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Original Article

Modeling the Altitudinal Variation in Secondary Metabolite Contents of *Hypericum orientale* from Turkey

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Hypericum orientale L. that is growing in high altitudes is used traditionally for hemorrhoids in Turkish folk medicine. In the present study, models were developed to estimate the altitudinal variation in secondary metabolite contents of *Hypericum orientale* growing wild in "Güldağı" mountain, Turkey. Aerial parts of 30 flowering individuals were collected at six different altitudes (500, 1150, 1650, 2100, 2720 and 3250 m). Actual secondary metabolite contents of plants were measured by high performance liquid chromatography method. Multiple regression analysis was performed for each altitude and chemical separately to develop multiple regression models. The R^2 coefficient values between the predicted and observed contents of secondary metabolites were determined as 0.89 for hyperoside and neochlorogenic acid, 0.94 for rutin, 0.95 for avicularin, 0.97 for quercetin, 0.98 for hypericin, pseudohypericin, cholorogenic acid, and 0.99 for hyperforin, 2,4-dihydroxybenzoic acid, amentoflavone, isoquercitrin, quercitrin. catechin, and epicatechin. All R^2 values and standard errors were found to be significant at the $P < 0.001$ level and a very close relationship was found between the actual and estimated values of secondary metabolites, tested. Prediction of secondary metabolite composition by using simple equations may represent a complementary important topic for phytochemical and taxonomical studies.

Keywords: Altitude, HPLC, *Hypericum orientale*, modeling, secondary metabolite

The genus *Hypericum* L. (*Hypericaceae*) consists of 484 species, occurring wild on every continent in the world, except Antarctica (1). *Hypericum* plants have traditionally been used as herbal remedy due to their anti-depressive and wound-healing properties (2). In particular, extracts of *Hypericum perforatum* L., the most abundant and well known species, are now widely used in Europe as a drug for the treatment of depression (3). The genus is represented in the flora of Turkey by 89 species from 19 sections, 43 of which are endemic (4). *Hypericum orientale* L., a widespread perennial herb growing wild in igneous slopes and rock ledges at high altitudes is one of the Turkish species of *Hypericum* (Figure 1). Its decoction has traditionally been used for hemorrhoids in Turkish folk medicine (5). Results from recent studies reporting high content of bioactive compounds in *H. orientale* (6, 7) serve as the basis for its effectiveness as a promising medicinal plant.

Naphthodianthrones, phloroglucinol derivate-ives, essential oils and several phenolics are thought to be main bioactive compounds of *Hypericum* extracts which possess a broad array of biological activity (8). Among the chemicals, hypericins and hyperforins are synergistically responsible for the observed antidepressant activity of *Hypericum* extracts (9). Anti-inflammatory (10), anti-tumoral

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Figure 1. A view from H. orientale plant at flowering.

(11) and anti-angiogenic (12) effects were also reported for hyperforins. Hypericins are well known pharmaceuticals with their documented anti-viral, anti-retroviral, photodynamic, anti-bacterial, anti-depressant and anti-tumoral activities (13). Although hyperforin and hypericins have been reported to mainly contribute to the pharmacological effects of *Hypericum* extracts, flavonoids and phenolic acids have also made an important contribution to the anti-depressant (14) and anti-microbial (15) activities, respectively.

A broad range of abiotic environmental factors such as radiation intensities, light, length of the vegetation period, temperature, wind velocity and precipitation are known to interfere in synthesis and accumulation of secondary metabolites in plants (16). All the factors vary greatly with the altitude of the natural growing locality, and for that reason altitude is considered as the most important ecological factor influencing chemical content variations in medicinal plants (17-19). In our previous paper, we reported a significant and positive relationship between bioactive compound accumulations and altitude of plant growing sites in *H. orientale* (20), and in the present study we aimed to develop, based on altitudinal ranking, mathe-matical models to estimate secondary metabolite contents in this medicinal plant for the first time.

Materials and methods

Plant materials and experimental procedures

Aerial parts of flowering 30 individuals were collected at six different altitudes (500, 1150, 1650, 2100, 2720, and 3250 m) of "Güldağı" mountain, Turkey in July 03-05, 2011. The voucher numbers and geographical data of collection sites are shown in Table 1. Species were identified by Dr. Samim Kayikci, Mustafa Kemal University, Faculty of Arts and Sciences, Department of Biology, Turkey. Voucher specimen was deposited in the herbarium of Ondokuz Mayis University Agricultural Faculty. The top of 2/3 plants was harvested between 11:00 am and 13:00 pm. The plant materials consisting of flowering aerial parts were dried at room temperature (20 ± 2 °C), and subsequently assayed for chemical contents by HPLC.

HPLC analysis and identification

Details of the analyses and identification were described previously (20). Briefly, air-dried plant material was mechanically ground with a laboratory mill to obtain a homogenous drug powder. Samples of about 0.1 g (weighed with 0.0001 g precision) were extracted in 10 ml of 100% methanol by ultrasonication at 40 ºC for 30 min in a Sonorex Super model RK 225H ultrasonic bath (Bandelin, Germany). The prepared extracts were filtered through a 0.22 µ membrane filter (Carl Roth GmbH, Karlsruhe, Germany) and kept in a refrigerator until analysis.

A Waters Alliance 2695 (Waters, Milford, USA) separation module system equipped with Waters 2487 UV / Vis and Waters 996 PDA diode-array detectors, was used for HPLC analysis. Chromatographic peaks were identified based on the retention time, UV spectra of the standard compounds using HPLC-PDA.

Quantification of compounds was carried out by the external standard method. A calibration curve for each of the compounds was constructed by plotting peak areas versus the respective compound concentration and calculated by linear regression analysis. The concentration of compounds was expressed as mg/g dry mass (DM). Solvents used were of HPLC grade and purchased from Roth

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GmbH (Karlsruhe, Germany).

Multiple linear regression analyses (MLR)

MLR is a statistical method used to investigate the relationship between several independent variables and a dependent variable. A linear regression model assumes that the relationship between the dependent variable and the *p*-vector of regressors is linear, where p is the number of independent variables. Thus the model takes the form $y_i = \beta_1\chi_{i1} + \ldots + \beta_p\chi_{ip} + \varepsilon_i = \chi_i'\beta + \varepsilon_i\, i = 1, \ldots, n,$ where $'$ denotes the transpose, so that $x_i'\beta$ is the inner product between vectors x_i and β. The y_i is called the dependent variable and the χi is called regressor or independent variable. The decision as to which variable in a data set is modeled as the dependent variable and which are modeled as the independent variables may be based on a presumption that the value of one of the variables is caused by, or directly influenced by the other variables.

Results

Hypericin, pseudohypericin, hyperforin, adhyperforin, caffeic acid and rutin were detected only in plants of growing sites with higher altitudes and the highest accumulation level for each compound was observed in the highest growing site (1.52, 3.57, 1.22, 0.01, 0.02 and 3.35 mg/g DM hypericin, pseudohypericin, hyperforin, adhyperforin, caffeic acid and rutin, respectively). The other compounds tested were accumulated in all growing sites and their contents increased significantly (P<0.01) with altitude. Similar to hypericins, hyperforins, caffeic acid and rutin, the highest accumulation level of these compounds were also observed in the highest growing site (1.03, 3.99, 0.48, 0.95, 7.22, 0.48, 0.29, 3.64, 1.97, 0.11 and 0.88 mg/g DM chlorogenic acid, neochlorogenic acid, 2,4-dihydroxybenzoic acid, amentoflavone, hyperoside, isoquercitrin, quercitrin, quercetin, avicularin, (+)-catechin and (-)-epicatechin, respectively) (Table 1). Further evaluation of the present results by performing

Table 1. Secondary metabolite contents (mg/g DM) in whole plant material of *Hypericum orientale* sampled in different altitudes of Güldağı mountain Turkey

Altitudes (m)	Compounds																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
500	0	0	0	0	0.08 c*	1.56 d	0	0.20 c	0.25 e	2.17 c	0.12 b	0.04 c	2.32 c	0.02 b	0	0.01 b	0.36 d
1150	0	0	0.01	0	0.10 c	1.68 d	0	0.22 c	0.25 e	2.53 c	0.14 b	0.10 b	2.35 c	0.02 b	0	0.01 b	0.43 d
1650	0	0	0	0	0.10 c	2.41 c	0	0.24 c	0.34 d	4.55 b	0.24 b	0.19 ab	2.88 b	0.05 b	0	0.03 b	0.52 c
2100	0	0	0.12	0.01	0.57 b	3.14 b	0.01	0.32 b	0.42 c	6.94 a	0.41 a	0.20 a	2.88 b	0.09 b	0.82	0.05 b	0.54 c
2720	0.34	0.52	0	0	0.69 b	3.45 a	0.01	0.39 a	0.52 b	7.01 a	0.43 a	0.24 a	3.51 a	1.12 a	2.97	0.09 a	0.65 b
3250	1.52	3.57	1.22	0.01	1.03 a	3.99 a	0.02	0.48 a	0.95 a	7.22 a	0.48 a	0.29 a	3.64 a	1.97 a	3.35	0.11 a	0.88 a

Hypericin (1), pseudohypericin (2), hyperforin (3), adhyperforin (4), chlorogenic acid (5), neochlorogenic acid (6), caffeic acid (7), 2,4-dihydroxybenzoic acid (8) and amentoflavone (9), hyperoside (10), isoquercitrin (11), quercitrin (12), quercetin (13), avicularin (14), rutin (15), (+)-catechin (16) and (-)-epicatechin (17). Values followed by different small letters in each column are significantly different (P<0.01) according to Duncan multiple range test.

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regression analyses revealed a positive and significant relationship between altitude and the content of chlorogenic acid (R^2= 0.86), neochlorogenic acid (R^2= 0.96), 2,4-dihydroxybenzoic acid (R^2= 0.92), amentoflavone (R^2= 0.79), hyperoside (R^2= 0.87), isoquercitrin (R^2= 0.91), quercitrin (R^2= 0.97), quercetin (R^2= 0.91), (+)-catechin (R^2= 0.91) and (-)-epicatechin (R^2= 0.92) (Figure 2). Coefficients, their standard errors and R^2 values of the predicting equations for the chemical tested are shown in Table 2 while their actual and predicted values are shown in Table 3.

Table 2. The coefficients, their standard errors and R^2 values of the predicting equation for each chemical tested in *H. orientale*

Secondary metabolites	Coefficients ± SE		
	a	b	R^2
Hypericin	-0.39±0.05***	0.42±0.02***	0.98
Pseudohypericin	-0.01±0.09***	0.11±0.006***	0.98
Hyperforin	-0.49±0.01***	0.51±0.01***	0.99
Cholorogenic acid	-0.01±0.06***	0.57±0.03***	0.98
Neochlorogenic acid	1.64±0.22***	0.04±0.008***	0.89
2,4-dihydroxybenzoic acid	-0.66±0.02***	0.71±0.01***	0.99
Amentoflavone	-0.41±0.04***	0.53±0.02***	0.99
Hyperoside	2.96±0.505***	0.003±0.0006***	0.89
Isoquercitrin	-0.71±0.01***	0.74±0.013***	0.99
Quercitrin	-0.83±0.02***	0.84±0.015***	0.99
Quercetin	1.93±0.095***	0.04±0.004***	0.97
Avicularin	-0.23±0.11***	0.32±0.036***	0.95
Rutin	0.06±0.21***	0.12±0.014***	0.94
Catechin	-0.94±0.006***	0.93±0.005***	0.99
Epicatechin	-0.37±0.03***	0.52±0.021***	0.99

R^2: regression coefficiency; SE: standard error; a and b are coefficients of produced equations; ***: significant at the level of P<0.001.

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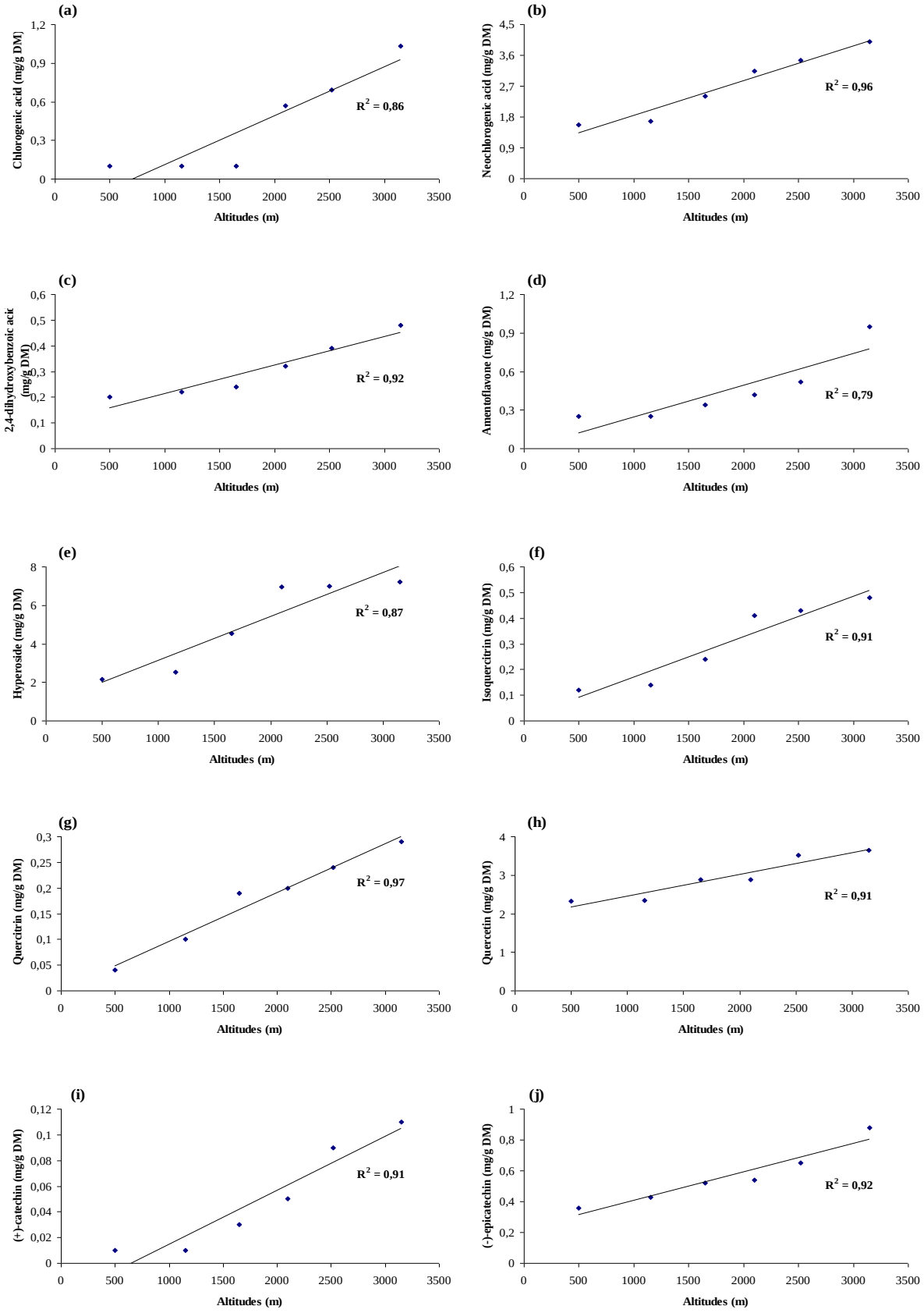


Figure 2. The relationship between altitude of plant growing sites and the content of secondary metabolites. Chlorogenic acid (a), neochlorogenic acid (b), 2,4-dihydroxybenzoic acid (c), amentoflavone (d), hyperoside (e), isoquercitrin (f), quercitrin (g), quercetin (h), (+)-catechin (i) and (-)-epicatechin (j) in *Hypericum orientale*. All R^2 values are significant at the level of $P < 0.01$.

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Table 3. Actual and predicted values of the compounds tested in *H. orientale*

cccSecondary metabolites	Altitude (m)						
	Values (mg/g) DM	500	1150	1650	2100	2720	3250
Hypericin	Actual	0.0	0.0	0.0	0.0	0.3	1.5
	Predicted	0.0	0.0	0.0	0.0	0.2	1.5
Pseudohypericin	Actual	0.0	0.0	0.0	0.0	0.5	3.6
	Predicted	0.1	0.1	0.1	0.1	0.2	3.6
Hyperforin	Actual	0.0	0.0	0.0	0.1	0.0	1.2
	Predicted	0.0	0.0	0.0	0.1	0.0	1.2
Cholorogenic acid	Actual	0.1	0.1	0.1	0.6	0.7	1.0
	Predicted	0.0	0.1	0.1	0.4	0.5	0.7
Neochlorogenic acid	Actual	1.6	1.7	2.4	3.1	3.5	4.0
	Predicted	1.9	1.9	2.2	2.8	3.2	4.3
2,4-dihydroxybenzoic acid	Actual	0.2	0.2	0.2	0.3	0.4	0.5
	Predicted	0.2	0.2	0.2	0.3	0.4	0.5
Amentoflavone	Actual	0.3	0.3	0.3	0.4	0.5	1.0
	Predicted	0.3	0.3	0.3	0.4	0.5	1.0
Hyperoside	Actual	2.2	2.5	4.6	6.9	7.0	7.2
	Predicted	3.0	3.0	3.3	6.6	6.8	7.7
Isoquercitrin	Actual	0.1	0.1	0.2	0.4	0.4	0.5
	Predicted	0.1	0.1	0.2	0.4	0.4	0.5
Quercitrin	Actual	0.0	0.1	0.2	0.2	0.2	0.3
	Predicted	0.0	0.1	0.2	0.2	0.2	0.3
Quercetin	Actual	2.3	2.4	2.9	2.9	3.5	3.6
	Predicted	2.4	2.4	2.8	2.8	3.5	3.7
Avicularin	Actual	0.0	0.0	0.1	0.1	1.1	2.0
	Predicted	0.1	0.1	0.1	0.1	0.8	2.1
Rutin	Actual	0.0	0.0	0	0.8	3.0	3.4
	Predicted	0.2	0.2	0.2	0.4	2.5	3.7
Catechin	Actual	0.0	0.0	0.0	0.1	0.1	0.1
	Predicted	0.0	0.0	0.0	0.1	0.1	0.1
Epicatechin	Actual	0.4	0.4	0.5	0.5	0.7	0.9
	Predicted	0.4	0.4	0.5	0.5	0.7	0.9

Discussion

In the present study, we have developed for the first time prediction models for the content of several *H. orientale* constituents belonging to different chemical classes. As the understanding of plant growth and development has been increasing, such mathematical models, performed in the present study could be very useful tools for the prediction of

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secondary metabolite profiles for many plants without using expensive analytical experimental plans. Due to the complexity of *Hypericum* spp. chemistry, prediction of secondary metabolite composition by using simple equations instead of expensive and time-consuming analytical procedures may represent a complementary important topic for phytochemical and taxonomical studies on the *Hypericum* genus. Hence, the models produced in the present study can be used safely by *H. orientale* researchers. On the other hand, different models can be developed for other *Hypericum* species and their relative phytochemicals which can be very different from those used for *H. orientale* in the present study.

Conflict of interest

The authors declared no conflict of interest.

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