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Estimation of the phenolics content of St. John's wort (*Hyperıcum perforatum* L.) grown under different water and salt levels based on reflectance spectroscopy

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ABSTRACT

Accumulation of phenolics were examined in greenhouse-grown *Hypericum perforatum* plants as effected by three different salts, which were $MgSO_4$, $CaCl_2$, and NaCl in the salt concentrations of 0.03 (control), 1, 2.5, 4, and 8 dS m^{-1} for each salt. Three different water levels, which were 80, 100, and 120% were applied to all three salts mentioned above. Multi regression analyses were performed to describe the effects of water and salt levels on phenolics accumulation. As a result of ANOVA and multi-regression analysis, it was found that there was close relationship between actual and predicted phenolic contents in *Hypericum perforatum*. HPLC analyses were used to determine quercetin, quercitrin, isoquercetin, hyperoside, rutin, and cholorogenic acid contents. The relationships between different water and salt levels, and phenolics accumulation were determined by spectral reflectance values. The r values for quercetin, quercitrin, isoquercetin, hyperoside, rutin, and cholorogenic acid were determined as 0.83, 0.82, 0.91, 0.88, 0.84, and 0.87, respectively. All r-values and standard errors of the equations were found to be significant at the $p<0.001$ level.

Keywords: *Hypericum*; modeling; phenolics; salt; water levels.

INTRODUCTION

Hypericum perforatum L. is a perennial medicinal plant known as "St. John's wort" and has been used in the treatment of several diseases (Odabas *et al.,* 2009a). It has already been used in naturopathic treatment of ear pain for children and results of current studies have indicated *Hypericum perforatum* L. as a promising medicinal plant for cancer treatment (Benkiki *et al.,* 2003). The species of *Hypericum* have great pharmaceutical potential, with their well-documented contents of hyperforin, hypericins, and flavonoids (Cirak *et al.,* 2007a, Ayan & Cirak, 2008) The plant contains a broad range of structurally diverse natural compounds (Cirak *et al.,* 2007b; Cirak *et al.,* 2010)

Salts management practices are usually done by applying enough water to satisfy crop requirements and leach out salts from the root zone (Rhoades, 1974). However, this approach is limited by agricultural application because of low quality of the irrigation water and economical cost (Tanji, 1990).

Salinity is one of the major factors that affect plant growth. It is a serious problem in many parts of world and cause considerable loss in agricultural production (Bray *et al*., 2000; Shao *et al.,* 2008; Wu *et al*., 2007; Lauchli & Epstain, 1990). Saline soils cover over 7% of the earth's land surface and demonstrate high levels of salinity due to the soluble salts in irrigation waters and fertilizers used in agriculture (Copeman *et al*., 1996). The deleterious effects of salinity on plant growth are associated with low osmotic potential of soil solution (water stress), nutritional imbalance, specific ion effect (salt stress), or a combination of them (Yildirim & Taylor, 2005). Saline soil is generally dominated by sodium ions, with the dominant anions being chloride and sulphate, it has a high sodium absorption rate with a high pH and electrical conductivities (>4 dSm^{-1}) (Flowers & Flowers, 2005). Soil salinity may reduce micronutrients uptake due to stronger competition by salt cations at the root surface (Marschner & Romheld, 1994; Page *et al*., 1990).

Developmental models are commonly explored using computational or simulation techniques (Odabas *et al*., 2008). The simulation software may be general-purpose, intended to capture a variety of developmental processes depending on the input files, or special-purpose, intended to capture a specific phenomenon. Input data range from a few parameters in models capturing a fundamental mechanism to thousands of measurements calibrated descriptive models of specific plants (species or individuals). Standard numerical outputs (i.e. numbers or plots) may be complemented by computer-generated images and animations (Prusinkiewicz, 2004). Most of the researches have investigations focused on plant developmental periods from seed sowing to reproductive stages and from reproductive stages to harvest. Environmental conditions affect the dry matter production rate, rooting percentage and the rooting degree of the plants. Different physiological processes occur at different stages of plant growth (Cirak *et al*., 2005).

Hypericum has already been used in naturopathic treatment for ear pain in children and results of current studies have indicated as a promising medicinal plant for cancer treatment (Odabas *et al.,* 2009b). Many of the pharmacological activities of *Hypericum* extracts appear to be attributable to their contents of hypericins and hyperforin (Cirak *et al*., 2013). Hypericin and pseudohypericin have been reported to exhibit important biological activities, namely photodynamic, antiviral, antiretroviral, antibacterial, antipsoriatic, antidepressant, and antitumoral activities (Guedes & Eriksson, 2005). Hyperforin is a prenylated phloroglucinol derivative that consists of a phloroglucinol skeleton with lipophilic isoprene chains (Medina *et al*., 2006). Results

from recent studies have indicated hyperforin as the main chemical responsible for the antidepressant effects of Hypericum extracts (Roz & Rehavi, 2004). It also exhibits antiinflammatory, antitumoral, and antiangiogenic effects (Dona *et al.,* 2004).

In this study, the Saint John's wort (*Hypericum perforatum* L.) phenolics under different water and salt levels based on reflectance spectroscopy were estimated.

MATERIAL AND METHODS

Plant material

Seeds, which were obtained from the Ondokuz Mayis University, Agriculture Faculty in Turkey, were germinated in float system, commonly used for seedling production of broad-leaves tobacco Burley and Flue-Cured-Virginia under a 16 h light/8 h dark cycle. Newly emerged seedlings were transferred to pots, 26 cm in diameter, and 19 cm depth, filled out with a peat, soil (Sand: 36%, Clay: 29%, Silt: 35%, texture class: CL, bulk density: 1.42g/cm^3) pearlite and (1:1:1) mixture. They were watered daily until reaching maturity. After maturation, the pots were moved to greenhouse conditions. Plants were subjected to experiment starting from June 1, for 92 days. Some climatic parameters since transfer to the greenhouse is shown in the Figure 1.

Salt stress and water levels experiments

The experimental design was a randomized factorial with 3 replications in a greenhouse environment. Five different types of water with varying EC were utilized (S0 = 0.40 dS m^{-1}, S1 = 1.0 dS m^{-1}, S2 = 2.5 dS m^{-1}, S3 = 4.0 dS m^{-1}, S4 = 8.0 dS m^{-1}. The doses were obtained from the previous publications (Morales *et al*., 1993; Said-Al Ahl *et al*., 2010; Kara & Kara, 2010). During the preparation of saline waters, sodium adsorption ratio (SAR) values of each treatment were maintained less than 1.0, in order to avoid the adverse effect of increasing SAR on soil structure. To do this, calculated amounts of $CaCl_2$, $MgSO_4$, and NaCl were mixed to prepare irrigation water with given salinity for each treatment.

For water deficiency experiments, control pots were irrigated using city water. Afterwards, the infiltration of the water in pots was observed. Then, the amount of irrigation water, which was not leaked, but held by pots was determined as the required water amount (W). The required water amounts for each experiment were named as W1, W2, and W3 and water contents were applied as 80, 100, and 120 %, respectively for the soil levels in each pot. The experimental design was a factorial experiment in completely randomized plots with 3 replications. Thus, 45 pots were used. The experimental factors were applied 18 times at an interval of 2-3 days.

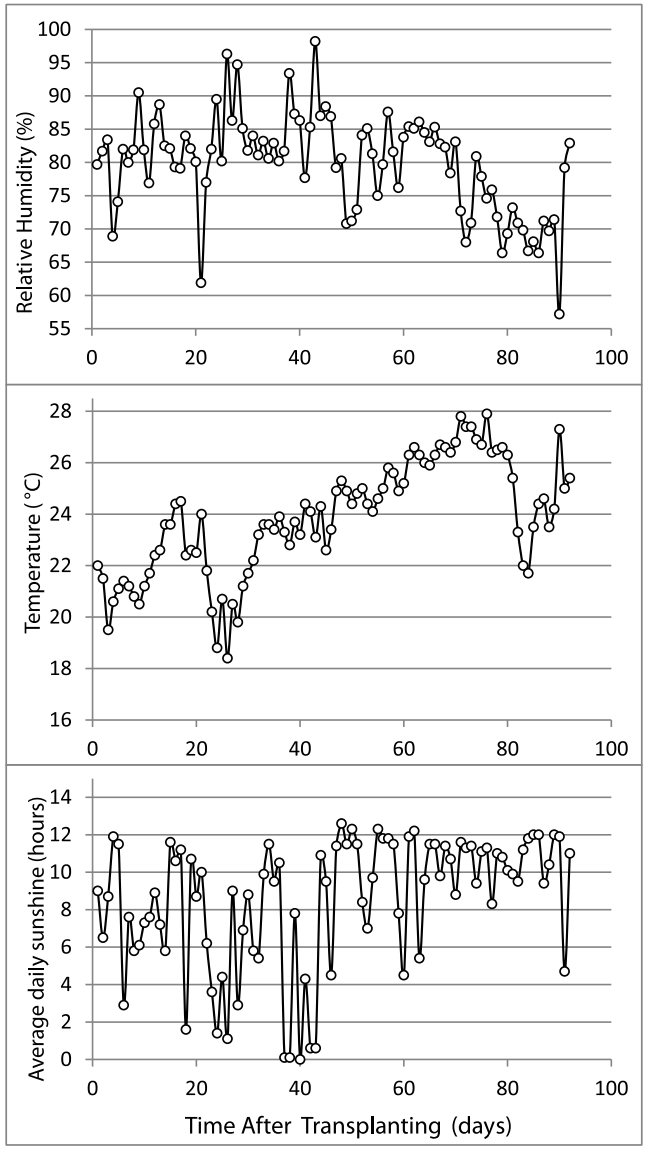

Fig. 1. Some climatic parameters belong the testing greenhouse

Preparation of plant extracts and HPLC analysis

The HPLC method, previously described by (Cirak *et al*., 2013) was used to determine phenolics in the plants. Air-dried plant material was mechanically grounded using a laboratory mill to produce a homogenous drug powder. About 0.5 g samples (weighed with 0.0001 g precision) were extracted in 50 ml of 100% methanol by ultrasonicatation at 40ºC for 30 minutes in a Sonorex Super model RK 225H ultrasonic bath. The

prepared extracts were filtered through 0.22 µm pore size membrane filter (Carl Roth GmbH, Karlsruhe, Germany) and kept in a refrigerator no longer than 3 hours until analysis.

A Shimadzu Prominence LC-20A (Shimadzu Europa GmbH, Duisburg, Germany) chromatographic system equipped with two LC-20AD model pumps, a SIL-20AC auto-injector, a thermostat CTO-20AC and a SPD-M20A detector was used for HPLC analysis. Separation of all compounds was carried out using a YMC Pack Pro-C18 (YMC Europe GmbH, Dinslaken, Germany) column (150 mm x 4 mm i.d.; 3 µm particle sizes) with 10 mm guard-precolumn. The mobile phase consists of solvent A (water containing 0.1 % trifluoroacetic acid, TFA) and solvent B (acetonitrile containing 0.1 % TFA). The following binary gradient elution program was used: 0–1 min (B 5→5%), 1–14 min (B 5→20%), 14–20 min (B 20→80 %), 20–30 min (B 80→100 %), 30–39 min (B 100→100 %), 39–39.5 min (B 100→5 %), and 39.5–45 min (B 5–5 %). The mobile phase was transferred with a flow rate of 1.0 mL min^{-1} and 10 µL volume of extract was injected in it. Detection was performed at 210–790 nm wavelength range with a constant column temperature at 40°C. The eluted compounds were determined on the basis of their retention time by comparison with retention time of reference standards and also confirmed with UV spectra of reference standards in the wavelength range from 210 to 790 nm.

The quantification of detected compounds was determined by using external standard method at the maximal absorption on the UV spectra of following corresponding compounds: chlorogenic acid at 325 nm, rutin at 353 nm, hyperoside at 353 nm, isoquercetine at 353 nm, quercitrine at 347 nm, and quercetine at 368 nm wavelengths. A six-point calibration curve was determined with pure standards dissolved in MeOH in the concentration range of 0.2–110 µg/ml. All calibration curves expressed high linear regression values ($r^2 > 0.999$) within the test range. All solvents and standards of reference substances were in HPLC grade and purchased from Roth Chemical Company (Karlsruhe, Germany).

Radiometer readings

Spectral measuremants were determined using handheld spectroradiometer (ASD fieldspec) and spectral reflectance readings of the plant leaves were conducted. Reflectance spectroscopy has the potential of providing a simple and cost-effective method (Arslan *et al*., 2014).

Experimental design and data analyses

All experiments were conducted using completely randomized designs that included ten treatments with three replications. Analysis of variance and regression models were implemented using the MATLAB software (Matlab ® 7.11.0.584 (R2010b)) that was used in many studies (Taner, 2012; Odabas *et al*., 2014; Dashti *et al*., 2014).

Model construction

The general purpose of multiple regression analysis is to learn more about the relationship between several independent or predictor variables and a dependent or criterion variables. A linear regression model assumes that the relationship between the dependent variable y_i and the p-vector of regressor's x_i is linear. This relationship is modelled through a so-called "disturbance term" ε_i — an unobserved random variable that adds noise to the linear relationship between the dependent variable and regressors. The model is:

$$y_i = \beta_1 x_{i1} + \cdots + \beta_p x_{ip} + \varepsilon_i = x_i' \beta + \varepsilon_i, \; i = 1, \cdots, n, \tag{1}$$

Where, apostrophe ($'$) denotes the transpose, so that $x_i' \beta$ is the inner product between vectors x_i and β. Often these n equations are stacked together and written in vector form as $y = X\beta + \varepsilon$, where,

$$y = \begin{pmatrix} y_1 \\ y_2 \\ \vdots \\ y_n \end{pmatrix}, x = \begin{pmatrix} x_1' \\ x_2' \\ \vdots \\ x_n' \end{pmatrix} = \begin{pmatrix} x_{11} \cdots x_{1p} \\ x_{21} \cdots x_{2p} \\ \vdots \ddots \vdots \\ x_{n1} \cdots x_{np} \end{pmatrix}, \beta = \begin{pmatrix} \beta_1 \\ \vdots \\ \beta_p \end{pmatrix}, \varepsilon = \begin{pmatrix} \varepsilon_1 \\ \varepsilon_2 \\ \vdots \\ \varepsilon_n \end{pmatrix} \tag{2}$$

Some remarks on terminology and general use:

y_i is called the dependent variable. The decision as to which variable in a data set is modelled as the dependent variable and which are modelled 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. x_i is called independent variable. Usually a constant is included as one of the regressors (Erper *et al*., 2011). In this research, for the best model to predict the phenolic contents was conducted with various subsets of the independent variables, namely, irrigation levels (%) and salt doses (dS m^{-1}).

RESULT AND DISCUSSION

The water and salt levels are one of the major environmental and soil factors affecting plant physiology, especially for photosynthesis and plant development. Main function of plant secondary metabolites is thought to be the adaptation of plants to their environment (Cirak *et al*., 2006). The physiological changes in plants in response to different stress factors may stimulate the secondary metabolite production for the restoration of the defensive systems. This is especially true for the genus *Hypericum* (Cirak *et al.,* 2012).

Figure 2 shows the raw reflectance spectra for phenolic contents in the leaves of Saint John's worts. Despite overall similarities in the graphs of phenolics, first order

derivative of spectra, significant variations could be observed within certain regions, namely: quercetin and rutin (467-612 nm); quercitrin, isoquercetin and cholorogenic acid (612-901 nm); hyperoside (559-612 nm). Figure 3 shows the first derivatives for leaf samples between 325-1075 nm.

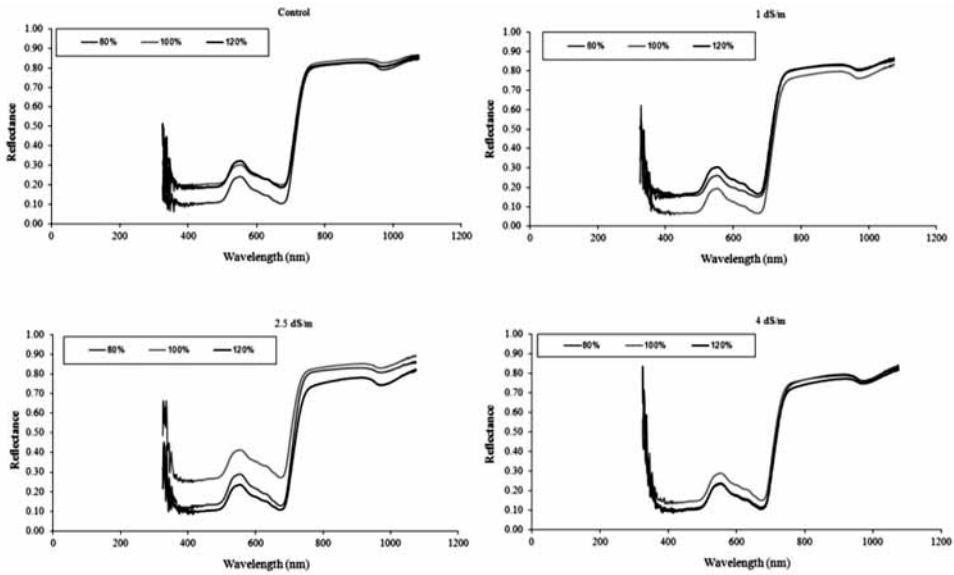

Fig. 2. Spectral reflectance for leaf samples (between 325-1075 nm).

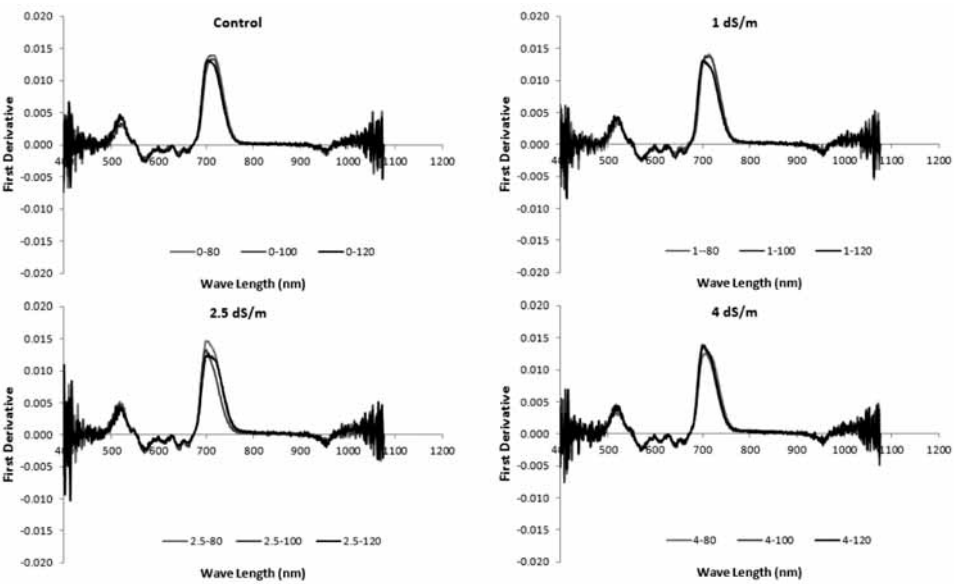

Fig. 3. First derivatives for leaf samples (between 325-1075 nm).

In the present study, the changes in secondary metabolite concentrations of plants under different water and salt levels may be attributed to physiological changes. The development of prediction models for the content of phenolics, namely, quercetin, quercitrin, isoquercetin, hyperoside, rutin, and cholorogenic acid in *Hypericum perforatum* L. has been used in the pharmacological treatments. The developed mathematical models could be applied as very useful tools for prediction of phenolic compounds content for *Hypericum perforatum* L. instead of using expensive devices and time-consuming analytical devices. The present results are also important for plant physiology, agronomy, and phytochemical studies on *Hypericum perforatum* L. HPLC analysis of the phenolics were shown Table 1.

Table 1. HPLC analysis of the phenolics according to water and salt level

	Water and Salt Level											
Phenolics	0-80	0-100	0-120	1-80	1-100	1-120	2.5-80	2.5-100	2.5-120	4-80	4-100	4-120
Quercetine	0.53	0.49	0.48	0.92	0.72	0.66	1.44	1.14	0.95	1.63	1.35	0.96
Quercitrine	0.54	0.57	0.51	0.95	0.77	0.67	1.21	0.91	0.78	1.34	1.06	0.79
İsoquercetine	4.54	4.25	4.48	10.61	8.25	6.74	13.12	10.31	8.46	12.62	11.55	8.32
Hyperoside	3.64	3.74	3.56	7.76	6.30	5.41	10.11	8.30	6.89	10.05	9.90	8.72
Rutin	0.96	1.02	1.03	1.68	1.42	1.32	2.79	2.05	1.54	2.99	2.48	1.83
Chlorogenic acid	1.56	1.45	1.36	5.81	5.14	4.49	8.31	6.12	5.71	8.36	6.98	5.62

Prediction equations were developed for estimation of the contents of quercetin, quercitrin, isoquercetin, hyperoside, rutin, and cholorogenic acid. Results of the statistical analysis revealed that most of variations in phenolic compound levels in plant material could be explained by differences in irrigation and salt levels.

The variation in content of quercetin was explained by 83% of changing environmental parameters. The equation for quercetin was explained as:

$$\text{quercetin} = (0.65)+(395.67 \times \rho 467)-(1205.02 \times \rho 612) \quad (3)$$

Where, ρ: first-order derivative spectra

Relationship between actual and predicted content of quercetin is presented in Figure 4. On the other hand, the variation in content of quercitrin, isoquercetin, hyperoside, rutin, and cholorogenic acid explained by 82%, 91%, 88%, 84%, and 87%, respectively. The equations for quercitrin, isoquercetin, hyperoside, rutin, and cholorogenic acid were explained as Table 2 and Figure 4.

$$\text{Quercitrin} = (-0.27)-(706.27 \times \rho 612)+(1547.72 \times \rho 901) \quad (4)$$

$$\text{isoquercetin} = (-6.77)-(10110.60 \times \rho 612)+(19285.81 \times \rho 901) \quad (5)$$

$$\text{Hyperoside} = (8.07)+(7875.96 \times \rho 559)-(8846.19 \times \rho 612) \quad (6)$$

$$\text{Rutin} = (1.16)+(709.55 \times \rho 467)-(2214.64 \times \rho 612), \text{ and} \quad (7)$$

$$\text{Cholorogenic acid} = (-6.37)-(7492.25 \times \rho 612)+(14571.60 \times \rho 901) \quad (8)\ .$$

The coefficients, their standard errors, and r-values of the newly produced equations predicting phenolic compounds contents in greenhouse-grown to estimation of water and salt levels effect on the *hypericum*'s phenolics with the best combinations of smoothed spectral reflectance and first-order derivative spectra (ρ) are shown Table 2.

Table 2. Statistical summary of the new produced equations predicting phenolic compounds contents.

Equations	Statistical Summary	
	r	SE
Quercetin = (-0.65)+(395.67 x ρ_{467})-(1205.02 x ρ_{612})	0.83	0.24
Quercitrin = (-0.27)-(706.27 x ρ_{612})+(1547.72 x ρ_{901})	0.82	0.17
isoquercetin = (-6.77)-(10110.60 x ρ_{612})+(19285.81 x ρ_{901})	0.91	1.45
Hyperoside = (8.07)+(7875.96 x ρ_{559})-(8846.19 x ρ_{612})	0.88	1.33
Rutin = (-1.16)+(709.55 x ρ_{467})-(2214.64 x ρ_{612})	0.84	0.41
cholorogenic acid = (-6.37)-(7492.25 x ρ_{612})+(14571.60 x ρ_{901})	0.87	1.36

Developmental models are commonly prepared by using computational or simulation techniques. The simulation software may be general-purpose, intended to capture a variety of developmental processes depending on the input files, or special-purpose, intended to capture a specific phenomenon. Input data range from a few parameters in models capturing a fundamental mechanism to thousands of measurements in calibrated descriptive models of specific plants (Odabas *et al.,* 2008). By using the data from chemical analyses and reflectance measurements in the present study, simple equations for predicting the contents of phenolics in plant material were developed.

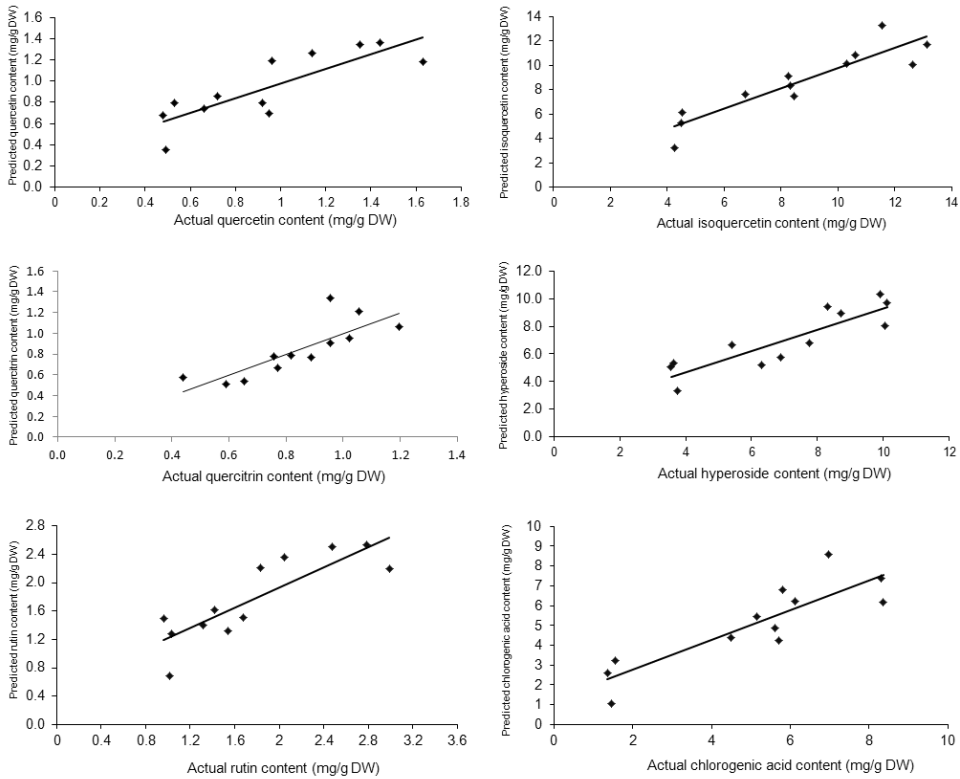

Fig. 4. Actual and predicted secondary metabolites content of *hypericum* leaves.

CONCLUSION

The prediction models for the content of phenolics, namely quercetin, quercitrin, isoquercetin, hyperoside, rutin, and cholorogenic acid during growth of *H. perforatum*, has potential use in fields of pharmacological treatments and botanical industry. The developed mathematical models could be applied as very useful tools for prediction of phenolics content not only for *H. perforatum* but also for other plants instead of using expensive devices and time-consuming analytical devices. The present results are also important for further studies on effect of different water and salt levels in chemical content of *Hypericum* plants.

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تقدير محتوى الفينولات من نبتة سانت جون (بيرفوراتوم) في ظل اختلاف مستويات الماء والأملاح استنادا إلى الانعكاس الطيفي

قادر ارسين تيمزيل
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خلاصة

تم فحص تراكم الفينول في نباتات سانت جون (بيروفوراتوم) التي نمت في بيت زجاجي و تأثرت بثلاثة أملاح مختلفة، والتي كانت كبريتات المغنيسيوم، وكلوريد الكالسيوم، وكلوريد الصوديوم في التركيزات الملحية 0.03 (السيطرة)، 1، 2.5، 4، و 8 ديسي متر−1 ل لكل ملح. تم تطبيق ثلاثة مستويات مختلفة من المياه، والتي كانت 80، 100، و 120% لجميع الأملاح الثلاثة المذكورة أعلاه. أجريت تحليلات الانحدار المتعدد لوصف آثار مستويات الماء والملح على تراكم الفينول. ونتيجة للتحليل التباين ANOVA وتحليل الانحدار المتعدد، تبين أن هناك علاقة وثيقة بين محتويات الفينول الفعلية والمتوقعة في نبتة بيرفوراتوم. واستخدمت تحليلات HPLC لتحديد كيرسيتين، كيرسترين، أيزوكيرسيتين، روتين، ومحتويات حمض الكلوروجين . وتم تحديد العلاقات بين مستويات المياه المختلفة ومستويات الملح، وتراكم الفينول بقيم الانعكاس الطيفي. تم تحديد القيم الاحتمالية (p values) للكيرسيتين، الكيرسيترين، الأيزوكيرسيتين، الهايبيروسايد، الروتين، وحمض الكلوروجين، حيث كانت 0.83، 0.82، 0.91، 0.88، 0.84، و 0.87، على التوالي. ووجدت الدراسة أن جميع القيم الاحتمالية و معدل الأخطاء القياسية من المعادلات لتكون اعتبارية عند مستوى القيمة الاحتمالية < 0.001.

كلمات البحث: عشبة، النمذجة ، الفينول ، الأملاح ، منسوب المياه.