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Full Length Research Paper

Influence of developmental stage on yield and composition of *Origanum syriacum* L. oil by multivariate analysis

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Accumulation of essential oil in herbs depends on development stage. The aim of the present study was to investigate the effect of growth stages (pre-flowering and blooming stages) on the yield and composition of essential oil of thyme (*Origanum syriacum* L.). Three harvesting times (morning at 8 am, noon at 12 pm and evening at 6 pm) and two harvesting date (growth stage (pre-flowering stage) and blooming stage) were studied. Total yield of oil was higher in the plant at blooming stage in comparison with that at pre-flowering stage. According to the results of multivariate analysis, higher levels of volatile compounds were noticed during the blooming stage, except carvacrol, gamma-terpinene, alpha-terpinene and para-cymene that were found higher in pre-flowering stage. Unexpectedly, carvacrol as structural isomer of thymol depicts higher concentration at pre-flowering stage. Moreover, an accurate valid prediction equation of oil samples was built to predict the growth stage and quality control purposes of the oil.

Key words: Carvacrol, essential oil, harvesting time, blooming stage, *Origanum syriacum*, thymol.

INTRODUCTION

Medicinal plants since times immemorial have been used in virtually all cultures as a source of medicine. Medicinal properties in plants are mainly due to the presence of secondary metabolites which these plants need in their natural environments under particular conditions of stress and competition (Schippmann et al., 2002). Cultivation of medicinal plants is widely viewed not only as a means for meeting current and future demands for large volume production of plants-based drugs and herbal remedies, but also as a mean for relieving pressure on wild populations (Lambert et al., 1997; Palevitch, 1991).

Plant essential oil extracts have been used for many thousands of years, especially in food preservation, pharmaceuticals, medicine and natural therapies (Hazzit and Baaliouamer, 2009). It has long been acknowledge that some plant essential oils exhibit antimicrobial properties and it is necessary to investigate these plants scientifically, which have been used in traditional medicine to improve quality of healthcare. Essential oils are potential sources of novel antimicrobial compounds, especially against bacterial pathogens (Azizi et al., 2009). Thyme (*Origanum syriacum* L.), a member of the Lamiaceae family, is an aromatic and medicinal plant of increasing economic importance for North America, Europe and North Africa, and Mediterranean (Letchamo and Gosselin, 1996). At present time, this plant is cultivated in large scale in Jordan. Evidently, thyme continues to command an important place in expanding world market. Thyme volatile phenolic oil has been reported to be among the top 10 essential oils (Letchamo et al., 1995), showing antibacterial, antimycotic, antioxidative, natural food preservative and mammalian

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age delaying properties (Letchamo et al., 1995; Jackson and Hay, 1994). The biosynthesis of secondary metabolites, although controlled genetically, is affected strongly by environmental influences (Yanivie and Palevitch, 1982).

Agricultural factors have a critical effect on quantitative and qualitative characteristics of thyme, which finally result in plant growth and yield increment. Spacing and harvesting schedule can be very effective factors in this area. One of the most important characteristics of oil accumulation is its dependence on the developmental stage of the plant per se as well as its concerned part (Sangwan et al., 2001). Another important factor influencing thyme oil production is harvesting time. Most oils were produced from flowering plants, because oil content was generally found to peak at that time (McGimpsey et al., 1994). A report on thyme grown in Northern Italy indicated that phenol content at full flowering varied from year to year (Piccaglia and Maroti, 1991). Oil yield and phenol content peaked after flowering had finished (McGimpsey et al., 1994). Rey (1991) has shown at Arbaz, Iran, that the best harvesting time was late September with cutting at 10 or 15 cm height, but at Bruson cutting in mid-August to a height of 15 cm was the best. However, harvesting time can be suggested according to area and its environmental conditions (Rey, 1991).

In several species of thyme, at flowering, essential oil is at the highest level, while in other species, flowering has a lower influence. The yield of plant material, the essential oil content and quantitative composition of *Thymus vulgaris* can be influenced by harvest time, ecological and climatically conditions (Cabo et al., 1982). *Origanum* species are underutilized since their genetic resources are not properly exploited, because little work has been done so far on their domestication or on their improvement. *Origanum* spp. are under serious threat of genetic erosion and are not adequately conserved, where the amount of genetic diversity that is being collected and maintained in genebanks or in botanic garden collection around the world is very limited (Heller and Padulosi, 1997).The objective of this study was to determine the most suitable harvest time and development stage for obtaining maximum drug yield, essential oil and essential oil components. The study aimed to determine the oil content and composition isolated from dried leave and whole aerial parts of *O. syriacum* L.

MATERIALS AND METHODS

Plant

Cultures of thyme (*O. syriacum* L.) were established from seeds planted in the greenhouse of the University of Jordan. Seedlings (30 days old) were grown direct in greenhouse, of which half were under shading. Three harvesting times (morning at 8 am, noon at 12 pm and evening at 6 pm) and two harvesting dates (growth stage (pre-flowering stage) and blooming stage) were studies during 2010/2011 growing seasons. The treatments were arranged in a spite-plot, in a randomized complete block design with repeated measurements, replicated three times.

Oil distillation

The oil of air-dried and finely ground whole aerial parts of *Teucrium polium* was obtained by hydrodistillation using a Clevenger-type apparatus. Distillation was performed using 50 g of dried plant material in 2.5 L distilled water for 4 h. The oils obtained were dried over anhydrous sodium sulfate and stored in a dark glass bottle at 4°C under N_2 until analysis. Three replicates were carried out. The yield of oil was 0.8 ± 0.04% w/w based on the dried weight of sample.

GC/MS analysis

Identification and quantification of volatile oils in gas chromatography-mass spectrometry (GC-MS) was performed on a ThermoQuest gas chromatograph coupled to mass spectrometer (QP2010) equipped with Rtx®-5MS polar capillary column stationary phases. About 0.1 µl aliquots of the tested oil without further modifications, or 1 µl of 0.1% oil solution (in n-hexane and dried over anhydrous Na_2SO_4), were injected into a TRACE GC2000 SERIES (ThermoQuest CE instruments, Austin, TX,USA) gas chromatograph equipped with a split-splitless injector. Split ratio of 1:30 for diluted and 1:100 for undiluted oil samples were used. The column was an Rtx-5MS fused silica capillary column (30 × 0.25 mm, 0.25 µm film thickness) consisting of crossbond (5% diphenyl, 95% dimethyl polysiloxane). Helium (He) was the carrier gas at a flow rate of 1.0 ml/min. The GC was interfaced with a GCQ plus (ThermoQuest, Finnigan) mass detector operating in the electron ionization (EI) mode (70 eV). The mass spectra were generally recorded over 40 to 650 amu full-scan mode that revealed the total ion current (TIC) chromatograms.

A linear temperature program was adapted to separate the different oil components as follows: initially, the column was maintained at 70°C for 1 min, ramped at 5°C min^{-1} to 130°C and maintained for 10 min, a second ramp was then applied at 8°C min^{-1} to final temperature of 210°C aqnd which was held isothermal for 2 min. The temperatures of the injector base, transfer line and ion source were maintained at 250, 250 and 200°C, respectively. The chemical identities of the separated components were determined by matching their recorded mass spectra with the data bank mass spectra (general purpose, terpene ThermoQuest and NIST libraries) provided by the instrument software, and by comparing their calculated retention indices with literature values measured on columns with identical polarity. Structures of α-pinene, β-pinene, myrcene, p-cymene, limonene, α-and γ-terpinene were further confirmed by chromatography of their authentic standards under the GC/MS conditions mentioned earlier.

RESULTS

Chemical composition of the essential oil

The plants were planted under plastic house condition, and the half part of the house was shading. The plants were planted in Jordan valley, and harvested in three harvesting times (morning, noon and evening). Several morphological characters were evaluated and oil content was also measured. The ration production under sunlight

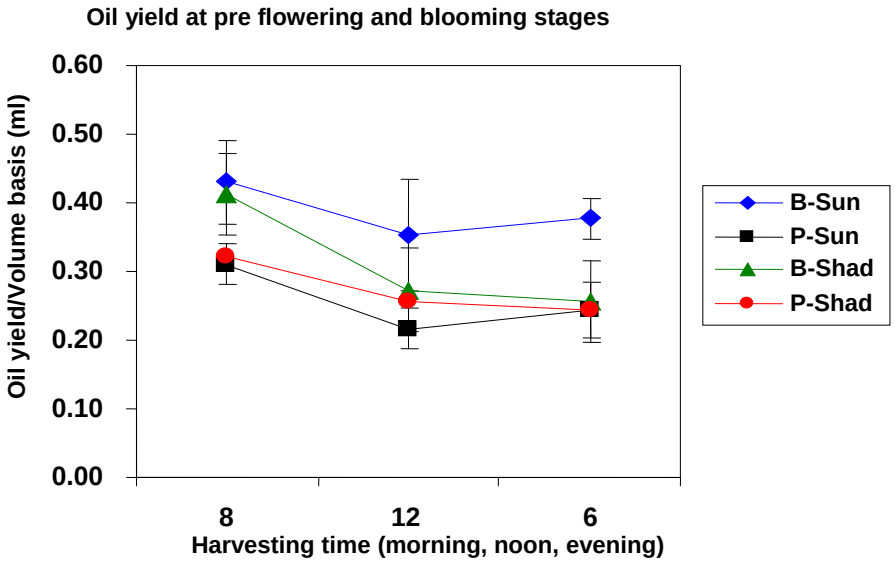

Figure 1. Effect of harvesting time on qualitative oil at pre-flowering (P) and blooming stage (B) of thyme under sunlight and shade.

during blooming stage was higher when compared with pre-flowering stage (Figure 1). On the other hand, the yield was higher in pre-flowering stage under shade condition. Harvesting time affected fresh herbage, oil content, thymol, carvacrol content yield and plant height and diameter. Lowest and highest plant diameter and height were in plants harvested at the beginning of blooming and fruit setting, respectively. Generally, as shown in Table 1 and Figure 1, the oils were characterized by dominant levels of thymol, carvacrol, γ-terpinene and *p*-cymene. The highest content of oil was observed in thyme growing in the blooming stage, whereas sunlight was generally associated with better oil yields (Figure 1).

All the tested thyme materials were found to satisfy, in terms of percentage yields, the compendial requirements as shown in Table 1. Freshly isolated essential oil was a yellow essential oil that was separated into five classes, which were monoterpene hydrocarbons, oxygenated monoterpenes, sesquiterpene hydrocarbons, oxygenated sesquiterpenes and others (Table 1). Based on GC and GC-MS analysis of the essential oil of thyme, 17 components were identified, which represented 99.95% of the total detected constituents. The major constituents of the oil were thymol and carvacrol, which were the major components of the oils from the populations (60.74 and 31.77%, respectively). Other major constituents (>1.0% of the identified portion) were β-Caryophyllene (1.48%), γ-Terpinene (1.76%) and p-Cymene (1.49%). Although, the presence of the latter two monoterpene ketones is not exceptional, their observed levels, particularly for α-Terpinene, are interesting.

As depicted from score plot (Figure 2), samples taken from blooming stage show noticeable clustering from those of pre-flowering samples. Principal component one (PC1) which was represented by x-axis is responsible of data clustering rather than PC2 (Y-axis) (Arceusz et al., 2010). This clustering is due to majority of the samples' data, because PC1 represents around 70% of the data, while PC2 represents only 20% of them. Thus, this separation emphasizes statistical differences between the component of volatile oils between blooming and pre-flowering stage. These differences are due to quantitative and qualitative variations between blooming and pre-flowering samples (Kaskoniene et al., 2011).

Loadings (volatile oils) which are located on the left part of the loadings plot (Figure 3) possess higher concentration in pre-flowering samples in comparison of blooming samples (Feng et al., 2009). This is due to mutual Cartesian coordination between the samples in score plot with volatile oils in loadings plot. α-terpinene, p-cymene and γ-terpinene are simple terpines found with higher concentrations in pre-flowering samples (Sefidkon et al., 2001, 2002, 2004; Sefidkon and Akbari-nia, 2009). This might be due to the fact that they are synthesized at early stage in terpine pathways when compared with other volatile oils such as thymol (Christensen and Grevsen, 2006).

It was decided to further fit the data by partial least square regression (PLS) after successful separation of the samples by using principal component analysis (Noorizadeh and Farmany, 2010; Pourbasheer et al., 2010; Noorizadeh et al., 2011). The idea was to build prediction equation by using PLS model. Since blooming and pre-flowering stages are qualitative characters, it was decided to use numerical values to facilitate their

Table 1. Chemical composition of essential oils hydrodistilled from thyme plants grown in Jordan valley.

RI*		Compound	Content ± SD (%)											
P	T		PH-8	PH-12	PH-6	PS-8	PS-12	PS-6	FH-8	FH-12	FH-6	FS-8	FS-12	FS-6
970	974	3-Octenol	0.26±0.13	0.31±0.13	0.27±0.16	0.11±0.04	0.14±0.03	0.08±0.03	0.21±0.07	0.33±0.04	0.18±0.05	0.27±0.07	0.34±0.07	0.35±0.15
990	988	3-Octanol	0.27±0.15	0.39±0.16	0.34±0.16	0.14±0.02	0.21±0.06	0.10±0.03	0.26±0.05	0.27±0.04	0.19±0.04	0.26±0.06	0.35±0.09	0.38±0.13
1011	1014	α-terpinene	0.47±0.28	0.24±0.13	0.25±0.07	0.08±0.03	0.07±0.06	0.06±0.05	0.16±0.04	0.08±0.07	0.28±0.17	0.17±0.04	0.28±0.03	0.18±0.03
1018	1020	ρ-Cymene	1.49±0.70	0.56±0.26	0.45±0.23	0.17±0.08	0.14±0.05	0.11±0.03	0.42±0.08	0.21±0.01	0.64±0.16	0.52±0.08	0.62±0.19	0.51±0.31
1055	1054	γ-Terpinene	1.76±0.70	0.80±0.41	0.83±0.22	0.28±0.13	0.27±0.08	0.14±0.08	0.21±0.06	0.18±0.07	0.71±0.76	0.21±0.06	0.55±0.20	0.34±0.20
1067	1065	Z-sabinene	0.35±0.21	0.44±0.18	0.51±0.20	0.30±0.09	0.53±0.11	0.36±0.07	0.52±0.14	0.80±0.08	0.45±0.10	0.54±0.14	0.43±0.14	0.94±0.44
1098	1095	Linalool	0.00±0.00	0.04±0.04	0.05±0.05	0.05±0.00	0.09±0.02	0.08±0.01	0.11±0.05	1.57±2.39	0.05±0.07	0.11±0.05	0.13±0.02	0.20±0.07
1100	1098	E-sabinene	0.06±0.11	0.21±0.05	0.23±0.06	0.14±0.07	0.25±0.03	0.19±0.04	0.21±0.05	0.34±0.04	0.20±0.05	0.21±0.05	0.20±0.02	0.33±0.16
1175	1078	Methyl iso-borneol	0.12±0.11	0.24±0.06	0.26±0.09	0.15±0.06	0.26±0.02	0.21±0.03	0.18±0.03	0.80±0.89	0.20±0.08	0.22±0.03	0.27±0.01	0.27±0.04
1180	1176	4-Terpinenol	0.43±0.09	0.45±0.15	0.47±0.13	0.31±0.17	0.48±0.07	0.38±0.03	0.47±0.11	1.05±0.67	0.40±0.02	0.62±0.13	1.03±0.19	0.88±0.12
1190	1186	α-terpineol	0.04±0.08	0.15±0.01	0.15±0.03	0.10±0.06	0.15±0.02	0.13±0.04	0.17±0.02	0.38±0.26	0.13±0.01	0.18±0.02	0.22±0.02	0.22±0.01
1290	1289	Thymol	60.74±11.64	69.07±6.73	56.13±12.93	35.26±19.70	60.16±3.68	69.13±4.01	68.51±11.16	53.20±8.54	71.75±6.39	74.62±4.54	71.59±14.48	65.15±6.11
1299	1298	Carvacrol	31.77±12.99	24.49±8.40	37.28±14.24	37.58±20.55	34.63±3.96	26.19±4.75	31.77±12.99	24.89±11.28	20.19±7.73	18.24±4.14	20.49±14.78	26.99±5.77
1415	1417	β-Caryophyllene	1.48±0.21	1.61±0.49	1.67±0.34	0.94±0.58	1.47±0.14	1.43±0.23	1.22±0.20	2.48±0.62	2.12±0.85	1.62±0.20	1.64±0.32	1.74±0.29
1452	1452	α-Humulene	0.20±0.11	0.16±0.02	0.17±0.03	0.09±0.05	0.16±0.03	0.19±0.03	0.16±0.00	0.25±0.03	0.18±0.10	0.16±0.00	0.19±0.04	0.14±0.03
1504	1505	β-Bisabolene	0.30±0.08	0.52±0.04	0.56±0.10	0.33±0.20	0.56±0.12	0.78±0.08	1.11±0.20	1.80±0.40	1.45±0.97	1.22±0.20	0.76±0.06	0.87±0.10
1580	1582	Caryophyllene oxide	0.25±0.01	0.31±0.09	0.37±0.04	0.31±0.23	0.45±0.02	0.44±0.03	0.81±0.11	0.66±0.01	0.89±0.74	0.84±0.12	0.91±0.04	0.52±0.03

description mathematically. Zero value was given to the pre-flowering stage, whereas value of one was given to samples from blooming stage. Minitab® software was used to implement regression analysis of the data using the growth stage numbers (0 and 1) as dependant variables. Furthermore, volatile oils were used as independent variables as described by the following equation.

$$y = a_1 x_1 + a_2 x_2 + a_3 x_3 \ldots\ldots\ldots + a_n x_n + c \quad (1)$$

Where "y" is the blooming stage, "x" is the type of the oil, "a" is the correlation coefficient which should be found and evaluated by the PLS and "c" as constant in y-intercept of the regression model found empirically.

A total of 31 out of 36 samples were used to build PLS model. The rest samples were left for validating the model. The percentage of area under the curve of each volatile compound to the total area of each sample was used as independent value, while binary values (0 and 1) were used to describe the growth stage of the samples and used as dependant values for the PLS model. All volatile oils as shown in Table 1 were used in the resulted prediction equation of PLS. In order to evaluate the significance of each volatile oil in predicting the growth stage, analysis of variance (ANOVA) was accomplished for the regression results. Volatile oils possess P-value above 0.05 that resulted from ANOVA of regressed data that were removed from the equation. Finally, only five descriptors (volatile oils) with P-value <0.05 remained in the prediction equation to get the following equation:

$$y = 49.8 - 1.15(\gamma\_Terpinene) - 2.77(Z\_Sabinene) - 0.504(Thymol) - 0.496(Carvacrol) - 0.842\beta(Caryophyllene) \quad (2)$$

As shown in Equation 2, it was found out that Z-sabinene is the most important component in predicting the growth stage, because it has the highest correlation value (2.77). Moreover, thymol and carvacrol as important components in thyme oil are also participating in the prediction model. In order to verify the accuracy of the prediction equation (Equation 2), it has been decided to

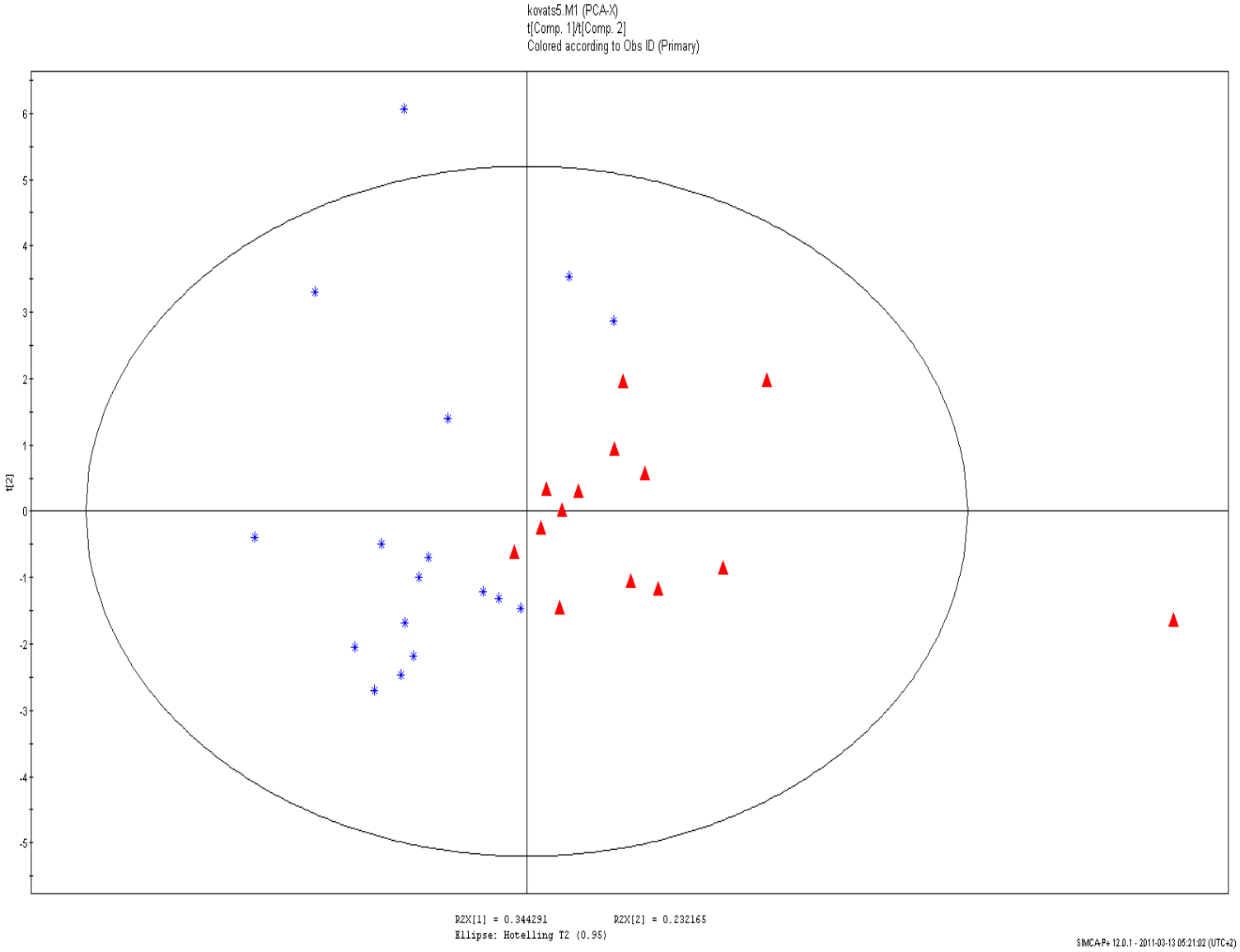

Figure 2. Scores plot derived from principal component analysis of GC-MS results of volatile compounds of thyme oil from pre-flowering and blooming stages. Samples harvested from blooming stage (▲) and pre-flowering stage (*). Data are reported as first (PC1) versus second (PC2) principal component for thyme samples.

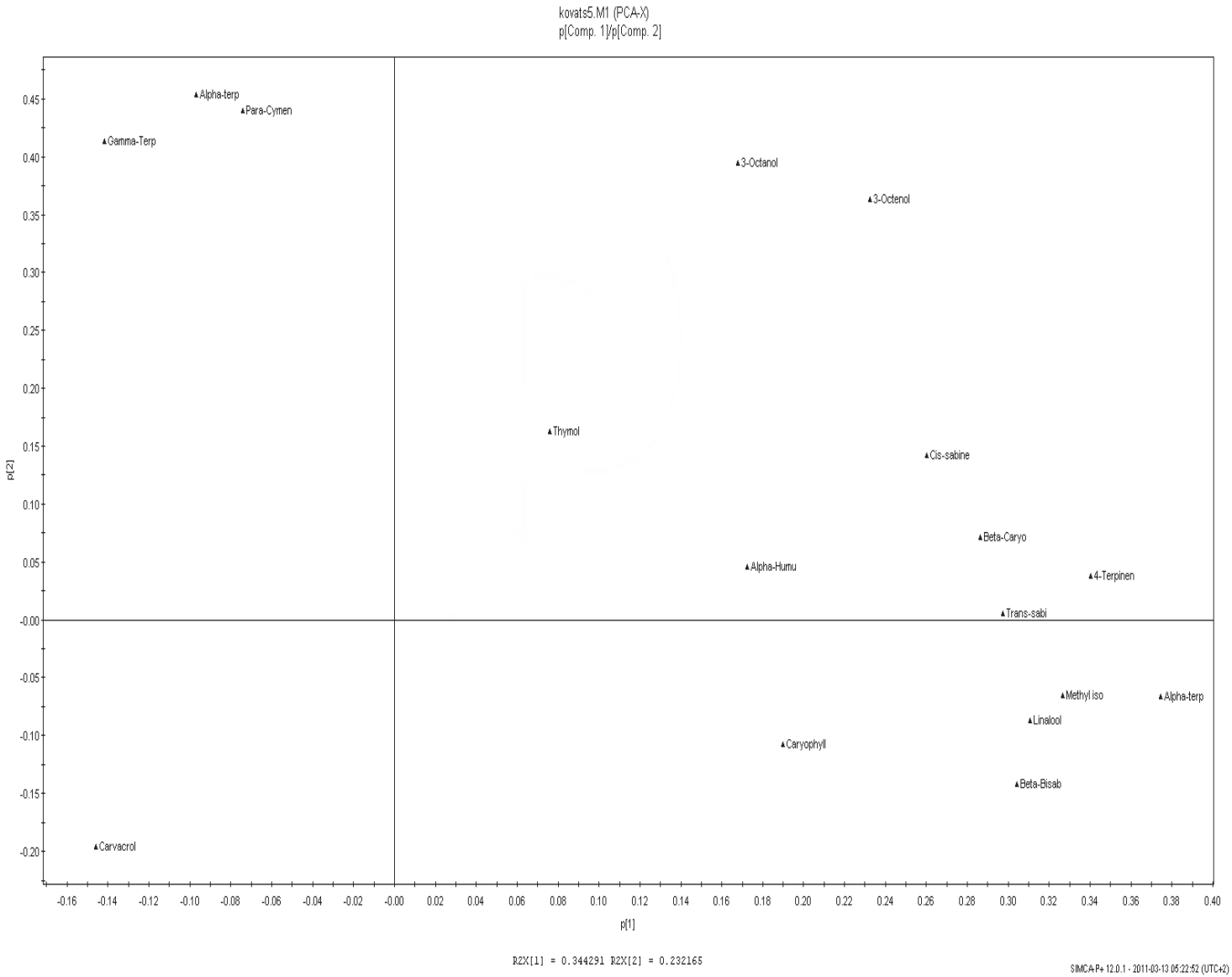

Figure 3. Loadings plot derived from principal component analysis of GC-MS results of volatile compounds of thyme oil from pre-flowering and blooming stages. Compounds with (+PC1) values represent higher concentration in blooming samples, while those with (-PC1) values represent higher concentration in pre-flowering stage.

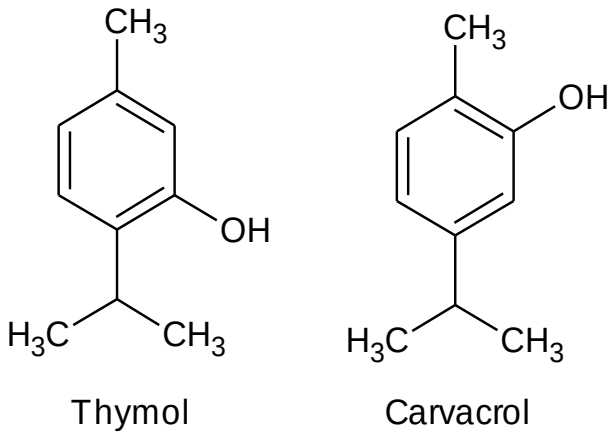

Figure 4. Structure of thymol and carvacrol.

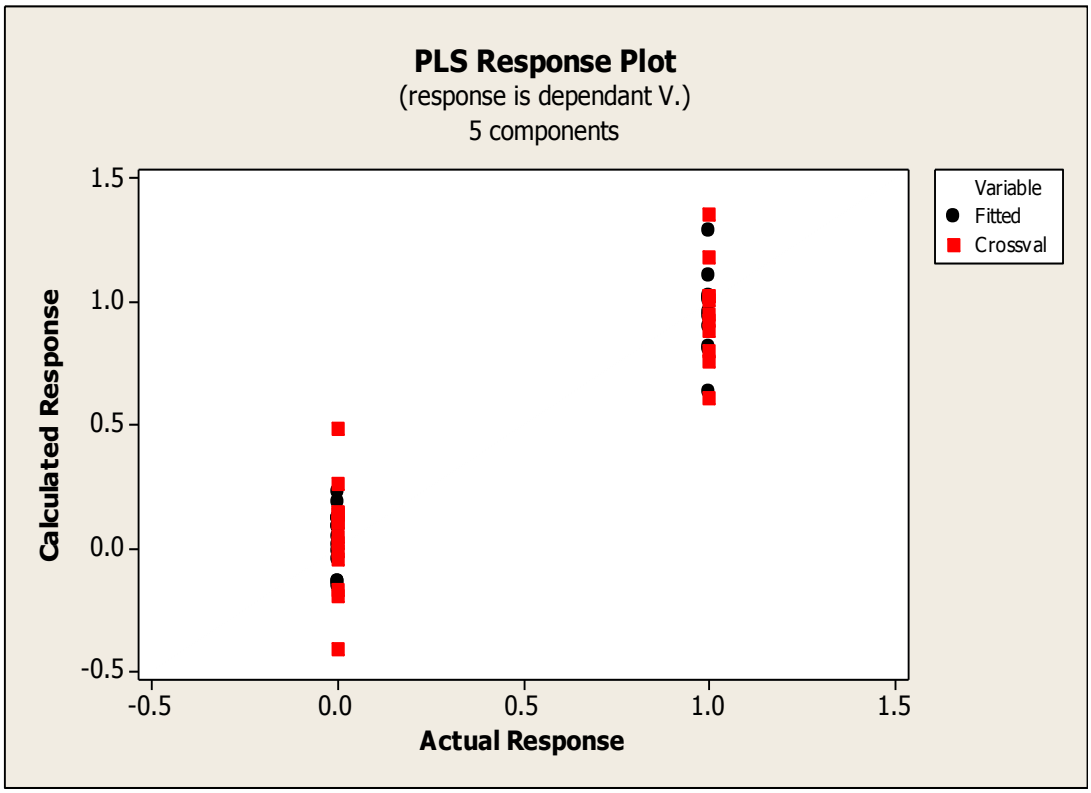

Figure 5. Internal validation of partial least square regression analysis (PLS), using 31 samples from original data of Table 1 as independent variable, while growth stages were given (“0” for pre-flowering and “1” for blooming values) as dependant variables. Calculated (Predicted) response of growth stages entirely match the actual stage of growth.

implement internal and external validations.Internal validation was accomplished by jackknifing procedure which is also called leave-one-out, because it relies on high number of mathematical perturbations in each time the model build from all samples except one chosen randomly (Du and Chen, 2009, Du and Chen, 2010). Then, the model calculated the left one. After many times of perturbations, the model graph the cross valid points with the fitted ones as shown in Figure 4. The cross valid points matches the fitted points which implicates the high

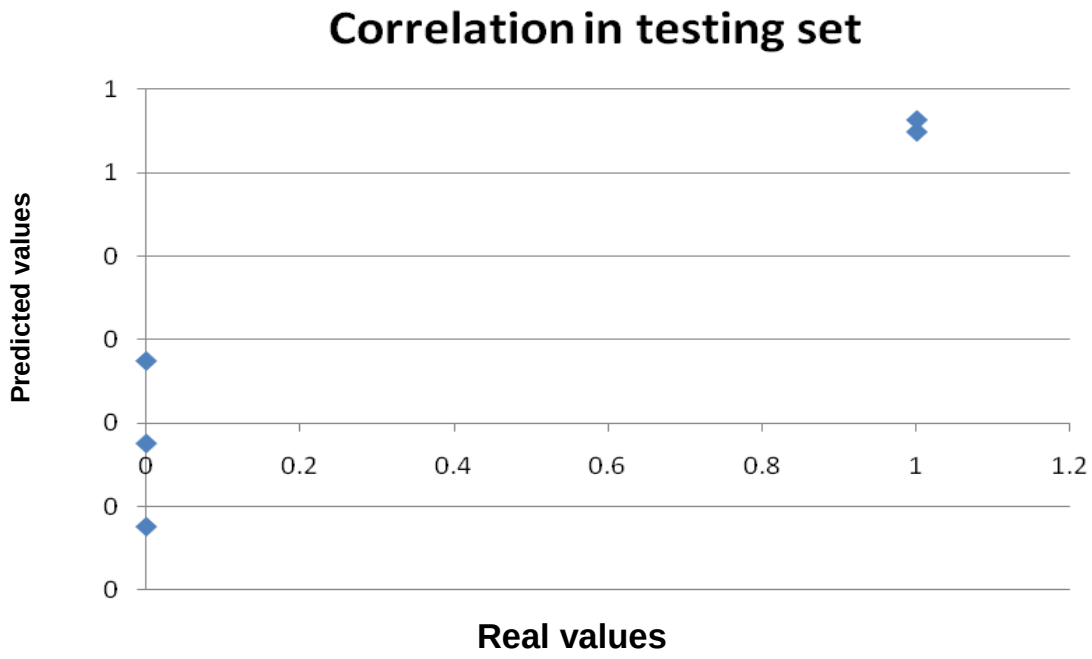

Figure 6. External validation using remained 5 samples from original data of Table 1 as independent variable, while growth stages were given (0 and 1) values as dependant variables. Calculated (Predicted) growth stages entirely match the actual stage of growth.

degree of accuracy of the model. Moreover, external validation of the prediction model has been fulfilled by comparing the outcome of the testing set of the samples with the training set from which the model built. The model was able, as shown in Figures 5 and 6, to predict the samples' growth stage accurately. The samples taken from pre-flowering stage (with zero values) are all predicted as pre-flowering samples by the model and the same thing for the blooming stage.

DISCUSSION

The essential oil in the Jordanian Za'atar (*O. syriacum* L.), is characterized for its thymol and carvacrol content using gas-liquid chromatography (Daouk et al., 1995). On the other hand, the essential oil (EO) of *O. syriacum* L. is characterized by the dominant occurrence of linalool, terpinen-4-ol and sabinene hydrate (Berna'th, 1997). It is used as folk remedy against asthma, indigestion, headache and rheumatism (Jun et al., 2001).

The results of this work are in agreement with previous reports on thyme. Felice (1996) reported that the best time to harvest thyme for both essential oil yield and phenol content is during or immediately after full bloom. McGimpsey et al. (1994) reported that the highest oil and phenol contents resulted in plants harvested after full blooming stage. McGimpsey et al. (1994) attributed this variation to the seasonal variations that affect chemical constituents and contents in thyme. Also, harvesting time of thyme may differ according to location (Rey, 1991). Harvesting at the beginning of blooming is the best treatments suggested for fresh and dry biomass, oil and thymol yields in this experiment (Figures 1 and 2). These results are also in agreement with the results of (Shalby and Razin, 1992). Highest oil and thymol yields resulted from plants harvested at the beginning of blooming. This is due to high yields of fresh and dry biomass and content of oil and thymol in this stage. On the other hand, carvacrol is a big structural isomer of thymol found in higher concentration in pre-flowering samples; this findings agreed with other researchers (Hazzit and Baaliouamer, 2009; Lakusic et al., 2011), whereas, thymol is higher in blooming samples matching other research findings (Kizil et al., 2008). This could be of interest because of close structural similarity between thymol and carvacrol (Figure 4). Shifting of the synthesis from carvacrol toward thymol during the blooming stage is a new finding and it might be good information for further exploration.

The differences in these reports from previous literatures may be associated with the variances in these factors, including genetic, seasonal, temperature, moisture, soil, day length changes on oil production and quality (Farooqi et al., 1999; Ceylan et al., 2003; Yaldiz et al., 2005; Baydar et al., 2004). Significant changes were in thymol and carvacrol content, due to the collection hours. It may be that the seeds used have a different genetic structure, because it is known that synthesis of essential oils may change plant's genetic structure (Ceylan et al.,

2003).

Conclusions

It has been known that agronomical factors have a great effect on both quality and quantity of essential metabolites. For this reason, it is necessary to determine optimum levels of agronomical factors affecting plant growth and production. Time of harvesting is the most important agronomical factors. The maximum yield of dry and fresh herbage, yield and content of oil and thymol yield were obtained in the beginning of blooming stage at morning. Maximum thymol content was observed in the beginning of blooming. However, harvesting in the beginning of blooming was the best treatment in respect of yield of dry matter, oil and thymol per unit area.

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