Study of Variation of Biochemical Components in *Hypericum perforatum* L. Grown in North of IRAN

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ABSTRACT

*Hypericum perforatum* L. is among important herbs worldwide, which growing wild in Iran various areas and altitudes particularly north of the country. However, according to different regional and genetic reasons, its secondary metabolites differ in its growth vegetative. Therefore, in order to define its best vegetative area, the most important biochemical components in five regions and three altitudes and four samples from each regions (60 samples all together) were sampled and analyzed. Experiment revealed that the highest values of hypericin (0.251% DW) and total phenols (412 mg/l) were obtained in Jamat Roodbar region in a height of 1218 m and the highest amounts of flavonoid (73 µg/ml) and carotenoid (0.13 mg/ml) were observed in Pole Zangoole region in a height of 2300 m. Regression equations have been demonstrated to have a positive linear relationship between hypericin and total phenols contents. While among hypericin and flavonoid as well as carotenoid, this relation forms a second degree equation. The relationship of hypericin and total phenols contents was a negative correlation as well.

**Keyword:** *Hypericum perforatum* L., Biochemical Components, Variation, Hypericin, Total phenol, Flavonoid

INTRODUCTION

*Hypericum perforatum* L., known by the name of St. John’s Wort, is an important customary medicinal plant native to Europe and Iran, but it is grown worldwide for commercial purposes. *H. perforatum* has gained international popularity mainly for the treatment of depression and wound healing (Xi-Hua and Chun-Hua, 2010). Studies on *H. perforatum* were shown to have potential as a source of novel anticancer compounds (Schempp et al., 2002). The active ingredients of this perennial herb are one of the top-selling phytopharmaceuticals in
North America (Zobayed and Saxena, 2003). The complex phytochemical profile of *H. perforatum* consists of several groups of phytochemicals including the phenolic acids (chlorogenic acid), flavonoids (rutin, hyperoside, isouercitrin, querectin), naphthodianthrones (hypericin, pseudohypericin), and the phloroglucinols (hyperforin, adhyperforin). Pharmacologic activity has been attributed to several phytochemicals within *St. John's Wort* (Butterweck, 2003; Silva et al., 2005; Susan et al., 2001). According to the US and the EC Pharmacopoeias, the crude drug consists of the dried flowering tops or aerial parts of the plant, at present coming almost exclusively from field-grown plants. Drug is used as an extract both in monopreparation and in multi-ingredient formulations (Bruni and Sacchetti, 2009). Three drug qualities are known: Hyperici herba (cuttings of the entire plant at flowering, including stem); hyperici herba flowering horizon (cuttings of the upper 30 cm of the entire plant at flowering, including stem) and almost pure flowers (Bruni and Sacchetti, 2009).

Today's market is supplied with *St. John's Wort* products from various herb producers as the plant is cosmopolitan. Therefore, available preparations of the *H. Perforatum* products may differ significantly in quality depending on a number of factors such as the different subspecies and varieties used, on the geographic location where the raw material is being grown and harvesting time (different plant development stages), and on the different and poorly controlled analysis conditions (Filippini et al., 2010). Several studies have reported variation of hypericin levels in *H. perforatum* in Australia (Campbell et al., 1997; Southwell and Campbell, 1991; Jensen et al., 1995) and Switzerland (Buter et al., 1998), but there are no reports documenting variation of Biochemical Components in *Hypericum perforatum* in Iran. There has been only some investigation attempting to dissect the chemical composition of field-grown clonal accessions of *Hypericum* as influenced by environment or as a result of genetic variation (Buter et al., 1998). The green parts and flowers of *St. John's Wort* contain a number of substances, including flavonoid, hypericin and other UV-B-absorbing secondary metabolites with biological effects (Erken et al., 2001). There have been several studies of the effect of environmental factors on hypericin concentrations in *St. John's Wort* (Zobayed et al., 2007; Zou and Wei, 2004). Hypericin is a component of the inducible plant defense response of *Hypericum perforatum* against fungal pathogens (Cirak et al., 2005). Umek et al. (1999) reported a positive correlation between the concentrations of some flavonoid in *H. perforatum* and the altitude of their growing sites. It is reported that some environmental factors such as light intensity and CO$_2$ concentration can significantly change the secondary metabolites synthesis and production in plants. Light is known to adjust not only plant growth and development, but also the biosynthesis of primary and secondary metabolites (Kurata et al., 1997; Zhong et al., 1991). The synthesis of medicinal components in herbs is affected by light intensity with changes in plant morphology and physiology characteristics (Kurata et al., 1997; Jaafar and Rahmat, 2008; Briskin and Gawienowski, 2001). Briskin et al. (2001) concluded that hypericin synthesis increased significantly in *H. perforatum* when grown under high light intensity (400 µmol m$^{-2}$s$^{-1}$). It seems that a high photosynthetic rate under high light intensity resulted in an increased amount of carbon assimilation and enhanced the secondary metabolites in the leaf tissues. Phenolic biosynthesis requires light or is enhanced by light, whereas flavonoid formation is absolutely light-
dependent, and its biosynthetic rate is related to light intensity and density (Xie and Wang, 2006). Previous studies showed that changes in light intensity are capable of changing the production of flavonoid and total phenols in herbs (Graham, 1998). Michel et al. (2001) reported TF production related to plant pigments (chlorophyll and carotenoid). In contrast with flavonoid, the xanthophyll cycle seems to be mainly relevant to the protection of photosynthesis against sudden increase in light intensity.

In this study we investigated *H. perforatum* populations sampled from a total of five sites and three elevations. For possible relationship between Hypericin, flavonoid and total phenols production and photosynthesis rate.

**MATERIALS AND METHODS**

*Plants and growth conditions*

Plant Materials of *H. perforatum* were collected between August and September of 2010 from five sites and three elevations with four samples (totally 60 samples) in west of Mazandaran province (North of Iran- Table 1). The top \( \frac{1}{3} \) of the plants crown were harvested between 9:00 AM and 1:00 PM. Conditions on the day of collection were clear and sunny at all sites. Temperatures ranged from 25°C to 30°C. Samples were placed on ice during transport to the laboratory of Islamic Azad University Tonekabon Branch (IAUTB), where they were dissected into tissue parts and dried overnight (or until constant weight) at 65°C, the current temperature used by laboratory employee. Reference specimens were placed in the (IAUTB) herbarium.

<table>
<thead>
<tr>
<th>Population</th>
<th>Altitude/m</th>
<th>Longitude and Latitude</th>
</tr>
</thead>
<tbody>
<tr>
<td>Marzanabad</td>
<td>500-1500</td>
<td>N 36 26 971</td>
</tr>
<tr>
<td>Pole Zangoole</td>
<td>2000 to up</td>
<td>N 36 46 123</td>
</tr>
<tr>
<td>Tonekabon-Shezezr</td>
<td>0-1500</td>
<td>N 36 47 627</td>
</tr>
<tr>
<td>Gennat</td>
<td>0-2200</td>
<td>N 36 48 118</td>
</tr>
<tr>
<td>Roudbar</td>
<td>0-2000</td>
<td>E 51 15 50.4</td>
</tr>
</tbody>
</table>

**Hypericin Determination**

Hypericin content was determined by a modified method of the European Pharmacopoeia (2008) as follows: 80 mg of powdered sample were extracted by 6 ml of 80% tetrahydrofuran in water at 65°C for 30 min. The samples were centrifuged at 2236g for 10 min, the supernatant was transferred to a fresh test tube and the sediment was extracted once more. After centrifugation, the supernatant was combined and 250 µl of the combined extract were transferred to a plastic microcentrifuge tube and evaporated under vacuum. The sediment was dissolved with 500 µl of methanol in an ultrasonic bath and then centrifuged at 11,269g for 10 min. Then, 300 µl of the supernatant were transferred to a microtitre plate vial and the absorbance was measured at 590 nm. The concentration of hypericin was calculated by comparison with a hypericin standard (Roth). For each treatment, six plants were analyzed, with one extraction per plant.

**Total phenolic determination**

Total phenols were determined by Folin Ciocalteu reagent (McDonald et al., 2001). A dilute extract of each plant extract (0.5 ml of 1:10 g ml-1) or Gallic acid (standard phenolic compound) was mixed with Folin Ciocalteu reagent (5 ml, 1:10 diluted with distilled water) and aqueous Na2CO3 (4 ml, 1 M). The mixtures were allowed to stand for 15 min and the total phenols were
determined by colorimetry at 765 nm. The standard curve was prepared using 0, 50, 100, 150, 200, 250 mg L⁻¹ solutions of Gallic acid in methanol: water (50:50, v/v). Total phenols values are expressed in terms of Gallic acid equivalent (mg g⁻¹ of dry mass), which is a common reference compound.

Total flavonoid determination

Total flavonoid contents were determined spectrophotometrically using AlCl₃ and vanillin - HCl reagents, respectively, as previously reported (Kreft et al., 2002). In short, 20 mg of powdered St. John’s Wort sample was extracted in 10 ml of 60% ethanol overnight on a shaker. For the determination of flavonoid, the sample was diluted 1:6 with 60% ethanol. Two aliquots of 180 ll of diluted sample were prepared in the wells of a microtitre plate and 20 µl of 5% AlCl₃ in methanol were added to the first aliquot and 20 µl of methanol were added to the second aliquot. After 30 min, the absorbance at 420 nm was measured in both solutions. The concentration was calculated from the differences in the measurements and compared with a rutin standard (Fluka, Sigma–Aldrich Corporation, St. Louis, USA).

Chlorophyll a, b and carotenoid determination

Chlorophyll was extracted in 80% acetone and the absorption at 663nm (Cₐ : Chlorophyll a), 645nm (Cₐ : Chlorophyll b) and 470 nm (Cₓ+c : Carotenoid) was read in an UV-160 spectrophotometer. Chlorophyll and carotenoid contents were calculated using the absorption coefficients (Arnon, 1949; Witham et al., 1971).

- Cₐ= 12.25 A₆₆₃-2.79 A₆₄₅
- Cₐ= 21.5 A₆₄₅-5.1 A₆₆₃
- Total Chlorophyll = Cₐ + Cₐ
- Cₓ+c= (1000 A₄₇₀-1.82 Cₐ -85.25 Cₐ) /198

Total protein extraction

A method suitable for high-quality protein extraction from H. perforatum tissues was optimized during the present study. The qualitatively and quantitatively optimized protocol based on sodium borate extraction and phenol / methanolic ammonium acetate.

The protein concentration of the extracted samples was measured according to a study by Bradford (1976). Analysis of proteins in their native form is carried out in polyacrylamide buffer gel. The classical disc electrophoresis using cylindrical gels has been described by Davis in 1964.

Statistical analyses

Correlation and regression analyses (SPSS version 13.0- SPSS Inc., Chicago, IL, USA) were performed and significance was accepted at P < 0.05.

RESULTS AND DISCUSSION

The results showed a high positive correlation between the amounts of hypericin and total phenols (Table 2) which would be stated by linear relation (Y= 0.07234+0.000444 X) (Figure 1). While hypericin increases, total phenols increases as well, and vice versa. This may be associated with hypericin and phenol contents. There is also a significant correlation between hypericin and flavonoid (Table 2). However, their relationship is as a second degree equation (Y= -76.58+974.7X-2468X²) in which at first, the mean value of flavonoid increases with increase in hypericin amount and after a peak, it decreases with decrease in hypericin content (Figure 2). This is may be ascribed to investing priority in order to produce antrons and flavonoid according to ecologic situation. Whereas, there is a negative correlation between hypericin
with a chlorophyll and total chlorophyll contents (Table 2), since secondary metabolites production while the plant needs them in any challenge means consuming substrates in order to produce secondary metabolites instead of pigment production, which is the result of different pathways like respiratory ones (Figure 4). Measuring total phenols demonstrated its positive correlation with flavonoid and following the second degree equation of ($Y = -25.39 + 0.3258X - 0.00058X^2$) (Figure 3). It should be noted that regression coefficients for equations of hypericin and total phenols with flavonoid is less than 90%. This can be interpreted as hypericin itself is composed of components such as pseudohypericin, protohypericin, etc. and total phenols itself is composed of other components such as caffeic, chlorogenic, paracoumaric, ferulic, parahydroxybenzoic, and vanillic acids) (Sirvent and Gibson, 2002). Flavonoid, on the other hand, is composed of flavonol (such as kaempferol, quercetin), flavones (such as hyperoside, isoquercetin, quercetin, rutin), biflavonoids as biapigenin, amentoflavone (a derivative of biapigenin) and catechines (flavonoid often together with tannins) (Barnes et al., 2001). Therefore, it is better to deal with relations ruling their components in order to distinguish their qualitative relations. The results shown that there is a negative correlation between total phenols and total chlorophyll (Table 2) and the result is the same as hypericin reaction. Measuring carotenoid reveals its positive relationship with chlorophyll a and total chlorophyll (Table 2) indicating stronger relation between chlorophyll a and minor pigments. On the other hand, chlorophyll a indicated positive correlation with chlorophyll a:b proportion, so that we could say chlorophyll a is a parameter having correlation whether positive or negative with most measured parameters i.e. chlorophyll a has a role in synthesis rate of most plant metabolites according to its critical role (Figure. 4). Such results were also in accordance with previous report by Khan et al. (2000).

b Chlorophyll variations indicated that this photosynthesizing pigment had positive correlation with total amount of chlorophyll (Table 2).

Current study demonstrated that the highest amount of hypericin (Figure 5) and total phenols contents (Figure 6) is related to Jannat Roodbar region with an altitude of 1218 higher than sea level and the highest amount of flavonoid, carotenoid and total chlorophyll contents collected samples was measured at 2300m altitude of Pole Zangoole (Figures 7,8).

Protein bands indicated the highest amount of protein accumulation in major plot number 13 (Jannat Roodbar region with an altitude of 1218 higher than sea level) which could be stated that protein accumulation is increased due to environmental challenges which most probably relates to reductase type enzymes and increase in hypericin and total phenols amount indicates high activity of anti-challenge mechanisms in this place (Figures 9, band number 13).

The study determined that change in environmental factors could change hypericin, total phenols, flavonoid and total protein synthesis in Hypericum perforatum L. (Southwell and Bourke, 2001). In this relation, differences in longitude and latitude would be an important factor in producing secondary metabolites since it is so much impressive in the subject of determining production position and potential in establishing a new pattern of herb cultivation.
Table 2. Correlation coefficients between various traits

<table>
<thead>
<tr>
<th>Variables</th>
<th>Total Phenols</th>
<th>Carotenoid</th>
<th>Flavonoid</th>
<th>a Chlorophyll</th>
<th>b Chlorophyll</th>
<th>Total Chlorophyll</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypericin</td>
<td>0.992**</td>
<td>-0.185**</td>
<td>0.992**</td>
<td>-0.322**</td>
<td>0.229**</td>
<td>-0.343**</td>
</tr>
<tr>
<td>Total Chlorophyll</td>
<td>-0.361**</td>
<td>0.56**</td>
<td>0.23**</td>
<td>0.707**</td>
<td>0.844**</td>
<td>-</td>
</tr>
<tr>
<td>b Chlorophyll</td>
<td>-0.263**</td>
<td>0.223**</td>
<td>0.114**</td>
<td>0.217**</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>a Chlorophyll</td>
<td>-0.31**</td>
<td>0.713**</td>
<td>0.268**</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Flavonoid</td>
<td>-0.903**</td>
<td>0.091**</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Carotenoid</td>
<td>-0.185**</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*, ** Significant at the 5% and 1% probability levels respectively.

Figure 1. Variations in amount of regression hypericin and total phenols content.

Figure 2. Variations in amount of Hypericin and Flavonoid contents in collected samples.

Figure 3. Variations in amount of regression total phenols and flavonoid content.

Figure 4. Variations in amounts of chlorophyll Pigments in samples of main plots.
Figure 5. Variations in amount of Hypericin content in samples of main plot

Figure 6. Variations in amount of total phenols content in samples of main plots

Figure 7. Variations in amount of Carotenoid content in samples of main plots

Figure 8. Variations in amount of Flavonoid content in samples of main plots
In addition to other environmental factors (humidity, soil and temperature) in the subject of altitude and effective materials, the most impressive factor is light and its quality, which affects the amount of aforementioned metabolites (Kefeli et al., 2003). Changes in amounts of chlorophyll a and b and dominant relations among them itself indicates variability of photosynthesis system by environmental situations which is followed by affecting production cycles of secondary metabolites (Khan et al., 2000).

Briskin and Gawienowski (2001) demonstrated that hypericin synthesis significantly increased under intensified light. Photosynthesis speed seems to increase in intensified light which consequently increases carbon assimilation and in turn, increases secondary metabolites in vegetative body. Phenol compound biosynthesis has also direct relation with amount of light while flavonoid formation relates to light wave length particularly ultraviolet light (Xie and Wang, 2006). The amount of chlorophyll a and b differs with change in altitude and light which affects the amount of photosynthesis and consequently metabolite amounts and quantitative relations among them. According to performed studies, flavonoid changes could change electron transport speed of photosynthesis photosystem which in turn affects photosynthesis speed (Fan et al., 1998). Any reason decreasing photosynthesis speed causes carbon to divert from photosynthesis cycle to shikimic acid and therefore increases flavonoid amount. The reaction indicates that it is possible for phenol compounds and flavonoid to regulate plant growth and improve photosynthesis efficiency and affect dividing photosynthesis materials through affecting resource-reservoir relation.

**Conclusion**

This study focused on analyzing the chemical composition of hypericin, total phenols, flavonoid and carotenoid from wild populations of *H. perforatum* collected in the north of Iran. Genetic, physiological, or environmental influences may affect hypericin yields, but such studies correlating hypericin concentrations to specific influences have not been well documented. Ecotypes genetic analysis will provide better results than a closer relationship between secondary metabolites, environmental factors and their interactions.

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