bacteria			
<i>Esherishia coli</i> ATCC 25922	30	50	0.012
<i>Salmonella typhimurium</i> ATCC 14028	25	40	0.010
<i>Salmonella enteritidis</i> ATCC 13076	20	40	0.010
<i>Pseudomonas tolasii</i> NCTC 387	30	50	0.016
<i>Pseudomonas aeruginosa</i> ATCC 27853	35	>50	0.016
<i>Proteus mirabilis</i> ATCC 14273	35	>50	0.005
Gram-positive bacteria			
<i>Staphylococcus aureus</i> ATCC 25932	20	30	0.016
<i>Bacillus cereus</i> ATCC 10876	20	40	0.005
<i>Micrococcus flavus</i> ATCC 14452	15	30	0.010
<i>Sarcina lutea</i> ATCC 10054	25	40	0.012
<i>Listeria monocytogenes</i> ATCC 15313	15	30	0.010

In the present study, both Gram-positive and Gram-negative bacteria were affected by the sage extracts tested, but with stronger action against Gram-positive strains, while Gram-negative bacteria were more resistant. This finding is consistent with previous studies on a range of herbs and spices (Shan et al., 2007; Wendakoon et al., 2012). Gram-positive bacteria also were more affected by the sage extracts and oils in other studies (Shelef et al., 1984; Shan et al., 2007), which could be explained by differences in membranes between bacteria. The cell wall of Gram-negative bacteria possesses differences in the outer membrane arrangement which serves as penetration barrier towards macromolecules (Nikaido, 1996, 2003). Hawas and El-Ansari (2006)

tested ether, chloroform, ethyl acetate, n-butanol, ethanol, and water extracts of Egyptian *S. lanigera*, among which the ether soluble fraction was found to be highly active against gram positive bacteria (*Streptomyces viridochromogenes*) while the other fractions and pure compounds showed low antimicrobial activities. An ether extract from *S. lanigera* growing in Egypt, which contained several flavonoid compounds, exhibited marked antimicrobial activity against Gram-positive bacteria, but a weaker effect on Gram-negative microorganisms (Ibrahim, 2012).

4.3.2.5.2. Antifungal activity

Antifungal activity of *Salvia lanigera* ethanol and water extracts was investigated using microdilution assay and results were shown in Table 19. Ethanol extract inhibited the growth of all tested *Candida* species (MIC were ranged from 16 to 64 mg/ml) while water extract affected only *C. parapsilosis*. Fungicidal effect was not detected for *Candida* species. *A. glaucus* was the only *Aspergillus* species which was sensitive to the both ethanol and water extracts (MIC were 8 and 64 mg/ml, respectively). Water extract showed the strongest effect on *Trichophyton mentagrophytes* growth (MIC/MFC were 8 mg/ml). Similar to the previous studies (Veli kovi et al., 2002; Tepe et al., 2004; Dulger and Hacıoglu, 2008), *Candida* species were generally sensitive to *Salvia* extracts. The both of *S. lanigera* extracts showed weaker antifungal effects comparing to standard antimycotic ketoconazole (MICs 0.0019-0.0078 mg/ml, MFCs 0.0039-0.0156 mg/ml).

Numerous studies showed the antimicrobial properties of plant extracts and essential oils, which proved that plant metabolites have potential for applications in medical procedures, cosmetic, food and pharmaceutical industries (Dorman and Deans, 2000). The essential oil of *S. lanigera* from Cyprus was studied for the antimicrobial properties (Tenore et al., 2011), as well as from Saudi Arabia (Al-Howiriny, 2003), and Egypt (Ibrahim et al., 2013). By inhibiting the growth of almost all the human pathogenic and/or food spoilage bacteria, moulds and the yeast tested, *S. lanigera* essential oil exerted a broad antimicrobial spectrum.

Table 19. Antifungal activity of *S. lanigera* extracts and standard antimycotic ketoconazole against selected micromycetes.

Micromycetes	Extracts				Ketoconazole	
	Ethanol		Water		MIC	MFC
	MIC	MFC	MIC	MFC		
<i>Candida krusei</i>	64	-	-	-	0.0078	0.0156
<i>Candida albicans</i>	32	-	-	-	0.0078	0.0156
<i>Candida parapsilosis</i>	16	-	32	-	0.0078	0.0156
<i>Aspergillus glaucus</i>	8	-	64	-	0.0078	0.0078
<i>Aspergillus fumigates</i>	-	-	-	-	0.0078	0.0156
<i>Aspergillus flavus</i>	-	-	-	-	0.0078	0.0078
<i>Trichophyton mentagrophytes</i>	32	-	8	8	0.0019	0.0039

^aMinimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC); values given as mg/ml; - MIC and MFC not detected at tested concentrations.

4.3.2.6. Acetylcholinesterase and tyrosinase inhibitory activities

Oxydative stress contributes to the development of neurodegeneration by modulating the function of biomolecules damaged by ROS (Gandhi & Abramov, 2012). Since the antioxidant activity and inhibition of enzymes involved in originating of neurodegenerative disease, such as Alzheimer's and Parkinson's diseases, could be related, we have examined the possible enzymes inhibitory effects of *S. lanigera* extracts.

In the present study, ethanol and water extracts of *S. lanigera* were tested for *in vitro* ability to inhibit acetylcholinesterase (AChE) and results are presented in the Table 20. In range of tested concentrations (25, 50 and 100 µg/ml), extracts performed lower activity (30.97-32.42%) than standard drug galanthamine (42.38-52.11%). Ethanol extract exhibited slightly stronger inhibition of AChE comparing to water extract.

Table 20. Acetylcholinesterase and tyrosinase inhibition activity of *S. lanigera* extracts and standards expressed as percentage (%).

Conc. (µg/ml)	AChE inhibition (%)			TYR inhibition (%)		
	Extracts		Galanthamine	Extracts		Kojic acid
	Ethanol	Water		Ethanol	Water	
25	31.54 ±	31.21 ±	42.38 ± 0.740	60.61 ±	69.97 ±	35.73 ±
	3.505	0.035		1.808	2.142	5.460
50	32.42 ±	31.79 ±	50.56 ± 0.510	56.35 ±	70.20 ±	33.93 ±
	2.638	0.293		2.083	2.142	3.780
100	31.03 ±	30.97 ±	57.11 ± 1.670	55.26 ±	63.98 ±	51.81 ±
	0.434	1.149		1.655	0.408	2.550

The most widely used treatment against the Alzheimer's disease is inhibition of acetylcholinesterase, the enzyme which hydrolysis ACh into choline and acetic acid (Giacobini et al., 2002). The essential oils of several *Salvia* species were proved for their AChE inhibitory activity, for instance, *S. lavadulaefolia* (Perry et al., 2000b, Kennedy et al., 2011), *S. officinalis* (Orhan et al., 2008). Mentioned species were also evaluated for the aromas of the essential oil potential to affect cognition and mood in healthy adults (Moss et al., 2010), and *S. officinalis* produced a significant enhancement effect for the quality of memory. The extracts of *Salvia* species were also investigated for treating neurological disorders, and *S. lavandulaefolia*, *S. officinalis*, *S. fruticosa* (Perry et al., 2000b; Howes et al., 2003; enol et al., 2010; Orhan et al., 2012, 2013) showed beneficial effects. The results in this study are first reported for *S. lanigera* extracts.

In this study the tyrosinase inhibitory potentials of *S. lanigera* ethanol and water extracts were also examined and results are shown in the Table 20. At concentrations of 25, 50 and 100 µg/ml, the both of tested extracts performed stronger inhibition (55.26-

70.20%) than standard kojic acid (33.73-51.81%). The inhibitory effects of water extract was stronger compared to ethanol extract.

Many screening studies are reported in the literature, with emphasis on those products made with tyrosinase inhibitors from African plants (Baurin et al., 2002; Kubo & Hori, 1999, Momtaz et al., 2008), Bolivia (Kubo et al., 1995), China (Iida et al., 1995; Masamoto et al., 1980; Miao et al., 1997), Japan (No et al., 1999), Bangladesh (Khanom et al., 2000) and others with positive results in relation to activity. Considering that current therapies have shown less than satisfactory results in the treatment of various dermatological disorders such as melasma, post-inflammatory or senile lentigo and ephelides, and that the side effects of the therapy include high cytotoxicity and mutagenicity, poor skin penetration and low stability of formulations (Grimes, 1999; Nerva et al., 2003), new drugs with enzyme inhibitory activity are needed (Su, 1999). Inhibition of tyrosinase by ethanol extracts of *Salvia cryptantha* and *Salvia cyanescens* was determined, but the activity at tested concentrations of 25, 50 and 100 µg/ml was low, 7.50% to 13.78% for *S. crypantha*, and from 1.92% to 11.95% for *S. cyanescens* (Suntar et al., 2011).

5. CONCLUSIONS

1. The analysis of three Libyan *Salvia* species (*S. aegyptiaca*, *S. fruticosa* and *S. lanigera*), revealed valuable results on micromorphological and cytological characteristics, as well as on bioactivities for samples grown in Libya.
2. This study is the first report on the foliar micromorphology and histology of *S. aegyptiaca*, *S. fruticosa* and *S. lanigera*. Additionally, the histochemical analysis was done for *S. aegyptiaca* and ultrastructural study for *S. fruticosa*.
3. The micromorphological study showed that the leaves of the investigated species bear glandular and non-glandular trichomes, and are rich from the point of trichome diversity and quantity.
4. On the leaves of *S. aegyptiaca* peltate trichomes composed of basal cell, short cylindrical stalk cell and broad head of eight or more secretory cells and short-stalked capitate trichomes and long-stalked capitate trichomes were determined. Histochemical tests showed that the secreted material in all types of *S. aegyptiaca* glandular trichomes was of a complex nature with positive reactions to lipids.
5. In *S. fruticosa* peltate trichomes consisted of basal cell, very short stalk cell and large round head of eight secretory cells arranged in a circle. Capitate trichomes can be divided into two main types, short- and long-stalked, and further into five subtypes according to the number of stalk cells, the morphology and the number of the glandular head cells. Digitiform trichomes consisted of one basal cell, one or two stalk cells and one narrow apical secretory cell, were also found.
6. *S. lanigera* possessed peltate trichomes consisted of one basal epidermal cell, short cylindrical stalk cell and large round or slightly flattened secretory head of eight secretory cells arranged in a single circle. Capitate trichomes were determined as short-stalked capitate trichomes.
7. Total phenolic content was the highest in the ethanol extract of *S. aegyptiaca*, and dichloromethane extracts of *S. fruticosa* and *S. lanigera*. Ethyl acetate

extracts of *S. aegyptiaca* and *S. lanigera*, and dichloromethane extract of *S. fruticosa* showed the highest flavonoid contents.

8. Methanol and water extracts of *S. fruticosa* and *S. lanigera* were quantitatively and qualitatively the richest in the phenolic components measured by HPLC-DAD. The extracts of *S. fruticosa* contained caffeic and rosmarinic acid, while in *S. lanigera* only caffeic acid was identified. Both species showed the highest content of kaempferol glycosides.

9. In the study of antioxidant activity of dichloromethane, ethyl acetate, methanol, acetone, chloroform, ethanol and water extracts, three Libyan *Salvia* species showed different results for antioxidant activity. Ethanol extract of *S. aegyptiaca* showed the strongest activity compared to other extracts in DPPH, ABTS and FRAP assays, while in the β -CB assay, ethanol extract showed low activity. The ethanol extract of *S. fruticosa* showed the strongest antioxidant activity in all applied assays. Among *S. lanigera* extracts, water extract exhibited better antioxidant activity in the applied assays, except in FRAP assay where methanol was better than water extract. In β -CB assay, water and ethanol extracts exhibited higher activity than standards BHA and BHT. The antioxidant activity was better correlated to total phenolic content than to flavonoid content.

10. The cytotoxic activity of *S. fruticosa* and *S. lanigera* ethanol and water extracts on human carcinoma cell lines was determined as low at tested concentrations.

11. Ethanol extract showed stronger antibacterial activity than water extract, particularly against Gram-positive bacteria. The antifungal activity of investigated *Salvia* species extracts was estimated as poor comparing to values obtained for commercial antimycotic ketoconazole.

12. In AChE inhibition assay, ethanol and water extracts exhibited weaker activity than standard galanthamine, while extracts performed stronger activity in tyrosinase inhibition assay in comparison to standard kojic acid at tested concentrations.

13. This study provides useful data about micromorphology and cytology, as well as for antioxidant, cytotoxic, antimicrobial and antineurodegenerative activities of Libyan *S. aegyptiaca*, *S. fruticosa* and *S. lanigera*. The investigated activities are connected with chemical data which could attribute to the understanding of mechanism of action of these traditionally used medicinal plants and their further application.

14. The samples of *S. aegyptiaca* and *S. fruticosa* could be considered as good natural antioxidants for use in food and pharmaceutical industries. The results for tyrosinase inhibition are promising, especially for water extracts of *S. fruticosa* and *S. lanigera*.

The potential therapeutic usage needs to be subjected to *in vivo* studies for further applications.

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Biography

Najat Beleed Mohammed Al Sheef was born in Labiar, Libya, on June 10th, 1975. She started her Bachelor studies in 1994. at the Faculty of Arts & Science, Al-Mergeb University, Al-Khoms in Libya, where she graduated in 1998.

From 1999. to 2005., Najat Beleed Al Sheef worked as a teaching assistant at the Faculty of Arts & Science, Al-Mergeb University, at Biology (Botany) Department, Al-Khoms. There she taught lectures laboratory in the following courses: Plant Taxonomy, Flora of Libya, Ecology, Medical and Aromatic plants, General Biology. During this period in 2002. she started Master studies at the same department, and graduated in 2005. It was the subject of research in Plant taxonomy, entitled "Taxonomical study of plants in Kaam region".

From 2005. to 2009. she worked as an assistant lecturer at the same department and taught the following courses: Plant Taxonomy, Flora of Libya, Ecology, Medical and Aromatic plants, General Biology, General Botany, Algae and Aquatic Plants.

In October 2009. she enrolled PhD studies at the Faculty of Biology, University of Belgrade, which is dealing with selected *Salvia* species from Libya and their microscopic characteristics and biological effects of different extracts.

Прилог 1.

Изјава о ауторству

Потписани-а _____ Najat Beleed Al Sheef _____

број индекса _____

Изјављујем

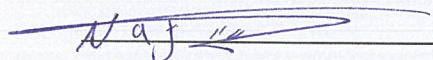
да је докторска дисертација под насловом

“Микроморфолошка и цитолошка анализа трихома и биолошки ефекти екстракта *Salvia aegyptiaca* L., *S. fruticosa* Mill. i *S. lanigera* Poir. (Lamiaceae) из Либије“ (Micromorphological and cytological analysis of trichomes and biological effects of extracts of *Salvia aegyptiaca* L., *S. fruticosa* Mill. and *S. lanigera* Poir. (Lamiaceae) from Libya)

- резултат сопственог истраживачког рада,
- да предложена дисертација у целини ни у деловима није била предложена за добијање било које дипломе према студијским програмима других високошколских установа,
- да су резултати коректно наведени и
- да нисам кршио/ла ауторска права и користио интелектуалну својину других лица.

Потпис докторанда

У Београду, 26.10.2015.

 _____

Прилог 2.

**Изјава о истоветности штампане и електронске
верзије докторског рада**

Име и презиме аутора ____ **Najat Beleed Al Sheef** _____

Број индекса _____

Студијски програм ____ **Биологија** _____

Наслов рада “**Микроморфолошка и цитолошка анализа трихома и биолошки ефекти екстракта *Salvia aegyptiaca* L., *S. fruticosa* Mill. i *S. lanigera* Poir. (Lamiaceae) из Либије**“ (Micromorphological and cytological analysis of trichomes and biological effects of extracts of *Salvia aegyptiaca* L., *S. fruticosa* Mill. and *S. lanigera* Poir. (Lamiaceae) from Libya)

Ментор ____ **Соња Дулетић-Лаушевић** _____

Потписани/а ____ **Najat Beleed Al Sheef** _____

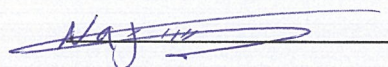
Изјављујем да је штампана верзија мог докторског рада истоветна електронској верзији коју сам предао/ла за објављивање на порталу **Дигиталног репозиторијума Универзитета у Београду**.

Дозвољавам да се објаве моји лични подаци везани за добијање академског звања доктора наука, као што су име и презиме, година и место рођења и датум одбране рада.

Ови лични подаци могу се објавити на мрежним страницама дигиталне библиотеке, у електронском каталогу и у публикацијама Универзитета у Београду.

Потпис докторанда

У Београду, ____ **26.10.2015.** ____



Прилог 3.

Изјава о коришћењу

Овлашћујем Универзитетску библиотеку „Светозар Марковић“ да у Дигитални репозиторијум Универзитета у Београду унесе моју докторску дисертацију под насловом:

“Микроморфолошка и цитолошка анализа трихома и биолошки ефекти екстракта *Salvia aegyptiaca* L., *S. fruticosa* Mill. i *S. lanigera* Poir. (Lamiaceae) из Либије“ (Micromorphological and cytological analysis of trichomes and biological effects of extracts of *Salvia aegyptiaca* L., *S. fruticosa* Mill. and *S. lanigera* Poir. (Lamiaceae) from Libya)

која је моје ауторско дело.

Дисертацију са свим прилозима предао/ла сам у електронском формату погодном за трајно архивирање.

Моју докторску дисертацију похрањену у Дигитални репозиторијум Универзитета у Београду могу да користе сви који поштују одредбе садржане у одабраном типу лиценце Креативне заједнице (Creative Commons) за коју сам се одлучио/ла.

1. Ауторство

2. Ауторство - некомерцијално

3. Ауторство – некомерцијално – без прераде

4. Ауторство – некомерцијално – делити под истим условима

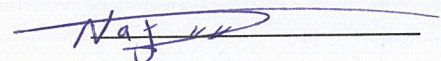
5. Ауторство – без прераде

6. Ауторство – делити под истим условима

(Молимо да заокружите само једну од шест понуђених лиценци, кратак опис лиценци дат је на полеђини листа).

Потпис докторанда

У Београду, 26.10.2015.



1. Ауторство - Дозвољавање умножавање, дистрибуцију и јавно саопштавање дела, и прераде, ако се наведе име аутора на начин одређен од стране аутора или даваоца лиценце, чак и у комерцијалне сврхе. Ово је најслободнија од свих лиценци.

2. Ауторство – некомерцијално. Дозвољавање умножавање, дистрибуцију и јавно саопштавање дела, и прераде, ако се наведе име аутора на начин одређен од стране аутора или даваоца лиценце. Ова лиценца не дозвољава комерцијалну употребу дела.

3. Ауторство - некомерцијално – без прераде. Дозвољавање умножавање, дистрибуцију и јавно саопштавање дела, без промена, преобликовања или употребе дела у свом делу, ако се наведе име аутора на начин одређен од стране аутора или даваоца лиценце. Ова лиценца не дозвољава комерцијалну употребу дела. У односу на све остале лиценце, овом лиценцом се ограничава највећи обим права коришћења дела.

4. Ауторство - некомерцијално – делити под истим условима. Дозвољавање умножавање, дистрибуцију и јавно саопштавање дела, и прераде, ако се наведе име аутора на начин одређен од стране аутора или даваоца лиценце и ако се прерада дистрибуира под истом или сличном лиценцом. Ова лиценца не дозвољава комерцијалну употребу дела и прерада.

5. Ауторство – без прераде. Дозвољавање умножавање, дистрибуцију и јавно саопштавање дела, без промена, преобликовања или употребе дела у свом делу, ако се наведе име аутора на начин одређен од стране аутора или даваоца лиценце. Ова лиценца дозвољава комерцијалну употребу дела.

6. Ауторство - делити под истим условима. Дозвољавање умножавање, дистрибуцију и јавно саопштавање дела, и прераде, ако се наведе име аутора на начин одређен од стране аутора или даваоца лиценце и ако се прерада дистрибуира под истом или сличном лиценцом. Ова лиценца дозвољава комерцијалну употребу дела и прерада. Слична је софтверским лиценцама, односно лиценцама отвореног кода.