International Journal of ChemTech Research
CODEN (USA): IJCRGG ISSN: 0974-4290
Vol.8, No.8, pp 53-60, 2015

Composition, *in Vitro* Antioxidant and Antileishmanial activities of *Vitex agnus-castus* L. and *Thymus syriacus* Boiss. Essential Oils

Faten Al Saka[1,*], Francois Karabet[1], Manal Daghestani[1], Chadi Soukkarieh[2]

[1]Damascus University, Faculty of Sciences, Department of Chemistry, Damascus, Syria.
[2]Damascus University, Faculty of Sciences, Department of Animal Biology, Damascus, Syria.

Abstract: The essential oils represent valuable sources for active molecules against *Leishmania* infections and natural antioxidant. In this present study, essential oils from fruits of Syrian *Vitex agnus-castus* L. (VAC), and leaves of *Thymus syriacus* Boiss. (TS) were analyzed by gas chromatography-mass spectrometry. The main constituents found in VAC essential oil were 1,8-Cineole (14.25%) and Sabinene (11.54%), while the major constituents in TS essential oil were thymol (40.61%) and *p*-Cymene (20.40%). The antioxidant activity of the essential oils were determined by their scavenging effect on 2.2-diphenyl-1-picrylhydrazyl and total phenolic contents. The result showed that antioxidant activity of TS essential oil was higher than the antioxidant activity of VAC essential oil. Finally, the biological activity, of both VAC and TS essential oils, against *L. tropica* were determined using 3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyl tetrazolium bromide, and The IC_{50} values were 211.62 µg/ml and 101.08µg/ml, respectively. Therefore, further work is needed to identify the compound(s) responsible for the effects of VAC and TS essential oils and their correlation with *in vivo* studies.
Keywords: *Vitex agnus-castus* L.; *Thymus syriacus* Boiss.; essential oil; antioxidant activity; *Leishmania.*

Introduction

Medicinal plants and their extracts have been used for many centuries to treat different diseases. Furthermore, their essential oils, which obtained by hydrodistillation or steam distillation, can be a source of alternative natural treatment of disease such as leishmaniasis.[1,2,3]

There is a growing interest in natural substances that exhibit antioxidant properties. The plant kingdom produces a wide range of antioxidants including phenolic compounds; which are commonly found in various plants as secondary metabolites, and have large variability of the physico-chemical properties and multiple biological effects such as antioxidant activity, since the excessive using of the synthetic antioxidant may cause numerous diseases.[4,5]

Leishmaniasis is a tropical and subtropical disease caused by protozoan parasites of the genus *Leishmania*. These parasites are transmitted by 30 different species of Phlebotomine sand flies as extracellular, flagellated promastigotes. Cutaneous leishmaniasis in Syria is caused by *L. tropica*, furthermore, it reported nearly 52,983 cases during 2012.[6,7] Currently, various treatment options are available for leishmaniasis

including meglumine antimoniate, and allopurinol.[8,9] However, treatment with these drugs causes many side effects, besides, poorly tolerated, expensive and need continuous administration, also. These observations imply an urgent and necessary developing of new natural drugs for the treatment.[10,11]

Syrian flora is well known for its diversity and richness, and it consists of numerous species for medicinal uses.[12] Among plants grown in Syria VAC and TS. VAC, Lamiaceae (placed in Verbenaceae, also) common names: Chaste Tree, Chaste berry and Monks Pepper. It is native to Mediterranean, European and Asian regions, beside it is grown for ornamental purposes in many countries. VAC is a shrub up to 4 m height. The leaves are crossed-opposite with pink-violet flowers.[13,14,15] This plant has a wide range of biological activities, including Premenstrual syndrome (PMS) and cancer cell lines.[13,16,17] In addition, it is has been used as antifungal.[18] TS, Lamiaceae (local name Zaatar), it is originated from Mediterranean region, more or less shrubby 30-50 cm. Leaves sessile, rigid 1-3 cm, with white flowers. TS is well known as medicinal plants because of their biological, and pharmacological properties. Also widely used as herbal tea, antiseptic and carminative as well as treating colds and cough. Besides, it is commonly used in pharmaceutical, cosmetic products, flavoring agents, food preservatives and antioxidant.[19,15]

The objective of this research is to determine the chemical composition of the essential oils from fruits of VAC and leaves of TS cultivated in Syria by using GC/MS system, and to study the antioxidant properties, as well as the biological activity against *L. tropica* of both essential oils.

Experimental section

Plant material

VAC was collected in July 2013 from local park (Tishreen park: 33°30'59.0"N 36°16'08.9"E), Damascus, Syria. The plant was identified by Prof. Anwar alkhateeb (Taxonomy and Ecology, Faculty of science, Damascus University, Syria). The fruits of VAC dried in the shade.

Isolation of essential oil and Preservation

VAC fruits were grounded in an electric grinder, the essential oil was isolated by hydro-distillation for 3 h using a Clevenger-type apparatus (0.809±0.059 %). The obtained oil was dried over anhydrous sodium sulfate and stored at +4°C in the dark until analyzed, following the procedure described in the European Pharmacopoeia fifth edition.[20] While TS essential oil was obtained from Bio-cham company (*Thymus syriacus* Boiss. Mars - 2013).

GC-MS analysis of essential oils

VAC and TS essential oils were analyzed by GC-MS, using an Agilent 7890A GC-MS system coupled with quadruple mass spectrometer (model 5975C). An HP-5MS 5 % Phenyl Methyl Silox column (30 m x 250 µm x 0.25 µm thickness) was used with helium as the carrier gas (1 ml/min). GC-MS interface, ion source, selective mass detector and injector temperatures were maintained at 280◦C, 230◦C, 150◦C, and 260◦C, respectively. The oven temperature was programmed from 60◦C to 200◦C at a rate of 4◦C/min, then at a rate of 8◦C/min up to 260◦C, finally maintained constant at 260◦C for 7.5 min. 1.0 µl of diluted oils in n-hexane (1/100, V/V) were injected with a split ratio 1:10.

Identification of constituents

Individual constituents were identified using mass spectrum and matching with mass spectral library (NIST), along with the retention data from analytical standards of available terpenoids (Sigma-Aldrich). As well, retention indices determined using a homologous series of n-alkanes C_8–C_{22}, confirmation was done by comparing calculated retention indices with literature.[21]

Antioxidant activity of the essential oils

- **Scavenging effect on 2.2 diphenyl-1-picrylhydrazyl (DPPH)**

One of the quick methods to evaluate antioxidant activity is the scavenging activity on DPPH, a stable free radical and widely used index. The effect of the essential oil on DPPH radical was estimated according to the literature.[22] 3 ml of freshly prepared ethanolic DPPH solution (45 µg/ml) was mixed with 300 µl of the

samples at varying concentrations (0.2-0.5-1 mg/ml). The mixture was shaken vigorously, and allowed standing for 30 min in the dark at room temperature. The decrease in absorbance (A) was measured at 517 nm with a spectrophotometer (Optizen 2120 UV Plus, Mecasys Co., Ltd, Korea). The inhibition percentage of the radicals (I %) was calculated according to the following formula:

Equation (1) $$\mathbf{I\ \%} = \frac{[(\mathbf{Acontrol} - \mathbf{Asample}) * \mathbf{100}]}{\mathbf{Acontrol}}$$

Where: $A_{control}$ is the absorbance of the control reaction (containing all reagents except the sample), and A_{Sample} is the absorbance of the sample. 50µl of 0.2 mg/ml solution of vitamin C, which was used as a control, treated as the sample and at the same condition.

- **Assay for total phenol**

Total phenolic contents of the essential oils were determined by employing the methods given in the literature,[23,24] involving Folin–Ciocalteu reagent, and gallic acid (Sigma) as standard. The absorbance was measured at 760 nm (λ_{max}) using the previous spectrophotometer against a blank. A calibration curve of gallic acid solutions were prepared in 70 % ethanol (0-125 mg/L; slope = 0.0043, and $R^2 = 0.9977$). Total phenolic compounds were determined according to the following equation obtained from the standard gallic acid graph

Equation (2) $$\mathbf{y = 0.0043\,x - 0.0089}$$

Parasite strain

leishmania strain used in this study was previously identified as *L. tropica* by molecular genotyping at Department of Animal Biology, Damascus University, Syria. Promastigotes forms were routinely cultured at 26◦C in RPMI 1640 medium (Sigma) supplemented with 10 % fetal bovine serum (Sigma), and penicillin-streptomycin (100 U/ml).

3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyl tetrazolium bromide (MTT) assay for Cell viability

Effects on viability were estimated by MTT colorimetric method, on the basis of the reduction of the tetrazolium-dye to insoluble formazan by the mitochondrial enzymes.[25,26] The VAC and TS essential oils were initially diluted in dimethylsulfoxide (DMSO; Sigma) to concentration of 100 mg/ml, then in culture medium RPMI 1640 in order to get a range of concentrations from 25 to 300 µg/ml, with stationary phase promastigotes of *L. tropica* (5×10^5 cells/ml), were incubated at 26◦C for 24 h. At the end of this period, 10 µl of MTT (5 mg/ml) was added to each well, and were again incubated for 2.5 h at 26◦C, the formazan crystals were solubilized by addition of 100 µL MTT solvent (Sigma) to each. All experiments were performed in triplicate and in three independent assays. Cell viability was measured by absorbance at 540 nm on an ELISA plate reader (HumaReader HS, Human), and calculated using the following formula:

Equation (3) $$(L2/L1) \times 100$$

Where L_1 is the absorbance of control cells, and L_2 is the absorbance of treated cells. And IC_{50} values were defined as the drug concentration that reduce absorbance to 50 % of control values.

Statistical analysis

Statistical Package for the Social Science (SPSS, 20) was used for statistical analysis. Data were expressed as mean ± SD of three different experiences. Comparisons for antioxidant activity and cell viability were performed by One-way ANOVA and tow-way ANOVA (Univariate analysis of variance) with post hoc test (Bonferroni test) respectively, the significance level was $P < 0.05$.

Results and Discussion

Chemical composition of the essential oil

Table 1, 2 show the GC–MS results which proved that 27 constituents represent 91.00 % of VAC essential oil, and 20 constituents represent 93.20 % of TS essential oil. Major components of VAC essential oil were 1,8-

Cineole (14.25 %), Sabinene (11.54 %), and (E)-β-Farnesene (7.19 %). While in TS essential oil major compounds were Thymol (40.61 %), *p*-Cymene (20.40 %), and γ-Terpinen (7.79 %). 1,8-Cineole and Sabinene

in VAC essential oil were the major components in most of the previous studies.[13,27,28,29] Whereas, the results obtained in TS essential oil are in agreement with some previous works only, where Thymol has the highest value of area, while Carvacrol was the main compound in other literatures.[30,31,32,33] It is necessary to mention, that the composition of these volatile oils varies according to the countries, or the places in the same country. These differences seem to depend on climate changes, and other factors like the method, and the time of extraction, which can influence essential oil composition.[34,35,29]

Table 1. The compositions of VAC fruit essential oil[a]

VAC constituents	RI $_{cal.}$	RI $_{lit.}$	%	Identified methods
á-Pinene	932	932	4.27	MS/RI/St.
Sabinene	975	969	11.54	MS/RI
â-Pinene	978	974	1.17	MS/RI/St.
â-Myrcene	991	988	1.38	MS/RI
(+)-Sylvestrene	1031	1025	2.14	MS/RI
1,8-Cineole	1034	1028	14.25	MS/RI/St.
ã-Terpinen	1059	1054	0.73	MS/RI
Terpinen-4-ol	1180	1174	1.55	MS/RI
(-)-á-Terpineol	1193	1186	2.51	MS/RI
ä-Elemene	1340	1335	1.81	MS/RI
Terpinyl acetate	1353	1346	4.99	MS/RI/St.
á-Gurjunene	1413	1409	0.66	MS/RI
â-Caryophyllene	1424	1417	4.35	MS/RI/St.
(E)-â-Farnesene	1461	1454	7.19	MS/RI
Aromadendrane <dehydro->	1464	1460	1.23	MS/RI
Germacrene D	1485	1484	2.16	MS/RI
Bicyclogermacrene	1502	1500	6.54	MS/RI
(-)-Spathulenol	1582	1577	1.71	MS/RI
Ledol	1607	1602	0.67	MS/RI
á-Cadinol	1646	1652	4.15	MS/RI
Unknown	1878	-	4.29	-
Biformene	1899	1931	1.07	MS
(Z,Z)-Geranyllinalool	1961	1960	1.17	MS/RI
5-(1-Isopropenyl-4,5-dimethylbicyclo[4.3.0]nonan-5-yl)-3-methyl-2-pentenol acetate	1993	n/a	1.12	MS
Manoyl oxide	1996	1987	0.99	MS/RI
Phyllocladene	2021	2016	4.44	MS/RI
7-Isopropyl-1,1,4a-trimethyl-1,2,3,4,4a,9,10,10a-octahydrophenanthrene	2047	n/a	2.90	MS
Total identified			**91.00**	

[a]Compounds listed in order to their elution on the HP-5MS column, RI $_{cal.}$ Retention indices on the HP-5MS column relative to C8–C22 n-alkanes, RI $_{lit.}$ Retention indices from the literature (21), St. stander terpenoids, n/a=not available.

Table 2. The compositions of TS leaves essential oil[a]

TS constituents	RI $_{cal.}$	RI $_{lit.}$	%	Identified methods
á-Pinene	932	932	1.45	MS/RI/St.
Camphene	948	946	1.22	MS/RI
â-Pinene	978	974	0.76	MS/RI/St.
â –Myrcene	991	988	0.89	MS/RI
á-Terpinene	1018	1014	1.27	MS/RI
P-Cymene	1026	1022	20.40	MS/RI
(+)-Sylvestrene	1031	1025	0.59	MS/RI
1,8-Cineole	1034	1028	1.33	MS/RI/St.
ã-Terpinen	1059	1054	7.79	MS/RI
â-Linalool	1102	1095	2.71	MS/RI
(-)-Camphor	1149	1141	0.96	MS/RI
Borneol	1168	1165	2.37	MS/RI
Terpinen-4-ol	1180	1174	0.78	MS/RI
Thymol methyl ether	1236	1232	1.28	MS/RI
2-Isopropyl-1-methoxy-4-methylbenzene	1246	1244	0.84	MS/RI
Thymol	1300	1289	40.61	MS/RI
Carvacrol	1306	1298	3.16	MS/RI
â-Caryophyllene	1424	1417	3.23	MS/RI/St.
ä-cadinene	1526	1522	0.62	MS/RI
Caryophyllene oxide	1587	1582	0.93	MS/RI
Total identified			**93.20%**	

[a] Compounds listed in order to their elution on the HP-5MS column, RI $_{cal.}$ Retention indices on the HP-5MS column relative to C8–C22 n-alkanes, RI $_{lit.}$ Retention indices from the literature (21), St. stander terpenoids, n/a=not available.

Antioxidant activity of the essential oils

Antioxidant activity of VAC and TS essential oils was determined by two different test systems DPPH, and total phenolics, as shown in table (3, 4). The scavenging effects of VAC and TS essential oils on DPPH radical increased with concentration, and the scavenging activity of TS essential oil was more effective than VAC. However, in the current study, none of the evaluated samples showed activity as strong as vitamin C; which is known by its radical scavenging activity. Total phenolic constituent of TS essential oil was about 13 times higher than VAC essential oil. Beside, the values of radical DPPH scavenging effect and total phenol showed a significant difference ($P<0.05$) between VAC and TS essential oils. The result shows that the essential oil from TS was higher antioxidant activity than VAC because it contains higher concentration of phenolic terpenoids especially, Thymol which is a natural antioxidant.[32] However, The previous studies showed that Thymus species are very interesting natural resources with antioxidant activity such as T. kotschyanus, T. eriocalyx, and T. daenensis subsp lancifolius.[36] While VAC extracts have an excellent antioxidant activity in comparison with its essential oil.[27]

Table 3. I% of VAC fruits and TS leaves essential oils at different concentrations [a]

Concentrations (mg/ml)	0.2	0.5	1
VAC	0.79±0.21	1.85±0.27	2.63±0.11
TS	1.47±0.24	4.05±0.52	8.86±0.68
Vitamin C	33.24±0.60		

[a] Values are expressed as means ± SD of three parallel measurements

Table 4. Total phenolic contents of VAC fruits and TS leaves essential oils[a]

Essential oils	total phenolic contents (µg GAEs/mg essential oil)[b]
VAC	33.29±5.02
TS	446.27±7.54

[a] Values are expressed as means ± SD of three parallel measurements.
[b] GAEs: gallic acid equivalents.

Cell viability Anti-Leishmania Activity Assay

Various studies revealed significant activity of essential oils against *Leishmania* species but to our knowledge, no studies have been conducted using VAC and TS essential oils as anti-leishmanial activity. In this study, the VAC and TS essential oils caused the death of *L. tropica* with values of IC_{50}: 211.62 µg/ml and 101.08 µg/ml respectively. These results may lead to the development of new drugs from natural sources.[11] The values presented in figure 1 show that no significant difference between 25, 50, 100, and 300 µg/ml of VAC and TS essential oils. While, there are significant differences ($P<0.05$) in other concentration 150, 200, and 250 µg/ml. However, the publications related to anti-parasitic protozoal essential oils have increased during the last few years. The interpretation of results and comparisons between studies need to take into account the *Leishmania* species, cell model and the plant species. *Cymbopogon citrates* and *Artemisia herba-alba* Asso. were evaluated against *L. tropica* (IC_{50} were 52 and 2 µg/ml, respectively).[37,38] Various species of *thymus* showed anti-leishmanial activity such as *Thymus capitellatus* essential oil against *L. Tropica* (IC_{50} value of 35 µg/ml),[39] another study tested anti-leishmanial activity of *Thymus vulgaris* L. against *L. major*.[38] A study in Turkey showed the cytotoxic and apoptotic activity of VAC essential oil against C6, A549 and MCF 7 cancer cell lines *in vitro* by MTT method,[13] but no studies showed The antileishmanial activity of VAC. In fact, the anti-leishmanial activity may be associated with the interaction between essential oil constituents, because 1,8-Cineole, the major compounds in VAC essential oil, do not have anti-leishmanial activity and do not seem to be responsible for the essential oil activity.[39] While Thymol, The main phenolic compounds in TS essential oil, was the most active against *Leishmania* and may be responsible for anti-leishmanial activity. In several studies, Thymol, had activity against the promastigote form of the parasite (*L. amazonensis*) with IC_{50} values of 19.47 µg/ml, after 48 h of incubation.[40] On the other hand, there are many coumpounds such as linalool, β-Caryophyllene, α-Pinene etc. have anti-leishmanial activity against other *leishmania* species.[38,40]

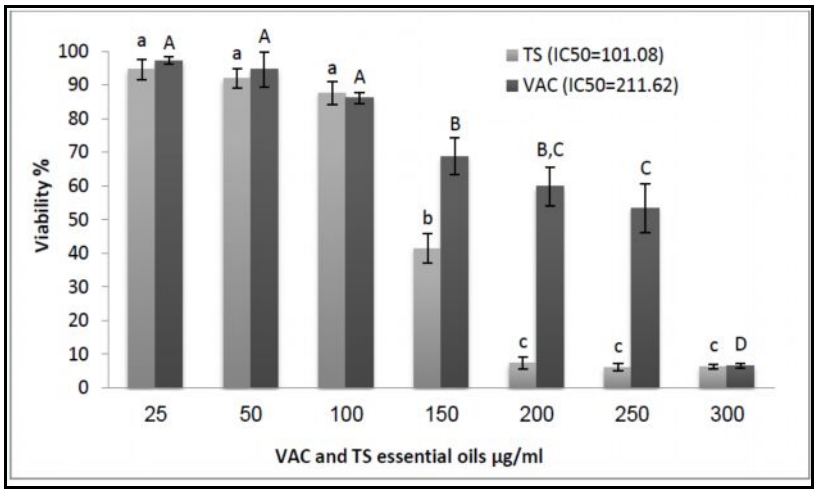

Figure 1. Effect of VAC fruits and TS leaves essential oils on *L. tropica* viability, as function of essential oil concentration. Values are expressed as means ± SD in triplicate, and in three independent assays, Means with different letters within a column for the same plant are significantly different ($P<0.05$).

Conclusions

VAC and TS are widely spread in Syria, and their essential oils have an obvious difference in the chemical constituents. They consider as source of natural products against *L. tropica* and antioxidant, especially TS essential oil for its potent antioxidant properties and contain significant amounts of phenolic terpenoids. To the best of our knowledge, this is the first study for anti-leishmanial activity of Syrian fruit VAC and leaves TS

essential oils. On the other, further studies are needed to clarify the bioactive compounds individually and fully understand the action of these essential oils.

Acknowledgements

The authors thank the Research Affairs team of Damascus University for the financial support. They thank Mr. Yahia Mahzia, Mohamad Osama AL Turkmani and Mr. Bashir Daoodi for their help in data analysis.

References

1. Machado M, Dinis AM, Salgueiro L, Custódio JBA, Cavaleiro C, Sousa MC. Anti-Giardia activity of *Syzygium aromaticum* essential oil and eugenol: Effects on growth, viability, adherence and ultrastructure. *Expt Parasitol*., 2011, 127 (4): 732-739.
2. Machado M, Santoro G, Sousa MC, Salgueiro L, Cavaleiro C. Activity of essential oils on the growth of *Leishmania infantum* promastigotes. *Flavour Fragr J*., 2010, 25(3): 156-160.
3. Edris AE. Pharmaceutical and therapeutic potentials of essential oils and theirindividual volatile constituents: a review. *Phytother Res*., 2007, 21(4): 308-323.
4. Naczk M, Shahidi FJ. Extraction and analysis of phenolics in food. *Chromatogr. A*., 2004, 1054: 95-111.
5. Proestos C, Sereli D, Komaitis M. Determination of phenolic compounds in aromatic plants by RP-HPLC and GC–MS. *Food Chem*., 2006, 95: 44-52.
6. Salam N, Al-Shaqha MW, Azzi A. Leishmaniasis in the Middle East: Incidence and Epidemiology: a review. *PLOS Negl Trop Dis*., 2014, 8(10): e3208.
7. World Health Organization, 2011. Leishmaniasis: background information. A brief history of the disease. In: WHO Technical Report Series949. World Health Organization, Geneva, Geneva, Switzerland.
8. Noli C, Auxilia ST. Treatment of canine old world visceral leish-maniasis: a systematic review. *Vet. Dermatol*., 2005, 16 (4): 213-232.
9. Baneth G, Shaw SE. Chemotherapy of canine leishmaniasis. *Vet. Parasitol*., 2002, 106 (4): 315-324.
10. World Health Organization, 2010. Control of the leishmaniases. In: Report of a Meeting of the WHO Expert Committee on the Control of Leishmaniases, Technical Report Series 949, Geneva.
11. Singh N, Mishra BB, Bajpai S, Singh KR, Tiwari KV. Natural product based leads to fight against leishmaniasis: a review. *Bioorg. Med. Chem*., 2014, 22: 18-45.
12. Sincich F. Bedouin Traditional Medicine in the Syrian Steppe, 2002. FAO,140.
13. Duymus HG, Akalın Çiftçi G, Ulusoylar Yıldırım S, Demirci B, Kırımer N. The cytotoxic activity of *Vitex agnus castus* L. essential oils and theirbiochemical mechanisms. *Ind Crops Prod*., 2014, 55: 33-42.
14. Tutin TG, Heywood VH, Burges NA, Valentine DH, Walters SM, Webb DA. *Flora Europaea*. Cambridge: Cambridge University Press, 1972. P.122.
15. Post EG. *flora of Syria, Palestine and Sinai.* second edition, Beirut: American Press, 1933.P.322.
16. Högner C, Sturm S, Seger C, Stuppner H. Development and validation of a rapid ultra-high performance liquid chromatography diode array detector method for *Vitex agnus castus*. *J. Chromatogr. B*., 2013, 927: 181-190.
17. Daniele C, Thompson Coon J, Pittler MH, Ernst E. *Vitex agnus castus*: a systematic review of adverse events. *Drug Saf* ., 2005, 28(4): 319.
18. Kuruüzüm-Uz A, Ströch K, Demirezer Ö, Zeeck A. Glucosides from *Vitex agnus-castus*. *Phytochem*., 2003, 63: 959-964.
19. De Martino L, Bruno M, Formisano C, De Feo V, Napolitano F, Rosselli S, Senatore F. Chemical Composition and Antimicrobial Activity of the Essential Oils from Two Species of *Thymus* Growing Wild in Southern Italy. *Molecules*, 2009, 14: .4614-4624
20. European Pharmacopoeia, 2004. Europe Department for the Quality of Medicines of the Council of Europe. fifth ed.
21. Adams PR. In: Carol Stream, Illinois. *Identification of essential oil components by gas chromatography/mass spectrometry*. USA: Allured Publishing Corporation, 2007.

22. Hatano T, Kagawa H, Yasuhara T, Okuda T. Two new flavonoids and other constituents in licorice root: their relative astringency and radical scavenging effects. *Chem. Pharm. Bull*., 1988, 36: 2090-2097.
23. AlHafez M, Kheder F, AlJoubbeh M. Polyphenols, flavonoids and (-)epigallocatechin gallate in tea leaves and in their infusions under various conditions. *Nutrition & Food Science* 2014; 44(5): 455-463.
24. Shaghaghi M, Manzoori J, Jouyban A. Determination of total phenols in tea infusions, tomato and apple juice by terbium sensitized fluorescence method as an alternative approach to the Folin-Ciocalteu spectrophotometric method. *Food chem*., 200, 108(2): 695-701.
25. Denizot F, Lang R. Rapid colorimetric assay for cell growth and sur-vival. Modifications to the tetrazolium dye procedure giving improvedsensitivity and reliability. *J Immunol Methods*, 1986, 89: 271-277.
26. Mosmann T. Rapid colorimetric assay for cellular growth and survival:application to proliferation and cytotoxicity assays. *J Immunol Methods*, 1983, 65: 55-63.
27. Sarikurkcu C, Arisoy K, Tepe B, Cakir A, Abali G, Mete E. Studies on the antioxidant activity of essential oil and different solvent extracts of *Vitex agnus castus* L. fruits from Turkey. *Food Chem. Toxicol*., 2009, 47: 2479–2483.
28. Stojković D, Soković M, Glamočlija J, Džamić A, Ćiric´ A, Ristić M, Grubišić D. Chemical composition and antimicrobial activity of *Vitex agnus-castus* L. fruits and leaves essential oils. *Food Chem*., 2011, 128: 1017-1022.
29. Sørensen JM, Katsiotis STh. Parameters influencing the yield and composi-tion of the essential oil from Cretan Vitex agnus castus fruits. *Planta Med*., 2000, 66: 245-250.
30. Al-Mariri A, Swied G, Oda A, Al Hallab L. Antibacterial activity of *Thymus Syriacus* Boiss essential oil and its components against some syrian gram-negative bacteria isolates. *Iran J Med Sci*., 2013, 38(2):180-186.
31. Zayzafoon G, Odeh A, Allaf AW. Determination of essential oil composition by GC-MS and integral antioxidant capacity using photochemiluminescence assay of two *Thymus* leaves: *Thymus syriacus* and *Thymus cilicicus* from different Syrian locations. *Herba Pol*., 2012, 58(4): 71-84.
32. Jamil DM, Brown JE, Driscoll D, Howell NK. Characterization and antioxidant activity of the volatile oils of *Thymus Syriacus* Boiss. *var syriacus* and *Thymbra spicata* L. grown wild in Kurdistan-Iraq. *Proc Nutr Soc.*, 2010, 69:104.
33. Tümen G, Baser KHC. Essential oil of *Thymus syriacus* Boiss. *J. Essent. Oil Res*., 1994, 6: 663-664.
34. Taziki S, Hamedeyazdan S, Pasandi AN. Variations in essential oils of *Vitex agnus castus* fruits growing in Qum, Khorasan and Tehran in Iran. *Ann Biol Res.*, 2013, 4 (2): 308-312.
35. Loziene K, Venskutonis PR. Influence of environmental and genetic factors on the stability of essential oil composition of *Thymus pulegioides*. *Biological System Ecology* 2005;33:517-525.
36. Amiri H. Essential Oils Composition and Antioxidant Properties of Three *Thymus* Species. *Evid. Based Complement. Alternat. Med*., 2011, 2012: 1-8.
37. Machado M, Dinis AM, Santos-Rosa M, Alves V, Salgueiro L, Cavaleiro C, Sousa MC. Monoterpenic aldehydes as potential anti-Leishmania agents: Activity of *Cymbopogon citratus* and citral on *L. infantum, L. tropica* and *L. major*. *Exp. Parasitol*., 2012, 130: 223–231.
38. Monzote L, Alarcón O, Setzer WN. Antiprotozoal Activity of Essential Oils: a review. *Agric. conspec. Sci.*, 2012, 77(4): 167-175.
39. Machado M, Dinis, AM, Santos-Rosa M, Alves V, Salgueiro L, Cavaleiro C, Sousa MC. Activity of *Thymus capitellatus* volatile extract, 1,8-cineole and borneol against *Leishmania* species. *Vet. Parasitol*., 2014, 200: 39- 49.
40. Pérez SG, Ramos-López MA, Sánchez-Miranda E, Fresán-Orozco MC, Pérez-Ramos J. Antiprotozoa activity of some essential oils: a review. *J. Med. Plants Res*., 2012, 6(15): 2901-2908.

***** *****