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ISSN print: 1224-5984
ISSN online: 2248-3942

Rom Biotechnol Lett. 2020; 25(6): 2111-2117
doi: 10.25083/rbl/25.6/2111.2117

Received for publication, December, 27, 2019
Accepted, February, 10, 2020

Original paper

Phytochemicals and antioxidant capacity of wild growing and *in vitro Hypericum heterophyllum*

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Abstract *In vitro* cultures of *Hypericum heterophyllum* Vent. was established by using MS and MS-B5 medium contained plant growth regulators such as kinetin, BAP, IAA, TDZ and picloram. Flower, leaf, stem and *in vitro* samples (callus and *in vitro* plantlets) of *H. heterophyllum* were analysed by LC-MS/MS for their chemical contents such as quinic acid, gallic acid, (+)-catechin, ferulic acid, vanillic acid, *p*-coumaric acid, caffeic acid, and quercetin; moreover their radical scavening activities conducted by DPPH and ABTS methods were evaluated. Among all the analysed samples, the *in vitro* plantlets shown the highest antioxidant activity (IC_{50}, 220 µg/mL for DPPH and 254 µg/mL for ABTS), probably due to the presence of phenolic acids and flavonoids, specifically the higher total phenolic content (64.4 mg GAE/g extract) than other samples. The phytochemical variation among all samples was discussed through principal component analysis (PCA) and hierarchical cluster analysis (HCA). The *in vitro* plantlets might offer possibilities for the production of high-value secondary metabolites as pharmaceuticals and food preservatives. This study is the first report on analyses and comparison of secondary metabolites and antioxidant activities in different plant parts and *in vitro* samples of endemic *H. heterophyllum*.

Keywords *Hypericum*, *in vitro* plantlets, antioxidant activity, phytochemical.

To cite this article: YAMAN C. Phytochemicals and antioxidant capacity of wild growing and *in vitro Hypericum heterophyllum*. *Rom Biotechnol Lett*. 2020; 25(6): 2111-2117. DOI: 10.25083/rbl/25.6/2111.2117

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Introduction

Hypericum genus belonging to the Hypericaceae (previously *Clusiaceae* or Guttiferae) family contains a wide variety of secondary metabolites known as mainly napthodianthrones (hypericin, pseudohypericin, protohypericin), flavonoids (campherol, quercetin, rutin, luteolin, hyperin, hyperoside), phenolic acid (chlorogenic acid), phloroglucinols (hyperforin, furohyperforin), xanthones and essential oils (Z.A.T FOBOFOU *&* al [1]). These components exhibit very important biological activities such as antioxidant, antidepressant, antitumor, antibacterial, antimicrobial, anti-inflammatory effects and others (C. ZORZETTO *&* al [2]).

At the present time, the most well-known member of this genus, *Hypericum perforatum* (St. John's wort), a rich source for flavonoids, widely consumed for medicinal purpose in all over the world. However, only a relatively few of them have been investigated in terms of their phytochemical content and biological/pharmacological activity, therefore, a large majority (about 60%) of the *Hypericum* species in need of attention due to their medicinal value (S.L. CROCKETT *&* al [3]). One of these species is *Hypericum heterophyllum* in which very few studies on chemical components such as essential oil content and its hypericin/pseudohypericin contents and biological activities of the species such as antioxidant and antimicrobial (A.K. AYAN *&* al [4]; E.L. UNAL *&* al [5]) were reported upto date. A.K. AYAN *&* al [4] stated that the aerial parts (flower, leaf and stem) of the species contain no hypericin and pseudohypericin.

Establishing *in vitro* cultures is a great tool for the production of desirable secondary metabolites under a controlled environment, independent of seasonal and geographical conditions. In previous studies, significant secondary metabolite production such as total phenols, flavonoids, hyperoside, hypericin, pseudohypericin and others were obtained specially from shoot, root, hair, callus and suspension culture of *H. perforatum* were reported (O. TUSEVSKI *&* al [6]; K. NIGUTOVÁ *&* al [7]). But, there are no reports about *in vitro* production of secondary metabolite production and analysed for *H. heterophyllum*, to this point.

The aim of the present study was to investigate endemic *H. heterophyllum* in terms of (1) production of undifferentiated callus and shoot culture; (2) content of eight secondary metabolites, such as gallic acid, quinic acid, quercetin, vanillic acid, (+)-catechin, *p*-coumaric acid, caffeic acid, ferulic acid, in different parts of the wild-growing plant and *in vitro* samples for the first time by LC-MS/MS; (3) characterization and comparison of the callus, *in vitro* plantlets, the plant parts extracts with respect to their to determine total phenolic and flavonoid contents, moreover antioxidant properties using DPPH and ABTS radicals; (4) examination whether or not there is a relationship between the samples of the plant by using principal component analysis, multiple comparison test, and cluster analysis.

Materials and Methods

Plant material

The aerial parts of wild growing *H. heterophyllum* Vent. representing a total of 40 shoots were collected at full flowering period between 11:00 a.m. and 13:00 p.m. from Yozgat/Turkey (39º46′ N, 34º47′ E, 1332 m). The species was identified, and the herbarium specimen was deposited in a herbarium (voucher number: 28281) placed in Selcuk University. The seeds of *H. heterophyllum* were handpicked from 30 randomly selected *Hypericum* plants and stored at 4 ± 2°C in sealed plastic bags until used for *in vitro* cultures.

Source of explants for *in vitro* cultures

The seeds of *H. heterophyllum* were surface sterilized by treatment by immersion in 20% sodium hypochlorite for 20 min, and followed by 3-4 times rinses in sterile distilled water. After sterilization, the seeds were germinated on the Murashige and Skoog (MS) basal media containing 2 mg/L GA_3 (gibberellic acid), 30% (w/v) sucrose and 0.7% (w/v) agar. Twenty five seeds were cultured in each petri dishes and incubated in a growth chamber under photoperiod of 16 h at 24 ± 1°C. Two week-old seedlings served as the source for further explants.

Shoot production

The nodal segments were cultured on MS medium supplemented with three different types of plant growth regulators (PGRs) (0.25 mg/L BAP (6-benzylaminopurine), 1.0 mg/L IAA (indole-3-acetic acid) and 0.5 mg/L kinetin). The explants were subcultured at the end of the two weeks and after two months of culture, secondary metabolite contents and antioxidant activities consisting of shoot were determined.

Callus production

Nodal segments of *H. heterophyllum* were placed on MS-B5 (medium supplemented with 1.0 mg/L TDZ (thidiazuron) and 0.10 mg/L picloram. The explants were subcultured every two weeks, 45 day old callus cultures were analysed.

Extraction

The aerial parts (flower, leaf and stem) of the wild-growing *H. heterophyllum* and *in vitro* samples (callus and *in vitro* plantlet) were used for the extraction. The aerial parts were dried under shade and mechanically ground with a blender while callus was ground with a mortar and pastle in a liquid nitrogen. 4 g (three replicate) of each grounded plant materials were extracted individually in 40 ml of 100% methanol at 40°C for 24 h. The resulting solutions were filtered through whatman paper and the solvent was removed on a rotary evaporator at temperature bellow 40°C. Extract yields of flower, leaf and stem, *in vitro* plantlet, callus were recorded 16.6%, 19.8%, 16.9%, 11.0%, 3.4%, respectively.

Total phenolic content (TPC)

The total phenolic content was analysed according to the Folin-Ciocalteu reagent (FCR) by the methodology of V.L. SINGLETON *&* al [8] with a slight modification. For the analysis, 200 µL extract was mixed with 9.0 ml distilled water, and added 200 µL FCR. After shaken vigorously for 3 min, 600 µl of a 20% Na_2CO_3 solution was added to the mixture. The total phenol content was calculated at 760 nm after the incubation in dark at room temperature for 2 h. The results were expressed as mg equivalents of gallic acid (GAE) per gram of extract according to the equation obtained from the standard gallic acid graph ($y = 0.0089x - 0.0003$, $R^2 = 0.999$).

Total flavonoid content (TFC)

Total flavonoid contents were determined according to A. ARVOUET-GRAND *&* al [9]. In brief, 200 µL extract was mixed with 100 µL of a 10% aluminium nitrate and 100 µL of a 1.0 M potassium acetate. Total volume of the solution was adjusted 4.0 mL with ethanol. Absorbance measurements were recorded at 417 nm after 40 min incubation at room temperature in dark. Total flavonoid contents were expressed as mg equivalents of quercetin (QE) per gram of extract according to the equation obtained from the standard quercetin graph ($y = 0.0122x + 0.065$, $R^2 = 0.998$).

Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) analysis and identification

Quantitative analysis of compounds was performed by LC-MS/MS, Thermo Scientific - Dionex Ultimate 3000 – TSQ Quantum with Thermo ODS Hypersil 250 × 4.6 mm, 5 µm column. The injection volume was 10 µL. The mobile phase included eluent water with 0.1 % formic acid (A) and methanol (B). The flow rate was 0.7 mL/min, and the column temperature was set to 40°C. The gradient programme was fixed as follow: 0-5 min, 100% A, 5-20 min, 95% A, 20-20.1 min, 20% A, 20.1-26 min, 5% A, 26-30 min 100% B. Total process time was 30 min. The injection volume was 10 µL. Method Validation parameters of compounds are also shown in Table 1.

The analytical method was validated to determine the linearity, limits of detection (LODs), limits of quantification (LOQs) and precision. The relationship between peak area and concentration was linear from 50 to 100 µg/mL (ppb) for each compound. Linearity was assessed using linear regression analysis of six points for each compound. Linear plot consists of triplicate per point. The correlation coefficients (R^2 values) were found to be ≥ 0.99. Linear regression equations of the compounds are also reported in Table 1. LOD and LOQ were determined by using measurements of reagent blanks spiked with low concentrations of analyte according to Eurachem Guide (Laboratory Guide to Method Validation and Related Topics, 2014). The blanks solution was spiked to 5 ppb standard. Calculate LOD and LOQ as $LOD = 3 \times S_0$ and $LOQ = 10 \times S_0$. The repeatability in the intra-day values (relative standard deviation, RSD%) for compounds, using the corresponding peak areas of three replicate analyses at all the concentration levels. The trueness was examined as recovery of each compound from mixed stock standard solutions in spiked plant extracts. The recovery was evaluated by means of three replicate measurements in a day. The average recovery data of the compounds were determined using the following formula:

$$\text{Recovery } (100\%) = \left(\frac{\text{Measured concentration}}{\text{Spiked concentration}}\right) \times 100$$

The concentration of compounds in samples of the plant was obtained from either one of the corresponding calibration curves. Finally, each bioactive amount for each samples were calculated mg/100 g DW (A. SHRIVASTAVA *&* al [10]).

Table 1. Linear regression equation and correlation coefficient, precision of each detected compounds by LC-MS/MS analysis on *Hypericum heterophyllum*

Composition (µg/g)	Linear regression equation	R^2	LOD (µg/L)	LOQ (µg/L)	RSD (%)	Recovery (%)
Quinic acid	y=483.60×-10563	0,999	5.52	6.92	4.07	97.75
Gallic acid	y=464.58×-10423	0.999	5.38	6.78	4.45	96.45
Vanillic acid	y=603.27×-19881	0.997	4.99	6.11	4.12	101.25
Caffeic acid	y=1219.67×-7914	0.999	5.45	7.13	2.74	98.55
p- coumaric acid	y=4773.03×+86775	0.996	7.33	11.11	3.14	99.85
Ferulic acid	y=322.29×-4272	0.998	6.62	9.00	4.90	100.15
(+)-catechin	y=598.80×+882	0.997	5.12	6.31	2.85	99.14
Quercetin	y=18760×-131657	0.998	6.62	11.59	2.21	99.90

R^2 – regression coefficient, LOD – limit of detection, LOQ – limit of quantification, RSD – relative standard deviation

DPPH• scavenging activity

Radical scavenging activity of the extracts was determined using the DPPH radical (M.S. BLOIS [11]). Briefly, 200 µL of methanolic solution of all tested extracts at five different concentrations was mixed with 3.2 mL of a 0.004% solution of DPPH• in 100% methanol. Ascorbic acid (AA) and trolox as standard were used. After a 30 min incubation at room temperature in dark, absorbance of the sample was measured at 517 nm.

ABTS•+ scavenging activity

ABTS was used for evaluation of radical cation scavenging activity according to the method described by R. RE & al [12]. The absorbances were recorded at 734 nm after a 30 min incubation at room temperature in the dark. AA and trolox as standard were used.

Statistical analysis

All data was statistically analysed using one-way ANOVA, and comparison of the means was carried out by Duncan's multiple range tests at a significance level of 0.01 and the data were given as the mean ± standard error. The data matrix of the plant samples was obtained from various statistical evaluations, including principal component analysis (PCA) and hierarchical cluster analysis (HCA).

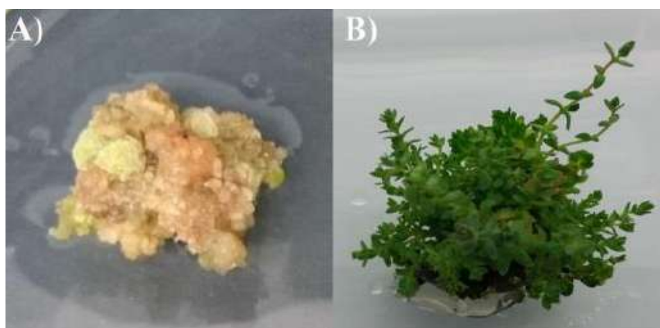

Figure 1. *In vitro* samples of *Hypericum heterophyllum*. **A**) Callus formation from nodal segments cultivated on MS-B5 + 1.0 mg/L TDZ + 1.0 mg/L picloram. **B**) Shoot induction from nodal segments cultivated on MS + 0.25 mg/L BAP + 0.5 mg/L kinetin +1.0 mg/L IAA.

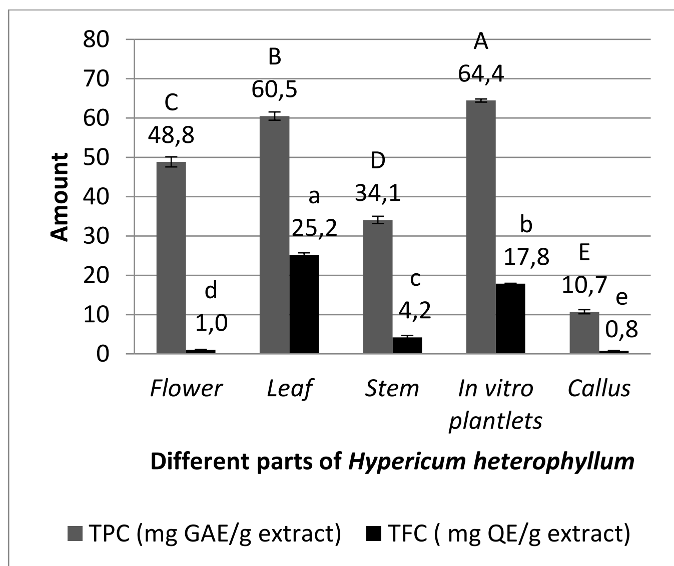

Figure 2. Total phenolic and flavonoid contents in flower, leaf, stem, *in vitro* plantlet, callus of *Hypericum heterophyllum*. Big letters for TPC and small letters for TFC and on the columns indicate Duncan at $P<0.01$.

Results and Discussion

Callus and shoot induction

Callus formation was determinated in the MS-B5 medium supplemented with 1.0 mg/L TDZ and 0.10 mg/L picloram. The calli were yellowish in color and fragile (Fig. 1A). Also, callus development was observed to be slow and poor. Many researchers notified that induction and growth of callus of *Hypericum* species were slow and compact (A.K AYAN & al [13]; S.A KHAN & al [14]). But, Kinetin and BAP has been found favourable for multiplication of some *Hypericum* species *in vitro* shoots (A.K AYAN & al [13]; H. SOOD & al [15]; I. BĂCILĂ & al [16]). We found that Kinetin and BAP were very effective on shoot induction from nodal segments of *H. heterophyllum* (Fig. 1B).

Total phenolic content (TPC) and total flavonoid content (TFC)

The total quantity of TPC and TFC were recorded in Fig. 2, and found statistically significant differences ($p<0.01$). The highest TPC was determined *in vitro* plantlets cultivated in media containing 0.25 mg/L BAP, 1.0 mg/L, IAA and 0.5 mg/L kinetin, and this media was found to be very effective for increasing TPC in *H. heterophyllum*. X.H. CUI & al [17] reported that combination of IBA (indole-3-butyric acid) and IAA was more effective than NAA (1-naphthaleneacetic acid), moreover, kinetin was better than BAP and TDZ for increasing phenolic contents in a different *Hypericum* species. On the other hand, the level of TPC and TFC in the callus culture cultivated in MS-B5 media supplemented with 1.0 mg/L TDZ and 0.10 mg/L picloram demonstrated the lowest among the other samples. Similarly to this study, V. KUMAR & al [18] stated that combination effect of picrolam (different concentrations) and 1.0 mg/L TDZ on TPC in callus of *Pelargonium sidoides* was exhibited a very strong negative effect compared to control and other concentration of TDZ. K. DANOVA & al [19] reported that MS medium was the most effective for production of flavonoids than B5 medium. Therefore, these results may attributed to the PGRs or the excess number of the leaves of the *in vitro* plantlets (Fig. 1B). Because, among all the samples, the leaf had the highest TFC, and followed by the *in vitro* plantlets.

Phytochemicals of *Hypericum heterophyllum*

The amounts of the identified phytochemicals by LC-MS/MS in methanolic extracts of flower, leaf, stem, *in vitro* plantlets and callus of *H. heterophyllum* is presented in Table 2.

Table 2. Phytochemicals in flower, leaf, stem, *in vitro* plantlet, callus of *Hypericum heterophyllum* (mg/100g DW)

Compounds	Flower	Leaf	Stem	*In vitro* plantlet	Callus
Quinic acid	38.7c	145.4a	58.1b	6.6d	0.2e
Gallic acid	0.46b	1.90a	0.44b	0.53b	0.08c
Vanillic acid	1.09c	1.45b	1.05c	2.14a	0.05d
Caffeic acid	1.55a	1.17b	0.45d	0.68c	0.01e
p-coumaric acid	0.13b	0.03c	0.01d	0.21a	0.0d
Ferulic acid	0.58b	0.44c	0.22e	1.72a	0.33d
(+)- catechin	0.09d	0.24c	2.46b	6.59a	0.02e
Quercetin	9.03b	3.18c	9.65a	0.87d	0.02e

Statistically. each column was evaluated separately and indicated in small letters ($P<0.01$)

Among all the samples, generally lower amount of the compounds were recorded in the callus. In comparison of the compounds tested, quinic acid was found in all samples as a major product except callus that contained the highest amount of ferulic acid (0.33 mg/100g DW) among other compounds. Caffeic acid was found the highest amount with 1.55 mg/100g DW in the flower. The stem consisted of the highest quercetin level (9.65 mg/100g DW) among all samples.

PCA was applied to the data matrix of chemical contents (8 chemical x 5 cases) of the *in vitro* samples and different parts of *H. heterophyllum* growing wild. The principal components, which have eigenvalues higher than 1 were extracted. This led to the formation of two principal components. The PC1 accounted for 44.26%, the PC2 for 33.67% of the total variation of all chemical data. The chemical components were rotated using Varimax rotation. Fig. 3 shows the two-way score and loading plots for PC1 and PC2. Fig. 3B shows a cluster of chemical components with large positive loadings on PC1. It includes *p*-coumaric, vanillic acid, ferulic acid, and (+)-catechin. It can be commented from the loading and score plots for PC1-PC2 (Fig. 3A and B) that (+)-catechin, vanillic acid, *p*-coumaric and ferulic acid concentrations are higher for the *in vitro* plantlets (6.59, 2.14, 0.21 and 1.72 mg/100 g DW, respectively). In addition, quinic acid and gallic acid had the highest value on PC1. This can be interpreted from PC1 and PC2 that quinic acid (144.11 mg/100 g DW) and gallic acid (1.98 mg/100 g DW) concentrations are highest for the leaf.

A hierarchical clustering by applying the *Between-groups linkage* method, which uses the squared Euclidean distance as a similarity measure, was applied using the SPSS package. In dendrogram, according to the phyto-chemical compounds tested, the flower and stem were in the same group, and the leaf was in the group close to them while the *in vitro* plantlets alone formed a separate entity from other samples (Fig. 4). The callus was in the group close to the *in vitro* plantlets. The parallelism between the distribution of PCA and the dendrogram suggests an appreciable degree of congruence between the two.

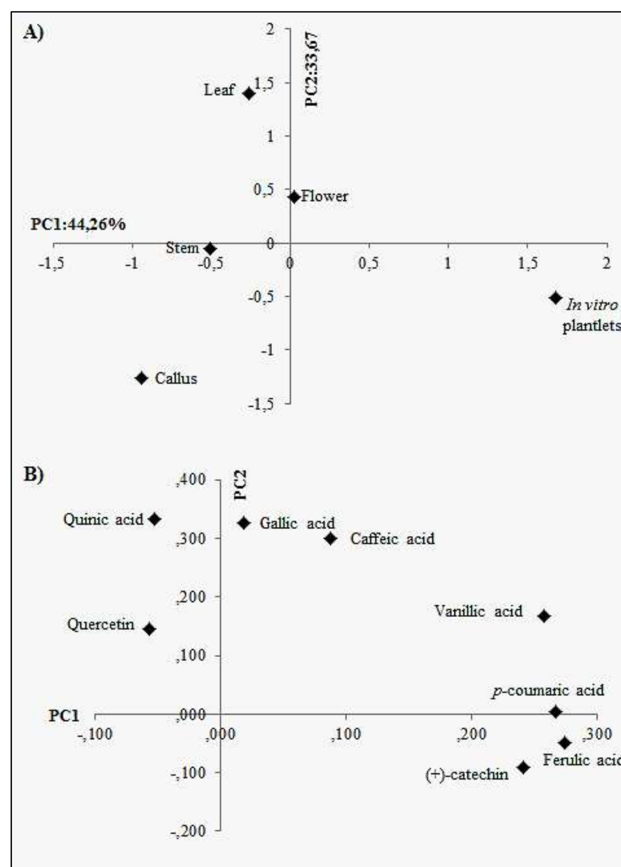

Figure 3. The score (**A**) and loading (**B**) plots for phytochemical contents of *H. heterophyllum.*

In this study, we found that ferulic acid, p-coumaric, vanillic acid, and (+)-catechin among the compounds tested for *H. heterophyllum* could be produced much more than those in different parts of the plant growing wild by *in vitro* culture. The *in vitro* plantlets synthesized 2 times more (+)-catechin than the stem that contained the highest (+)-catechin among different parts of H. heterophyllum growing wild. Also, the amount of *p*-coumaric acid in the in vitro plantlets was found about 2 times higher than in the flowers, 7 times abundant than in the leaf, 21 times more than in the stem samples.

The amount of vanillic acid of the *in vitro* plantlets was about 2 times increase as compared to the plant parts. Vanillic acid, a dihydroxybenzoic acid derivative has very weak antioxidant activity, but used widely as a fragrance, flavoring and food preserving agent. The price has increased about 20-fold between 2012 and 2018 for this compound, which has a global market in several areas. In this way, it opened the route to alternative vanillin production methods R. CIRIMINNA & al [20]. One of these alternative productions, vanillic acid, is synthesized from ferulic acid as natural product, and the price of this biotechnological vanillic acid is also considerably high R. DELISI & al [21]. The *in vitro* plantlets was notable for the presence of high amounts of ferulic acid, and found about 4 times more abundant than in the flower and leaf, 8 times more abundant than in the stem.

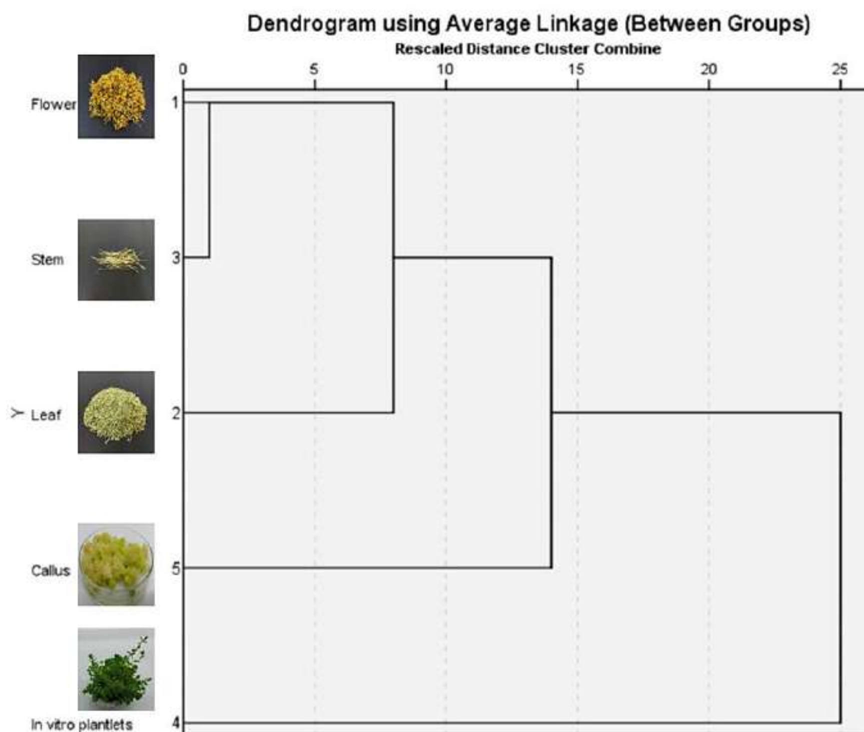

Figure 4. Dendrogram of *H. heterophyllum* samples (versus each of the eight metabolites under study) obtained by hierarchical cluster analysis using square Euclidean distance.

The radical scavenging activity

In this study, the radical scavenging activity of all samples of *H. heterophyllum* growing wild and *in vitro* samples was evaluated by means of the DPPH and ABTS assays, and expressed as IC_{50} values (Table 3). The results were compared with those of trolox and ascorbic acid (AA) used as standard.

Table 3. Free radical scavenging activities of flower, leaf, stem, *in vitro* plantlet, callus of *Hypericum heterophyllum* (IC_{50} values)

Samples	DPPH		ABTS	
Flower	800±3.1	e	782±1.7	f
Leaf	342±1.0	c	420±0.3	d
Stem	544±3.4	d	690±1.2	e
In vitro plantlets	220±0.4	b	255±0.9	c
Callus	1457±10.4	f	8863±1.6	g
Trolox	109±1.3	a	101±0.2	b
AA	59±0.0	a	56±1.9	a

Statistically. each column was evaluated separately and indicated in small letters (P<0.01)

The *in vitro* plantlets with IC_{50}, 254.95±0.9 µg/mL for ABTS and 220.14±0.4 µg/mL for DPPH showed the stronger activity in both assays, which was significantly lower than that of standards such as ascorbic acid (55.68±1.9 µg/mL and 59.06±0.1 µg/mL, respectively) and trolox (100.66±0.2 and 108.88±0.13 µg/mL, respectively). For both activities, the *in vitro* plantlets demostrated the strongest activity, followed by the leaf, stem, flower and callus. Similarly, among the aerial parts of *H. perforatum* L., the leaf shown the higher DPPH activity than other parts

of the plant, and the lower than AA as a standard N. SEKEROGLU & al [22]. In previous studies, similar to results were reported that many *Hypericum* species displayed the lower antioxidant activity than the standards C. Zorzetto & al [2]. The variations of antioxidant activity in H. heterophyllum samples may be attributed to differences and amounts of chemical compounds in the samples. Because, the callus that contained lowest TPF and TFC exhibited the lowest antioxidant activity.

Conclusion

This study provides novel information about phytochemical compositions, total phenolics, flavonoids and radical scavenging activities of *H. heterophyllum.* Furthermore, statistical analysis of PCA and HCA for the phytochemicals of the plant was conducted for the first time. Among the samples, the *in vitro* plantlets on MS medium supplemented with BAP, IAA and kinetin was very effective for the productions of ferulic acid, vanillic acid, (+)-catechin and *p*-coumaric acid. Consequently, statistical analyses have demonstrated that these compounds could be synthesized and accumulated more *in vitro* cultures than that in different parts of *H. heterophyllum* growing-wild. The *in vitro* production of these compounds used for many purposes may be very important. Especially, *in vitro* production of ferulic acid and vanillic acid may be considered to be important for the natural production of vanillin. Conclusively, further studies are necessary to exploit the biosynthetic potential, different extraction methods and to reveal production of other specific bioactive metabolites of *H. heterophyllum.*

Acknowledgement

Author would express their deep thanks for Dr. Ufuk KOCA-CALISKAN, Gazi University, Turkey for technical support during interpretation of data.

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