Journal homepage www.jzs.univsul.edu.iq
Journal of Zankoy Sulaimani
Part-A- (Pure and Applied Sciences)

Variation in production of some bioactive molecules of Hypericum triqutrifolium Turra, during plant developmental stages

Hoshyar A. Azeez[1], Ahmad H. Ameen[2] and Shewa A. Faqe[1]

[1] *Biology Dept., College of Science, Sulaimani University, Bakrajo street, Sulaimaniyah-Iraq, E-mail: hoshyar.azeez@univsul.edu.iq and shewa.faqe@univsul.edu.iq*
[2] *Filed Crop Dept., Faculty of Agricultural Science, Sulamani University, Bakrajo campus- Awall Street, Sulaimaniyah- Iraq; ahmad.hamarashid@univsul.edu.iq*

Article info

Original: 24 August 2016
Revised: 28 December 2016
Accepted: 9 January 2017
Published online:
20 June 2017

Key Words:
Hypericumtriquetrifolium, Phenolic compoundsHyperforin, Hypercicn, Plant development.

Abstract

In this research work some bioactive molecules of *Hypericum triqutrifolium* Turra., were evaluated at vegetative (V), flowering (Fl) and fruiting (Fr)} developing stages during the growing seasons as natural products. HPLC was used for qualitative and quantitative of bioactive compounds, which compared with expected compounds such as: Sirginic, Querctrin, Apeginin, Karmeferol, Hypericin and Hyperforin. Different diversity and phytochemical composition among (V), (Fl), and (Fr) were observed. The highest concentration of quercitrin was obtained when plants fruited, whereas apigenin, kameferol and sriginic acid showed highest levels during flower development, while hyperforin in fruiting stage accumulated the highest concentration compared to vegetative and flowering. On the other hand, vegetative stage accumulated larger amounts of hypericin. Regarding to the bioactive accumulation in the individual plant parts, fresh fruit accumulate the highest level of hyperforin at fruiting stage, while it was not detected in stem during flowering and fruiting, whereas leaves produce more hypericin at flowering time.

Introduction

Higher plants are the main sources of bioactive molecules used as pesticides,pharmaceuticals, flavor and fragrance ingredients, food additives, and agrochemicals [1]. Hypericum genus includes approximately 400 species, some are considered as traditional medicinal plants many years ago because of their positive effects on wound-healing, as bactericides, as anti-inflammatory, as diuretic and for their sedative properties [2].
Extracts from vegetative and reproductive organs (stem leave and flower) of Hypericumplants typically contain different concentration of flavonoids: hyperoside, rutin, quercitrin, quercetin,biapigenin,caffeic acid, chlorogenic acid, hypericin,pseudohypericin, and the derivatives hyperforin and adhyperforin [3]. It is commonly used for antimicrobial effect, neuroprotective effect, anti-depressive effect, antioxidant effect, menopause, dental practice, anti inflammatory, wound healing effect, anti-cancer effect, anti-herpes effect, phototoxicological effect [4].The biological activity of Hypericum extracts is mainly attributed to the presence of phenolic compounds which work as antioxidant agents, some of which exert therapeutic antiviral, anti-platelet, anti-allergic,and anti-inflammatory activities [5]. Flavonoids have been used as dietary constituents.Clinical studies they indicated their possible role in preventing cardiovascular diseases and several types of cancer [6]. Based on the results of [7], crude extract of *H. perforatum* decreases the serum lipid profile, low density lipoprotein – cholesterol and triglyceride among rats, while extracts contain hypericins and hyperforin from Hypericum herb are widely used in Europe as a drug for the treatment of

depression [8]. Several studies reported that the production and accumulation of bioactive molecules may change during its phonological stages [9, 10 and 11].

H. triquetrifolium Turra., a perennial herbaceous species, which is widely distributed in Northern-East of Iraq, usually found in rocky mountain slopes, stony hillsides, and steep plains, sometimes near streams, in vineyards, gardens and field, on sandstone, clay, loam, gravel, etc.; alt. 250-1200 (m.a.s.l.). The species *triquetrifolium* has great pharmaceutical potential, with well documented contents of phenolics [12], hypericin [13], phloroglucinols [14] and essential oils [15].The present work deal with the variability of some bioactive compounds concentration three developmental stages namely; (vegetative, flowering and fresh fruiting) among plant parts such as; leaves, stem, flowers and fresh fruits.

Material and Methods

A. Plant collection and identification

Samples of *H. triquetrifolium* Turra., were collected from dry rocky within the Gllazarda mountain district of Sulaimania province, Iraq (35°21'49.35"N, 45°29'37.31 E; 1100 m.a.s.l.) during July - September 2015., (Fig. 1). Plant material was collected at three times of phenological stages namely; vegetative, full flowering and fresh fruiting. The top two thirds of the plants were harvested at 10 -12 PM in clear and sunny day at every collection sites. The mean temperature during the sampling period was 37.5 °C. Harvested plants were identified by the plant Taxonomist, Shewa A. F. Mahmood, Biology Dept. College of Science/ Sulaimani University. Plant samples were dried at room temperature 25 ± 2°C for 7 days until the stable moisture content of 10% was obtained. Dried plant material was then packed in paper bags and kept in a dark, dry and cool place.

Figure 1: *Hypericumtriquetrifolium*Turra (a) Plant grown naturally in the field (b) Plant at the full blooming stage

B.Phenolic and flavonoid extraction

The dried material were ground to fine powder using a mechanical blender and passed through 24 mesh sieve. Powdered plant samples (1 g) were extracted using a maceration method with 50 ml of 70% ethanol (EtOH) for 72 h at room temperature [16] and [17]**.** After the maceration, the extracts were collected, filtered and evaporated to dryness using a rotary evaporator. Analysis of the phenolic and flavonoid compounds conducted by (HPLC), the crude extracts were accurately weighed and dissolved in the mobile phase, to make 10% (w/v) stock solutions.

C. Hypericin and Hyperforin extraction

A quantity of 75 mg of the extract was transferred to analytical glasses and diluted in 25 ml of methanol. All samples were maintained 20 minutes in an ultrasonic bath at room temperature in order to improve the hypericin and hyperforin extraction. The resulting products were centrifuged 10 min at 4000 rpm and the clear supernatant was then filtered and transferred into brown HPLC vials. The prepared extracts were kept in a refrigerator until used [18]. Before HPLC separation, the extracts were filtered through a membrane filter with a pore size of 0.22 μm.

D. HPLC analysis

HPLC analysis of phenolic acid, flavonoids, hypericin and hyperforin were performed using a A Shimaduz liquid chromatograph (Shimaduzcorp, Kyoto, Japan) consisting of a LC-20AT quaternary pump, a DGU-20A3 degasser, an SPD-M20Adiode array detector and a manual rheodyne injector with a 20µl loop were used.Quantification of secondary metabolites was based on peak area (retention time and UV-VIS spectrain the range of 200-800 nm) in comparison with the standard curves (Table 1). The standard curves were obtained by plotting the peak areas of standard concentrations for phenolic acids (0.05, 0.1, 0.2, 0.3, or 0.5 $mgml^{-1}$), for flavonoids (0.0062, 0.0125, 0.025, 0.05, or 0.1 $mgml^{-1}$), and for hypericin (0.025, 0.05, 0.08, or 0.1 $mgml^{-1}$) and for hyperforin(0.025, 0.05, 0.1, or.0.12 $mgml^{-1}$).Standard solutions were kept in darkat -20°C HPLC conditions, LC time program: 35 min.,Total of mobile phase: 1ml/min,Injection volume: 20µl, Temperature of column: room temp. Pressure on pumps: (A, B, C) 7.9 – 9.0 milpaskal (Mpa) Mobile phase: A: water 99% - phosphoric acid 0.3%, B: Acetonitrile 100%, C: Methanol 100%.

Table (1): Retention time, area, concentration of standard compounds

Active Molecules	Retention Time(Min.)	Area (MAU)	Concentration (mnml-1)
Sirginric	19.42	7490426	0.1
Quercitrin	21.28	7308406	0.1
Apeginin	23.2	3824368	0.1
Kameferol	23.31	8990763	0.1
Hypericin	27.05	7080880	0.05
Hyperforin	27.602	280643	0.12

E. Statistical analysis

For Statistical analysis, the (one- way) analysis of completely randomized design was used, with three replicates using Duncan's multiple range tests and the means followed by different letter are highly significant ($p<0.01$). For this purpose (**SPSS**, version 17) was employed [19].

Results and discussion

Composition of bioactive molecules present in the samples is reported in Fig 2a to f. The major compounds detected were Quercitrin, Apigenin, Kameferol, and Sirginric acid, Naphtho dianthrones (hypericin) and Phloroglucinols (hyperforin). These compounds were identified when ethanol was used as a solvent for phenolics and flavonoids, while methanol was used for hypericin and hperforin extraction during phenological cycle of *H. triqutrifolium* Turra. Differences in chemical composition among (V), (Fl) and (Fr) stages were found in the present study. The highest level of phenolics and flavonoids were present in Fl stages compared to V and Fr stages, except the production of hyperforin in Fr stage which was higher than those in V and Fl, but V accumulated more hypericin compared with the Fl and Fr. Fluctuations and biochemical diversity at different growth stages confirmed by [3, 9, 10 and 11], also results in the current study was supported by [20], who reported the production of higher concentration of hypericin at the vegetative stage which might be correlated with the higher dark gland densities in where they sequestered. Hypricum plants at fruiting stage produced higher amount of quercitrin that record 1.159 mg g^{-1}dw. compared to lower levels at V and Fl. The highest Apigenin, kameferol and sriginric acid concentrations were recorded in Fl (0.720, 0.484 and 0.558 mg g^{-1} dwt.) respectively compared to V and Fr growth stages. Data also exhibited a significant increase in hypericin during V stage which was 0.114mg g^{-1} dwt. Compared with plants at Fl and Fr stages which recorded 0.097 and 0.036 mg g^{-1} dwt., hypericin respectively.

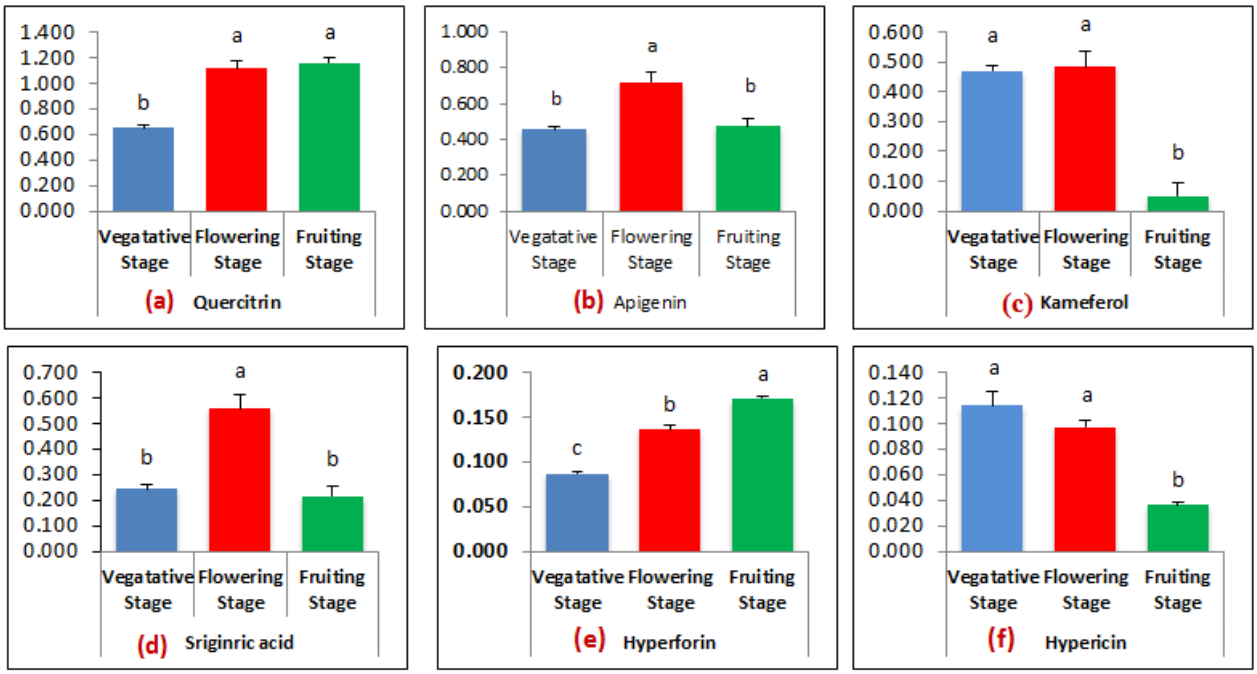

Figure 2: Various contents of compounds (a) Quercitrin, (b) Apigenin, (c) Kamferol, (d) Sirginric, (e) Huperforin, and (f) Hypericin (mg g^{-1} dw.) of *Hypericum triquetrifolium* during plant development.
(V) = Vegetative stage, (F) = Flowering stage, (Fr) = Fruiting stage

Columns with different letters, represent highly significant difference ($p < 0.01$) between the concentrations, according to Duncan multiple range test.

On the other hand, hyperforin production in (Fr) increased and siginificantly recorded 0.171 mg g^{-1} dwt., compared with those produced during both V and Fl stages which produce 0.087 and 0.137 mg g^{-1} dwt., respectively. Results obtained from this study are in agreement with those of [21], [22] and [23] whose reported that the accumulation of hyperforin occur most abundantly in *H. perforatum* flowers, especially in pistils and subsequently forming fruits, but they are also present in leaves but in lower concentrations [22]. Hyperforins have been shown to accumulate in the translucent glands of *H. perforatum* [24] and [25].

Hence, the higher dark gland density at the vegetative stage (leaves and stems) resulted in an increase in hypericin content with respect to the flowering and fruiting stages. Such association of hypericin with dark glands has been reported in *H. perforatum* [26] and [27], *H. elodes* [28], *H. avicullariifolium* var. depilatum, *H. perforatum*and *H. pruinatum* [29], and *H. triquetrifolium* [30]. Biosynthesis of hypericin and its derivatives seems related to cell differentiation, attaining its highest level during leaf morphonogenesis [31]. In addition, hypericin was most abundant in oil glands of leaves [32].Thus, leaves are the preferred organs for hypericin extraction [33].

Differences in bioactive molecules were found in the present study among plant parts during plant development. Results indicate the presence of quercitrin in fresh fruit at fruiting stage recording a high level 2.456 mg g^{-1}dw., (fig. 2 a), whereas flower parts during flowering stage produce large quantities of apigenien, kameferol and siginric acid compared to other parts which reached 1.951, 0.931 and 1.2 mg g^{-1}dw respectively (fig. 3 b, c, and d).

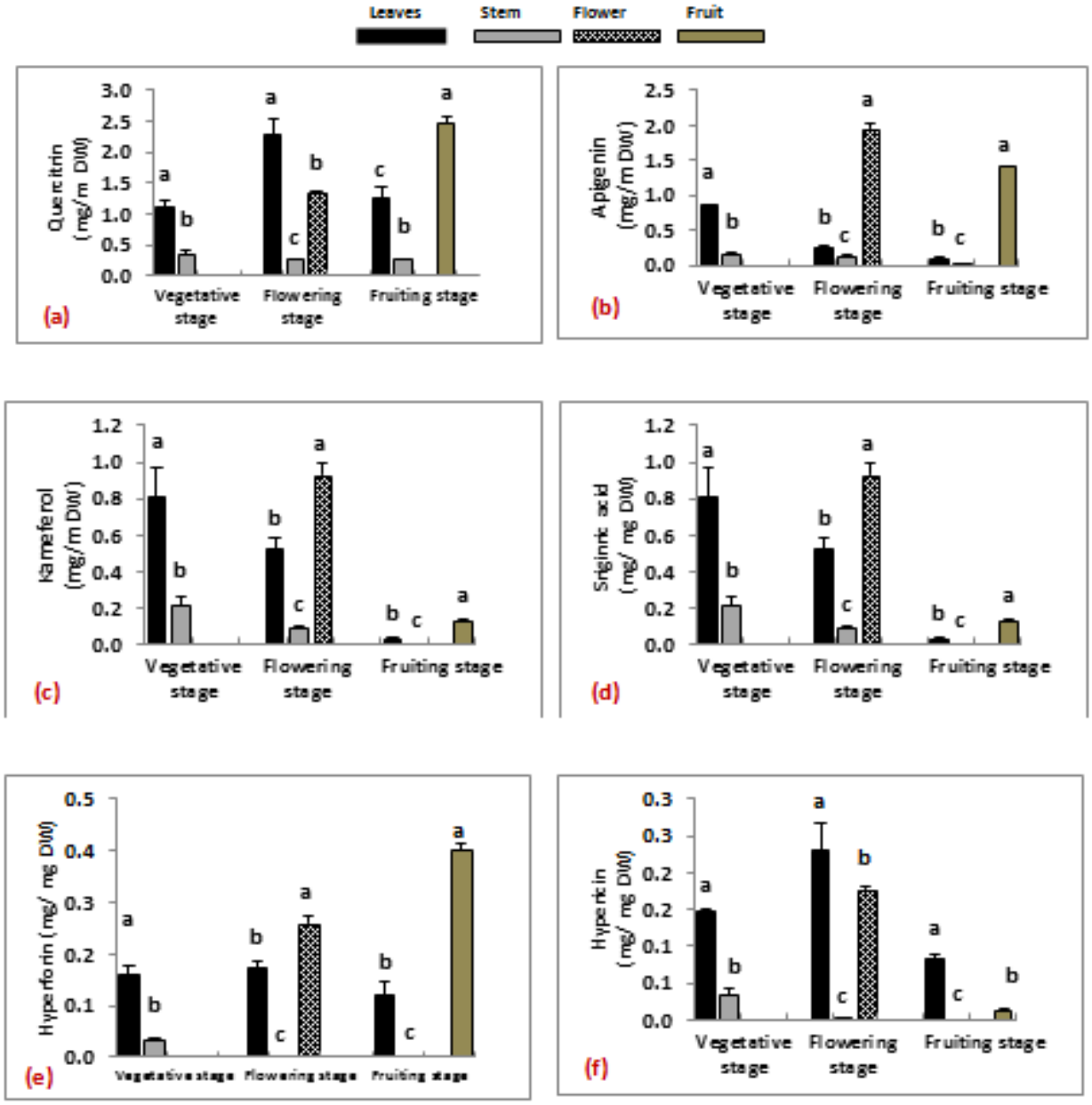

Figure 3: Quercitrin (a), Apigenin (b), Kameferol (c), Sirginric (d), Hyperforin (e) and Hypericin (f) contents (mg g^{-1}dw.) in leaves, stem, flower and fresh fruit of *Hypericumtriquetrifoliu* during plant development.
Columns with different letters, represent highly significant difference ($p < 0.01$) between the concentrations, according to Duncan multiple range test.

In this study, the highest concentration of hyperforin was observed in fresh fruit harvested at fruiting stage recording 0.400 mg g^{-1}dw., (fig. 3 e), but in the case of *H. perforatum* the plant tissues at full blooming stage accumulated higher amount of hypericin when compared to other plant parts [34], whereas the hyperforin did not detected in stem during flowering and fruiting time. Although leaves generally contained more hpericin at flowering stage than vegetative and fruiting ontogenesis, the highest level of this compound was found in leaves recording 0.231 mg g^{-1}dw., (fig.3 f),Similar results were obtained from other *Hypericum* species which reported the yield of hypericin in leaves at flowering stage was higher than others, because the leaves were covered by dark glands, mainly in margins, containing hypericin during this developmental stage [35] and [36], but this result disagree with several studies carried out on different species of Hypericum such as *H. maculatum* [37], *H. pruinatum*and *H. aviculariifolium* [38] were reported to accumulate more hypericin in flowers than in leaves.

Conclusions

It is concluded that plant secondary metabolites accumulate differently at different developmental stages of the different plant parts with a noticeable variation in the diversity of biochemicals, thus it is advisable to collection different plant parts at the proper growing stage to obtain the highest concentration of these constituents.

References

[1] Balandrin, M.J. and J.A. Klocke. *"Medicinal, aromatic and industrial materials from plants. In* Y.P.S". Bajaj (ed.), Biotechnology in Agriculture and Forestry. Medicinal and Aromatic Plant, Vol. 4. Springer-Verlag, Berlin, Heidelberg, pp. 1-36, (1988).

[2] Dias A.C.P., Seabra R.M., Andrade P.B., Ferreres F. and Fernandes-Ferreria M. "*Xanthone biosynthesis and accumulation in calli and suspended cells of Hypericumandrosaemum".* Plant Sci. Vol. 150, pp. 93 – 101, (2000).

[3] Patocka J. "*The chemistry, pharmacology, and toxicology of the biologically active constituents of the herb Hypericumperforatum*L". J. Appl. Biomed.Vol. 1, pp. 61– 73, (2003).

[4] Rahimi, R,and Kiani, S. *"Chemical compound and Therapeutic effects of Hypericumperforatum".* Der Pharmacia Lettre, Vol. 8, No. 9, pp. 237-241, (2016).

[5] Isfahlan A. J., Mahmoodzadeh, A. ,Hasanzadeh, A. "*Antioxidant and antiradical activities of phenolic extracts from Iranian almond (Prunusamygdalu*s L.*)* hullsand shells". Turk J. Biol. Vol. 34, pp. 165-173, (2010).

[6] Chu YH, Chang CL, Hsu HF. *"Flavonoid content of several vegetables and their antioxidant activity".* J Sci Food Agric. Vol. 80, pp. 561-566, (2000).

[7] GhosianMoghaddam, M. H., Roghani, M., and Malek, M. "*Effect of Hypericumperforatum aqueous extracts on serum lipids, Aminotransferases, and Lipid Peroxidation in Hyperlipidemic Rats".* ResCardiovasc Med. Vol. 5, No. 2, e31326, (2016).

[8] Gastpar, M, and Zeller K. *"Hypericum-extrakt STW3 und sertralinzurbehandlung der mittelschweren depression*". Psychopharmakotherapie Vol. 12, pp. 146-153, (2005).

[9] Cirak, C., Radusiene, J., Janulis, V. and Ivanauskas, L. "*Secondary metabolites in Hypericumperforatum: variation among plant parts and phonological stages*". Bot. Helv., Vol. 117, pp. 29-36, (2007).

[10] Hosni, K., Msaada, K., Taarit, M.B. and Marzouk, B. *"Phonological variations of secondary metabolites from HypericumtriquetrifoliumTurra".* Biochemical Sys.and Ecol. Vol. 39, pp. 43-50, (2011).

[11] Ghavamaldin, A., Aptin, R., Khalil, P., Mansour, G. and Mariamalsadat, T. *"Study of variation of biochemical components in Hypericum perforatum* L. *growm in north of Iran".* J. of Medicinal Plants Res., Vol. 6, No. 3, pp. 366-372, (2012).

[12] Azeez, H. A., and Ibrahim K. M. *"Hypericum Triquetrifolium Callus Cultures a Potential Source of Phenolics and Flavonoids".* Journal of Zankoy Sulaimani- Part A, Special Issue, Vol. 16, (2014).

[13] Azeez, H. A., and Ibrahim K. M. *"Effect of Biotic Elicitors on Secondary Metabolite Production in Cell Suspensions of Hypericum triquetrifolium Turra*". Bulletin UASVM Horticulture,.Print ISSN 1843-5254; Electronic ISSN 1843-5394, Vol 70, No. 1, pp. 26-33, (2013).

[14] Benkiki, N., Kabouche, Z., Tillequin, F., Verite, P., Chosson, E. & Seguin, E. *"A new polyisoprenylatedphloroglucinolderivativefrom*Hypericumperfoliatum *(Clusiaceae)".* Z Naturforsch Vol. 58, pp. 655–658, (2003).

[15] Couladis,M.,Baziou,P., Petrakis,P.V., Harvala, C. "*Essential oil composition of HypericumperfoliatumL". growing in different locations in Greece*, Flavour Fragrance J. Vol. 16, pp. 204-206, (2001).

[16] EMA/HMPC. "*Community Herbal Monograph on Hypericumperforatum*L., *Herba (Well-Established Medicinal Use)"*. Available online: http://www.ema.europa.eu/docs/ en_GB/document_library/Herbal-Community_herbal_monograph/2010/01/WC500059145,(2013).

[17] "European Directorate for the Quality of Medicines & Health Care". European Pharmacopoea, 6th ed.; Council of Europe: Strasbourgh, France; Vol. 2, pp. 2958–2959, (2007).

[18] Tortoriello, J., Zamilpa, A., Herrera, A., Romero-Cerecero, O., and Mental, S. "*Determination of Hypericin and Hyperforin content in different phytopharmaceuticals and dietary supplements containing Hypericumperforatum extract"*. Salud Mental,. Vol. 26, No. 4, pp. 59-63, (2003).

[19] Duncan, D.B. *"Multiple Range and Multiple F-test"*, Biometrics, Vol. 11, pp. 1-42, (1995).

[20] Cirak, C., Saglam, B., Ayan, A.K. and Kevseroglu, K. *"Morphogentic and diurnal variation of hypericin in some Hypericum species from Turkey during the course of ontogenesis*", Biochem. Syst. Ecol., Vol. 34, pp. 1-13, (2006).

[21] Tekel'ova, D., Repcak, M., Zemkova, E. and Toth, J. "*Quantitative changes of dianthrones, hyperforin and flavonoids content in the flower ontogenesis of Hypericumperforatum"*. Planta Med., Vol. 66, pp. 778−780, (2000).

[22] Sirvent, T.M., Krasnoff, S.B. and Gibson, D.M. "*Induction of hypericins and hyperforins in Hypericumperforatum in response to damage by herbivores"*. J. Chem. Ecol. Vol. 29, pp. 2667-2681, (2003).

[23] Beerhues, L. "*Hyperforin. Phytochemistry", Vol.* 67, pp. 2201–2207, (2006).

[24] Soelberg, J., Jørgensen, L. B.andJäger, A. K. *"Hyperforin accumulates in the translucent glands of Hypericumperforatum*". Ann Bot. Vol. 99, pp. 1097−1100, (2007).

[25] Holscher, D., Shroff, R., Knop, K., Gottschaldt, M., Crecelius, A., Schneider, B.,Heckel, D. G, Schubert, U.S.andSvatos, A. *"Matrix-free UV-laser desorption/ionization (LDI) mass spectrometric imaging at the single-cell level: distribution of secondary metabolites in Arabidopsis thaliana and Hypericum species*". Plant J Vol. 60, pp. 907−918, (2009).

[26] Briskin, D.P. and Gawienowski, M. C. *"Differential effects of light and nitrogen on production of hypericins and leaf glands in Hypericumperforatum"*. Plant Physiol. Biochem., Vol. 39, pp. 1075−1081, (2001).

[27] Cirak, C., Radusiene, L. and Arslan, B. *"Variation of bioactive substances in Hypericummontbetii during plant growth*". Nat. Prod. Res., Vol. 22, pp. 246- 252, (2008).

[28] Piovan, A., Filippini, R., Caniato, R., Borsarini, A., Maleci., L.B. and Cappelletti, EM. "*Detection of hypericin in the "red glands" of Hypericumelodes by ESI-MS/MS*". Phytochemistry, Vol. 65, pp. 411-414, (2004).

[29] Cirak, C., Saglam, B., Ayan, A.K. and Kevseroglu, K. "*Morphogentic and diurnal variation of hypericin in some Hypericum species from Turkey during the course of ontogenesis"*, Biochem. Syst. Ecol., Vol. 34, pp. 1-13, (2006).

[30] Ayan, A.K. and Cirak, C. "*Variation of hypericin in HypericumtriquetrifoliumTurra. growing in different locations of Turkey during plant growth"*. Nat. Prod. Res., Vol. 22, pp. 1597-1604, (2008b).

[31] Kirakosyan, A., Vardapetyan, H. and Charchoglyan, A. *"The content of hypericin and pseudohypericin in cell culture of Hypericumperforatum*L". Russian J. Plant Physiol., Vol. 47, pp. 301-306, (2000).

[32] Ciccarelli, D, Andreucci AC &PagniAM. *"Translucent glands and secretory canals in Hypericumperforatum L. (Hypericaceae): morphological, anatomical and histochemical studies during the course of ontogenesis"*. Ann Bot Vol. 88, pp. 637−644, (2001).

[33] De-Smet, P. and Nolen, W.A. *"St. John's wort as an antidepressant"*. British Med. J., Vol. 313, pp. 241-242, (1996).

[34] Cirak, C., Radusiene, J., Janulis, V., and Ivanauskas. *"Pseudohypericin and Hyperforin in Hypericumperforatum from Northern Turkey: Variation among Populations, Plant Partsand Phenological Stages"*. Journal of integrative plant biology,Vol. 50, Issue 5. pp. 575–580, (2008).

[35] Cirak, C., Radusiene, J., Janulis, V. and Ivanauskas, L. *"Secondary metabolites in Hypericumperforatum: variation among plant parts and phonological stages"*. Bot. Helv., Vol. 117, pp. 29-36, (2007).

[36] Ayan, A.k., and Cirak, C. "*Hypericin and psudohypericin contents in some Hypericum species growing in Turkey*". Pharmaceutical Biology, Vol. 46, No. 4, pp. 288–291, (2008 a).

[37] Radušienė, J., Bagdonaite, E., and Kazlauskas, S. *"Morphological and chemical evaluation on Hypericumperforatum and H. maculatum in Lithuania*". ActaHort (ISHS) Vol. 629, pp. 55-62, (2004).

[38] Cirak, C., Saglam, B., Ayan, AK., and Kevseroglu, K. *"Morphogenetic and diurnal variation of hypericin in some Hypericum species from Turkey during the course of ontogenesiss"*. Biochem. Syst. Ecol. Vol. 34, No. 1, pp. 1-13, (2006).