

## An Optimization Study for The Production of *Origanum onites* Tincture by Response Surface Methodology: Effect of Liquid/Solid Ratio, Ethanol Concentration and Storage Period

*Origanum onites* Tentürü Üretim Şartlarının Tepki Yüzey Yöntemi ile Optimizasyonu: Sıvı/Katı Oranı, Etanol Konsantrasyonu ve Depolama Süresinin Etkisi

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### Abstract

In this study, *Origanum onites* was used for the production of tincture, a hydroalcoholic extract, and manufacturing conditions were optimized to determine the processing factor variable levels to increase the final quality of samples. For this purpose, response surface methodology study was applied and Box Behnken design was used for the optimization. Three factors were selected namely liquid/solid ratio, ethanol concentration and storage period for the preparation of samples. Total phenolic content, antiradical activity and efficient of concentration (EC50) values were determined to evaluate the bioactivity of tincture samples. It was observed that the total phenolic content of the tincture samples was affected by ethanol concentration and liquid/solid ratio. Total phenolic contents of tincture samples were in the range of 964.1-7020.8 mg GAE/ L samples while the efficient concentration (EC50) ranged from 44.88 to 89.56. Increase of solid level increased the bioactivity values of samples, but this increment decreased the tincture yield significantly. Optimization results showed that the highest total phenolic content would be at 50% ethanol concentration, 6 ml ethanol level which means the liquid/solid ratio is 1:5 and 15 days storage for the tincture samples.

**Keywords:** *O. onites*, tincture, bioactivity, optimization, response surface methodology

### Öz

Bu çalışmada, bir hidroalkolik ekstrakt tipi olan tentür üretimi amacıyla *Origanum onites* bitkisi kullanılmış ve son ürün kalitesini artırmaya yönelik olarak üretim şartlarını optimize etmek üzere proses faktör seviyelerinin belirlenmesi amaçlanmıştır. Bu amaçla, optimizasyon işlemi için tepki yüzey yöntemi tabanlı Box-Behnken deneme tasarımından faydalanılmıştır. Örneklerin hazırlanması amacıyla sıvı/katı oranı, etanol konsantrasyonu ve depolama süresi faktör olarak seçilmiştir. Tentür örneklerinin biyoaktif özelliklerinin belirlenmesi amacıyla toplam fenolik madde miktarı, antiradikal kapasite ve EC50 değeri hesaplanmıştır. Sıvı/katı oranı ve etanol konsantrasyonunun örneklerin toplam fenolik madde miktarı üzerinden etkili olduğu görülmüştür. Örneklerin toplam fenolik madde değerleri 964.1-7020.8 mg GAE/ L, EC50 değerleri ise 44.88-89.56 aralığında değişim göstermiştir. Örnek konsantrasyonunun artışı toplam fenolik madde miktarını artırırken, bu artış tentür veriminde düşüşe yol açmıştır. Optimizasyon sonuçları en yüksek toplam fenolik madde miktarının, %50 etanol konsantrasyonunda, 1:5 katı/sıvı oranında, 15 günlük depolama süresi sonunda elde edilebileceğini göstermiştir.

**Anahtar Kelimeler:** *Origanum onites*, tentür, biyoaktivite, optimizasyon, tepki yüzey yöntemi

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Oregano (*Origanum vulgare* L., fam. Lamiaceae) which is a medicinal and aromatic herb grows commonly in Europe and Asia, especially in the Mediterranean region (Josifovic et al., 1974). It is used as a spice by humans all around the world for ancient times and it is a good raw material for the production of tincture to use against some diseases like cold, for digestive and respiratory problems (Ivanova et al., 2005; Leporatti and Ivancheva, 2003). In folk medicine, to treat many health problem like respiratory disorders, dyspepsia, painful menstruation, rheumatoid arthritis and urinary tract disorders, *O. vulgare* is one of the commonly used as medicinal plant (Gruenwald et al., 2000). Oregano was reported that it had strong biological activity in many published papers (Barros et al., 2010; Economou et al., 1991; Kaurinovic et al., 2011; Kosar et al., 2005; Spiridon et al., 2011; Özbek et al., 2008; Sahin et al., 2004). For the biological activity of oregano, in general essential oil of the plant was characterized and in recent years biological activities of the essential oil of *O. vulgare* have intensively been studied (Castilho et al., 2012; Sahin et al., 2004). It was reported that the oregano essential oils and extracts include phenolic substances such as carvacrol and rosmarinic acid and they have strong antioxidant and antibacterial activities (Baydar et al., 2004; Koşar et al., 2003; Pizzale et al., 2002; Tepe et al., 2004). As is well known, antioxidants are compounds capable of delaying, retarding or preventing oxidation. They can react with free radicals or can interrupt the chain reaction in the propagation of oxidation (Valko et al., 2007). Botanical extracts are well recognized sources of antioxidants. The supplementation with phytochemicals such as polyphenols, including flavonoids, could be help to protect the body against diseases. Because they are free radical scavengers and antioxidant compounds having capacity to reduce the negative effect of oxidative stress and free radicals (Seifried et al., 2007). Tincture is a hydroalcoholic extracts of the medicinal plants and they are galenic preparation of many herbs which could be used for the medicinal treatments. This method of plant material extraction used are recommended by several Pharmacopoeias, e.g. European Pharmacopoeia and Polish Pharmacopoeia (Olech et al., 2012). Preparation of tinctures in phytherapy is very common technique to increase the bioactivity of plant on the disease. It is reported that the solid form of the plant powders had common usage in the medicinal treatment but liquid preparation of the herbs have many advantage compared to solid form like easy preparation of a unique formulation and another considerable advantage of liquids is that if properly prepared, they include minimal processing during manufacturing and so they reflect the chemical spectrum of the original herb. Also, tinctures which are the liquid form of the herbs have superior bioavailability because all important phytochemical constituents are already in solution compared to solid-dose preparation. It was also stated that the herbal liquids confer considerable dose flexibility and intake of liquids is every time easier by the children compared to solid ones (Bone, 2003). Due to the stated advantages of the above, tincture is preferred by the doctors compared to solid powder form.

According to our knowledge, there was no published paper regarding the optimization of production conditions for the oregano tincture. The main purposes of the current study was to determine the bioactivity of *Origanum onites* plant and its produced tinctures in terms of total phenolic content, antiradical activity and EC<sub>50</sub> values and optimized conditions for the production of tincture having high bioactivity. For this aim, three processing variables were determined as liquid/solid ratio, ethanol concentration and storage period and response surface methodology Box Behnken design was used for the determination of effective factors and optimized production conditions.

### Material and Methods

In the current study, *Origanum onites* was purchased from Boralife Medicinal Plant Company (Ankara, Turkey) as dried material. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was purchased from Sigma-Aldrich (Madrid, Spain), and ethanol was from Tekkim Co. (Istanbul, Turkey). Folin Ciocalteu reagent and sodium carbonate were procured from Merck (Germany). All of the reagents used were of analytical quality.

### Sample preparation and experimental design

In the current study, both bioactivity of plant and also its tincture were characterized. The plant samples were ground using a grinder for further analysis. To extract the total phenolics as active substance in the plants, the related amount of the plant material was weighed as 1 g (moisture: 9.27%) and placed in a glass jar. After that, ethanol at desired concentration (5 ml, 10 ml or 30 ml) according to the experimental design in Table 1 was incorporated and mixed for 1 minute. Finally, the jars were covered tightly and stored in dark conditions at room temperature during the determined storage time. At the end of the storage period, the samples were filtered using a filter paper and the filtrate was used for further analysis. The tincture production was performed as similarly. As is known, tincture is the ethanolic extract of the plant samples. So, the filtrated samples was used as tincture (Bone, 2003).

**Table 1. Box-Behnken design matrix used to evaluate the effects of processing variables on bioactive properties of *O. onites* plant extract and tincture**

Runs	Coded values			Actual values		
	X1	X2	X3	Liquid/solid (ml)	Ethanol concentration (%)	Time (day)
1	0.00	0.00	0.00	10	50	8
2	0.00	1.00	1.00	10	75	15
3	0.00	0.00	0.00	10	50	8
4	-1.00	1.00	0.00	30	75	8
5	0.00	1.00	-1.00	10	75	1
6	1.00	-1.00	0.00	6	25	8
7	1.00	1.00	0.00	6	75	8
8	0.00	-1.00	1.00	10	25	15
9	1.00	0.00	1.00	6	50	15
10	-1.00	0.00	-1.00	30	50	1
11	1.00	0.00	-1.00	6	50	1
12	0.00	0.00	0.00	10	50	8
13	-1.00	0.00	1.00	30	50	15
14	-1.00	-1.00	0.00	30	25	8
15	0.00	-1.00	-1.00	10	25	1

#### Determination of total phenolic content

To determine the total phenolic content of the samples, Folin Ciocalteu method suggested by Singleton and Rossi (1965) was used. For this purpose, 200 µl of sample extract was placed in a tube including 1800 µl of distilled water. Afterthat, 1 ml of Folin reagent (1/10 diluted) was incorporated and waited for 1 min. At the end, 2 ml of sodium carbonate (2% w/v) was added and the tubes were covered, vortexed and incubated for 2 h in a dark place at room conditions (24-25 °C). At the end of the incubation, the absorbance values of samples were measured at 765 nm by a UV-VIS spectrophotometer (Schimadzu, Japan) and the total phenolic content of the plant samples were calculated as mg GAE/g sample for plant material and mg GAE/L for tincture samples.

#### Determination of antiradical activity

To determine the antiradical activity of samples, 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was used as radical substance and scavenging activity of samples was determined as % inhibition. For this purpose, 100 µl of extract sample (diluted as 1/40) was mixed with 3900 µl of DPPH solution (0.1mM in methanol) and vortexed in tube. The absorbance values of the samples were measured at 517 nm after 30 min incubation in dark place at room conditions by using a UV-VIS spectrophotometer (Schidamzu, Japan). The % inhibition value was calculated using following equation:

$$\% \text{Inhibition} = [(Abs_c - Abs_s) / Abs_c] \times 100 \quad (\text{Eq.1})$$

where  $Abs_c$  is the absorbance of the control and  $Abs_s$  is the absorbance of the sample (Kaplan et al., 2018).

#### Determination of antiradical efficiency ( $EC_{50}$ )

To determine the  $EC_{50}$  values of the samples, the methodology described by Moreno et al. (1998) and Brand-Williams et al (1995) was used. For this purpose, 100 µl of the extract samples diluted at different concentrations was mixed with 3900 µl of DPPH solution (0.1mM in methanol) and vortexed in tube. The absorbance values of the each sample were recorded at 517 nm after 30 min incubation in dark place at room conditions by using a UV-VIS spectrophotometer (Schidamzu, Japan). The remaining DPPH concentration in the reaction medium was calculated from the following calibration curve equation (Eq.2) determined by linear regression:

$$DPPH_{REM}(\text{mg/ml}) = [37.92 \times Abs_{517} + 0.27] \times 1000 \quad (R^2 = 0.997) (\text{Eq.2})$$

The percentage of the remaining DPPH was also calculated by dividing the DPPH level at the end of the reaction to the DPPH level at the beginning as following equation:

$$\% \text{ DPPH}_{\text{REM}} = \frac{[\text{DPPH}]_t}{[\text{DPPH}]_{t=0}} \quad (\text{Eq.3})$$

The percentage of remaining DPPH against the standard concentration was then plotted to calculate the amount of antioxidant necessary to decrease the initial concentration of radical substance by 50%.

### Measurement of tincture yield

The tincture yield of the samples was determined by measuring the liquid volume after filtration of the sample by using a measuring cylinder and the results were given as ml.

### Data analysis, modeling and optimization

In this study, a 3-factor-3-level Box-Behnken experimental design (Box and Behnken, 1960) with three replicates at the center point was used to develop predictive models for studied parameters. The factors (processing variables), levels and experimental design in terms of coded and uncoded are presented in Table 1.

$$Y - \varepsilon = \beta_0 + \sum_{i=1}^N \beta_i x_i + \sum_{i=1}^N \beta_{ii} x_i^2 + \sum_{\substack{i=1 \\ i < j}} \sum_{j=i+1}^N \beta_{ij} x_i x_j ,$$

(Eq.4)

where  $Y$  is the corresponding predicted response value,  $\beta_0$  is the intercept term,  $\beta_i$  is the linear term,  $\beta_{ii}$  is the quadratic term,  $\beta_{ij}$  is the interaction term, and  $X_i$  and  $X_j$  are the coded levels of the independent variables. The regression coefficients of linear, quadratic and interaction terms were determined by using Design Expert package software for each output parameter.

In this context, ethanol level (6,10 and 30 ml for 1 g plant), ethanol concentration(25, 50 and 75%), time (1, 8 and 15 days) were selected as the processing variables. Design-Expert® Software Version 7.0 (Stat-Ease Inc., Minneapolis, USA) was used for the computational work including designation of experimental points, randomization, analysis of variance, fitting of the second-order polynomial models and graphical representations as well as optimization was performed.

Optimization procedure was performed by using desirability function of the Design-Expert® Software Version 7.0 (Stat-Ease Inc., Minneapolis, USA) and maximum and minimum values and their conditions were calculated.

## Results and Discussion

### *O. onites* plant properties

Table 2 shows the bioactivity values of all plant samples prepared according to the experimental design in Table 1.

**Table 2. Bioactivity of *O. onites* plant and tincture samples as affected by processing variables**

Runs	O. onitesplant		O. onitestincture		
	PTPC	TTPC	EC <sub>50</sub>	ARA	TY
1	45.25	4524.9	42.63	32.3	16
2	31.86	3186.1	80.66	16.4	13
3	45.29	4528.9	53.19	33.0	15.5
4	46.69	1556.4	51.44	10.6	22
5	38.71	3870.8	64.69	27.7	15.5
6	20.07	3345.5	84.99	28.8	15.5
7	34.05	5674.9	73.64	25.0	11.7
8	39.53	3953.2	57.63	22.6	15.9
9	42.12	7020.8	44.88	43.5	10
10	50.30	1737.2	44.97	13.3	21
11	34.61	5767.8	68.53	44.7	9
12	40.51	4050.5	48.72	31.0	14.5
13	46.31	1543.8	55.54	7.4	19.6
14	28.92	964.1	89.56	3.3	20

15	20.97	2096.9	83.22	22.0	13.5
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PTPC: Plant Total Phenolic Content (mg GAE/g sample), EC50: Efficient concentration, TY: Tincture yield (ml),  
 TTPC: Tincture Total Phenolic Content (mg GAE/L), ARA: Antiradical activity (% inhibition for 1/40 diluted samples)

Total phenolic content of samples showed a significant variation depending on the processing variables and it was observed that the lowest total phenolic content of *O.onites* plant was determined for the sample prepared with the lowest solvent level (6 ml) and 25% ethanol concentration for 1 g plant and stored eight days in dark conditions. The highest total phenolic content was determined for the sample prepared with the highest solvent level (30 ml) and 50% ethanol concentration for 1 g plant and stored 15 days in dark conditions (run 10 in Table 1). Ozkan et al. (2009) investigated the harvesting time on total phenolic content of the *O. onites* and reported that they had 106.1-149.4 mg GAE/g extract and they stated that the total phenolic level was significantly affected by the harvesting time. Lucina et al. (2013) also studied the effect of different solvent type on some bioactive parameters of *Origanum vulgare* and reported that the highest total phenolic content was determined for the only water and ethanol to be 235 and 135 mgGAE/g extract, respectively. Similar findings were reported by Teixeira et al. (2013) for the *O. vulgare* and they reported total phenolic content of cold water extract to be 17.8 mg GAE/g sample while the ethanolic extract of the sample was 13.5 mg GAE/g sample. The total phenolic level of the samples was affected by the species of oregano and solvent type significantly. Total phenolic content of *O.onites* plant was affected by the processing variables and especially liquid/solid level and ethanol concentration showed a significant effect on the phenolic level to be extracted from the plantleaves. It was concluded that the extractable phenolics increased by the increase of liquid/solid level ( $P<0.05$ , Table 3). Fig.1 shows the change of plant total phenolic content (PTPC) according to the change of processing variables and as it could be seen from the 3D surface plots in Fig.1, PTPC increased by the increase of solvent level which added to the constant plant amount (1 g). Ethanol concentration increase provided an increment in the phenolic level of plant samples to the constant extent significantly ( $P<0.05$ ) and more increase of ethanol in the extraction solvent caused a decrease of the extractable phenolic content. Storage period increase also increased the PTPC but this increase was found to be insignificant ( $P>0.05$ , Table 3) showing that the phenolics could be extracted easily in short time by addition of suitable ethanol concentration which was obtained from the optimization results.

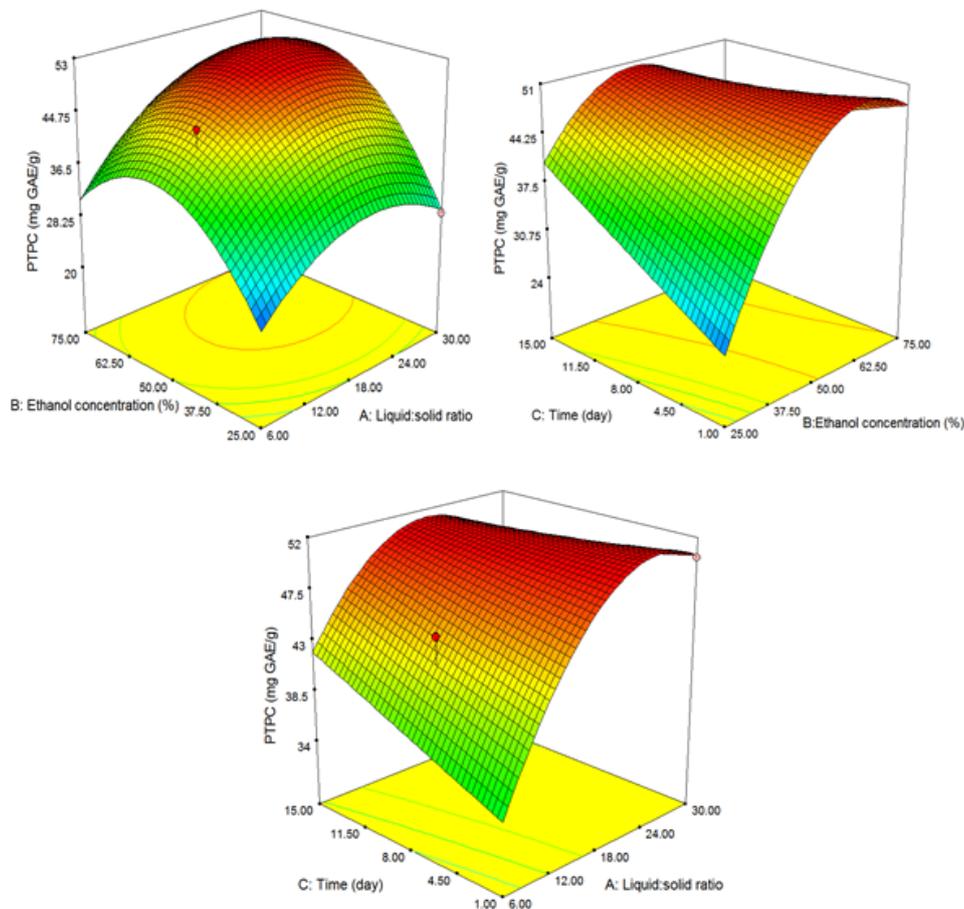


Fig. 1 Effect of processing variables on plant total phenolic content (PTPC) of *O. onites*

**Table 3. Significance of the regression models (F values) and the effects of processing variables on bioactive properties of *O. onites* plant extract and tincture**

	Df	Parameters <sup>§</sup>				
		PTPC	EC <sub>50</sub>	TY	TTPC	ARA
Model	9	0.007 <sup>a</sup>	0.028 <sup>a</sup>	0.007 <sup>a</sup>	0.010 <sup>a</sup>	0.001 <sup>a</sup>
<i>Linear</i>						
<i>A</i> <sup>φ</sup>	1	0.014 <sup>a</sup>	0.031 <sup>a</sup>	0.001 <sup>a</sup>	0.002 <sup>a</sup>	0.001 <sup>a</sup>
<i>B</i>	1	0.005 <sup>a</sup>	0.409	0.706	0.116	0.138
<i>C</i>	1	0.243	0.503	0.808	0.368	0.033 <sup>a</sup>
<i>Cross product</i>						
<i>AB</i>	1	0.186	0.101	0.130	0.499	0.016
<i>AC</i>	1	0.105	0.110	0.448	0.370	0.251
<i>BC</i>	1	0.012 <sup>a</sup>	0.043 <sup>a</sup>	0.135	0.121	0.071 <sup>b</sup>
<i>Quadratic</i>						
<i>A</i> <sup>2</sup>	1	0.097	0.082 <sup>c</sup>	0.066 <sup>b</sup>	0.056 <sup>b</sup>	0.215
<i>B</i> <sup>2</sup>	1	0.001 <sup>a</sup>	0.003 <sup>a</sup>	0.330	0.026 <sup>a</sup>	0.009 <sup>a</sup>
<i>C</i> <sup>2</sup>	1	0.998	0.824	0.071 <sup>b</sup>	0.956	0.807
Residual	5					
Lack of fit	3	0.396	0.269	0.180	0.096	0.059
Pure error	2					
Cor error	14					
<i>R</i> <sup>2</sup>		0.955	0.919	0.955	0.947	0.986

<sup>φ</sup>A: Liquid/solid (ml), B: Ethanol concentration (%), C: Time (day)

<sup>§</sup>PTPC: Plant Total Phenolic Content (mg GAE/g sample), EC<sub>50</sub>: Efficient concentration, TY: Tincture yield (ml), TTPC: Tincture Total Phenolic Content (mg GAE/L), ARA: Antiradical activity (% inhibition for 1/40 diluted samples), <sup>a</sup> *p*<0.01, <sup>b</sup> *p*<0.05, <sup>c</sup> *p*<0.1

### ***O. onites* tincture properties**

Tincture is the hydroethanolic extract of the plant samples and produced with the processing of constant level of plant added with ethanol:distilled water for a while like 15 days in dark conditions. Bioactivity of tincture samples such as total phenolic content, efficiency concentration and antiradical activity of diluted at constant level for the determination of optimized values for the tincture production were given in Table 2. Tincture phenolics varied within the wide range depending on the processing variables. The highest tincture total phenolic content was determined to be 7020.8 mg GAE/L for the sample with the addition of 6 ml 50% of solvent for 1 g plant and stored for 15 days while the lowest value (964.1 mg GAE/L) for the tincture samples was for the sample prepared with the addition of 30 ml of 25% ethanol and stored for eight days (run 14 in Table 1). Total phenolic content of tincture sample was significantly affected by the solvent level in the mixture (*P*<0.01, Table 3). Fig.2 shows the change of total phenolic level of tincture samples depending on the plant concentration used for the preparation of tinctures. Increase of liquid level used for the extraction decreased the phenolic concentration of tincture samples significantly (*P*<0.05) because of the dilution in the active substances levels. It could be concluded that the amount of plant used for the preparation of tincture had a significant effect on the bioactivity of tinctures and so, to produce highly concentrated sample in terms of phenolic substance, the ratio between the liquid (solvent) and solid (plant) should be considered and minimized. As could be seen in the Fig.2, phenolic content of tincture samples was recorded to be low when the water or ethanol level was set to be high in the mixture, but the mixtures containing both of liquids at equal level showed higher phenolic level.

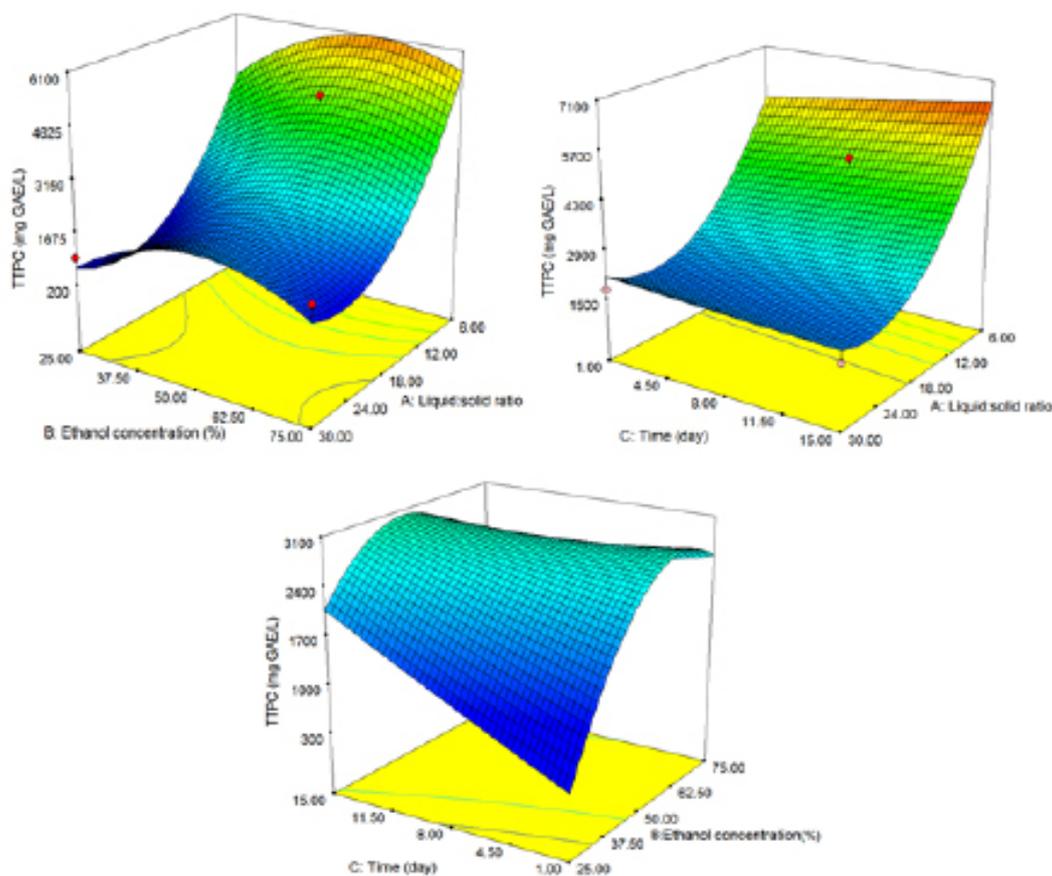


Fig. 2 Effect of processing variables on tincture total phenolic content (TTPC) of *O. onites*

Efficient concentration ( $EC_{50}$ ) of tincture samples was given in Table 2. As is known, efficient concentration level is desired to be low and its decrease means that the bioactivity increase for the extract samples. The highest  $EC_{50}$  was determined for the tincture samples prepared with the addition of 30 ml of 25% ethanol and stored for eight days (run 14 in Table 1) which was the sample having the lowest total phenolic content. The lowest  $EC_{50}$  was calculated for the sample having the highest total phenolic content level as is seen in Table 2.  $EC_{50}$  values were affected by the liquid/solid ratio significantly ( $P < 0.05$ , Table 3). Increase of liquid level compared to solid one increased the  $EC_{50}$  value showing that the active substance dilution decreased the bioactivity of tincture samples and caused the increase for the  $EC_{50}$  value which means that the level of extract amount should be increased to scavenge of 50% of antiradical substances.  $EC_{50}$  values were also affected by the ethanol concentration and time and the lowest  $EC_{50}$  value was calculated for the tincture samples having the highest total phenolic substances (Fig. 3). A negative correlation was determined between total phenolic substances and  $EC_{50}$  value of the tincture samples. Increase of the total phenolic substances caused to the decrease of  $EC_{50}$  value.

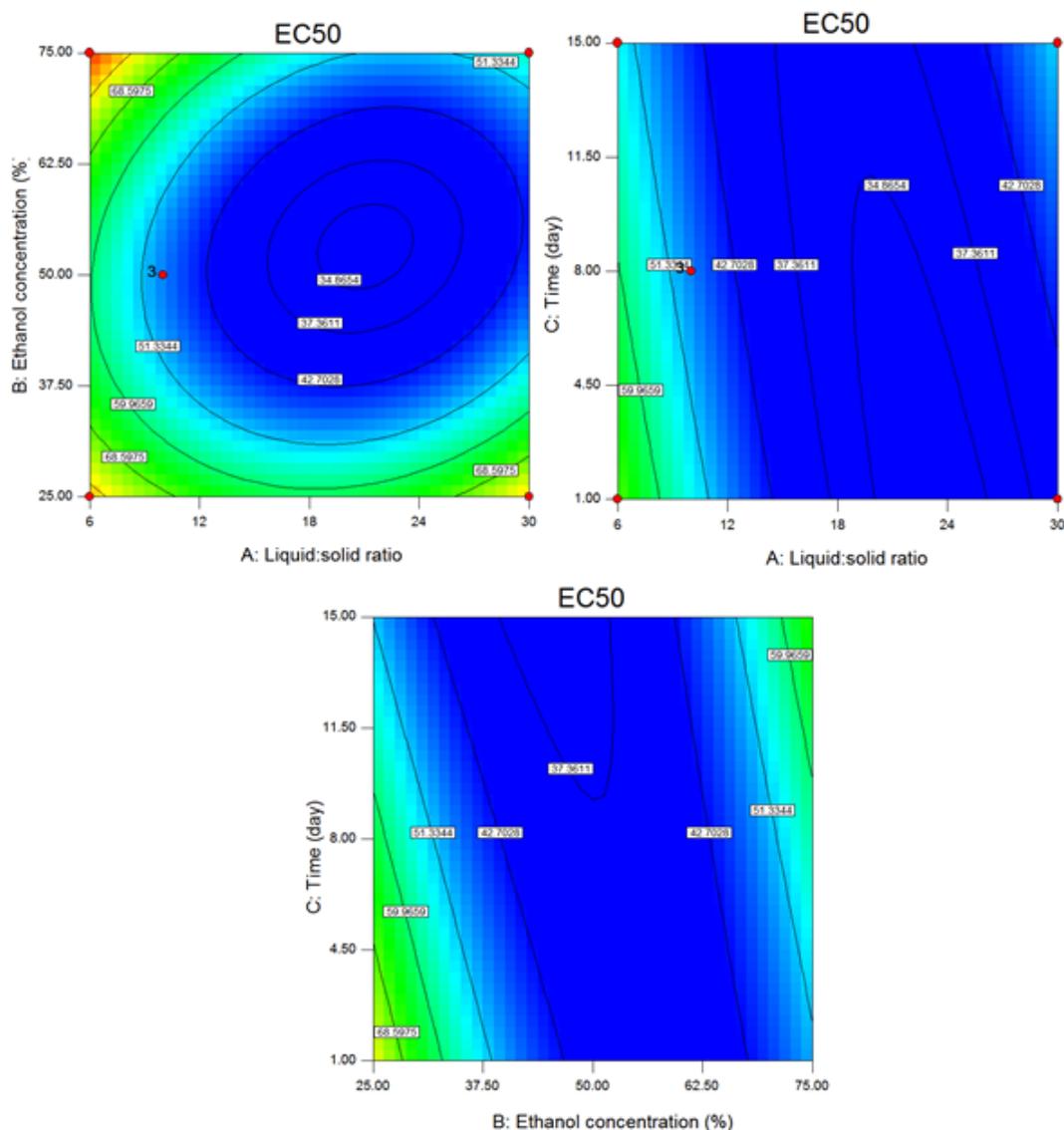


Fig. 3 Effect of processing variables on EC50 values of *O. onites*

Antiradical activity as percentage inhibition of all tincture samples diluted as 1/40 to compare was given in Table 2. The highest antiradical activity was calculated as 44.7% while the lowest value was 3.3%. The lowest antiradical activity value was determined for the tincture sample having the lowest total phenolic content level (run 14 in Table 1). Increase the total phenolic content of the tincture samples increased the antiradical activity and a positive and significant correlation between them was determined ( $r=0.899$ ). The liquid/solid ratio and timeshowed a significant effect on the antiradical activity of tincture samples and as is shown in Fig.4, increase in the liquid level decreased the antiradical activity of the samples. Increase or decrease after 50% ethanol concentration caused a decrement in the antiradical activity of tincture. It was reported that the antiradical activity of *O.onites* comes from its active substances isolated by Kikuzaki and Nakatani (1989) namely as protocatechuic acid, caffeic acid, rosmarinic acid, a phenyl glycoside and 2-caffeoyloxy-3-[2-(4-hydroxybenzyl)-4,5-dihydroxyphenyl] propionic acid.

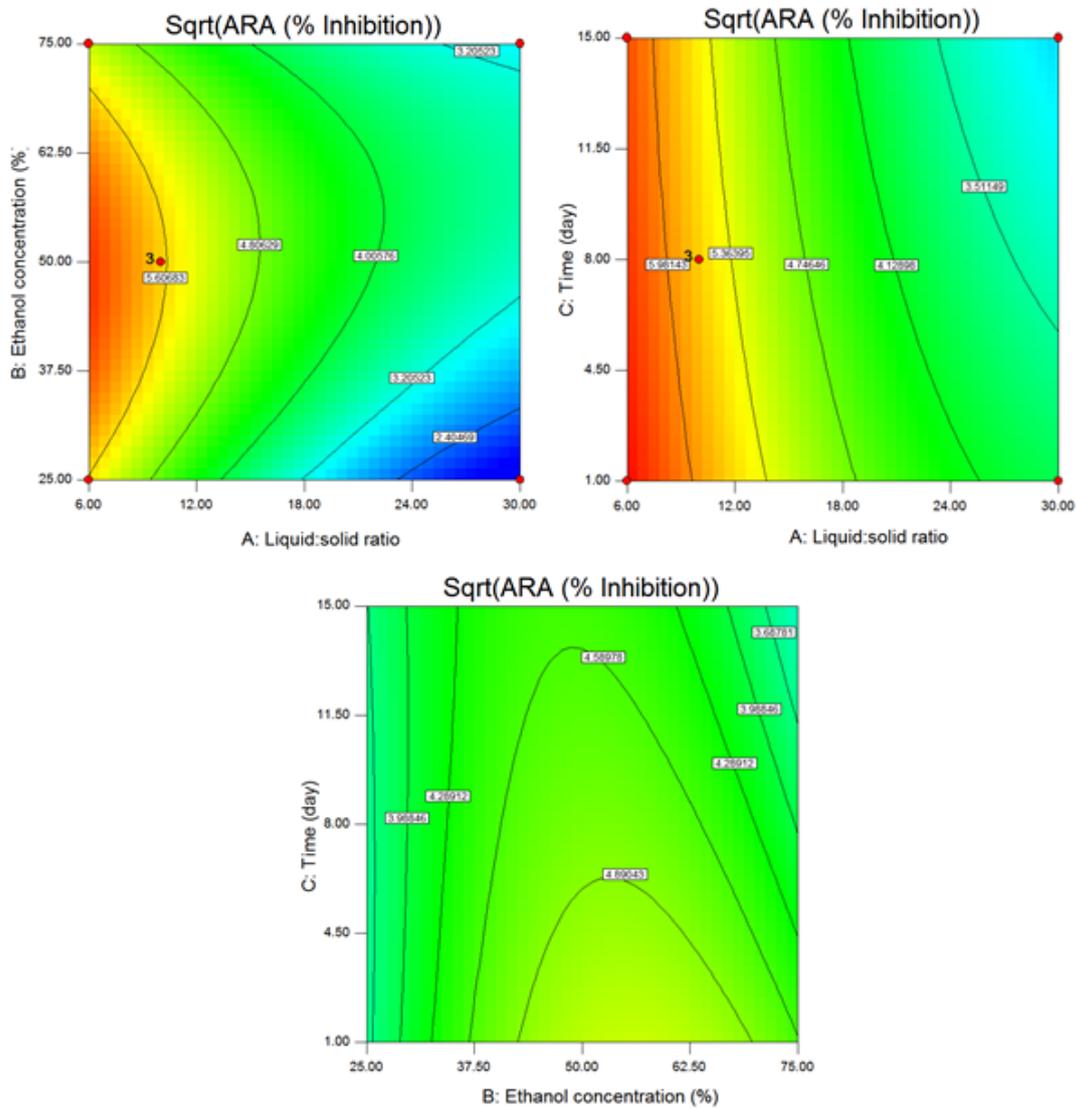


Fig. 4 Effect of processing variables on antiradical activity (ARA) of *O. onites*

The extract yield which is important for the economic reasons in the tincture manufacturing process was also considered. It was measured after filtration of the plant samples for each sample and given as volume in Table 2. As is seen in the table, the lowest tincture yield (9 ml) was calculated for the sample prepared by the addition of 6 ml ethanol to the sample. The highest one was 30 ml ethanol. As expected, tincture yield was determined to be directly related with the addition of ethanol level (Table 3), linear effect of concentration was determined to be significant statistically ( $P < 0.05$ ) while the other two factors (ethanol concentration and time) did not show a significant effect on the tincture yield values of the samples ( $P > 0.05$ ). Fig. 5 shows the change of tincture yield values of samples depending on the processing factors and it is clear from the figure that the tincture yield increased with the increase of liquid level in the samples.

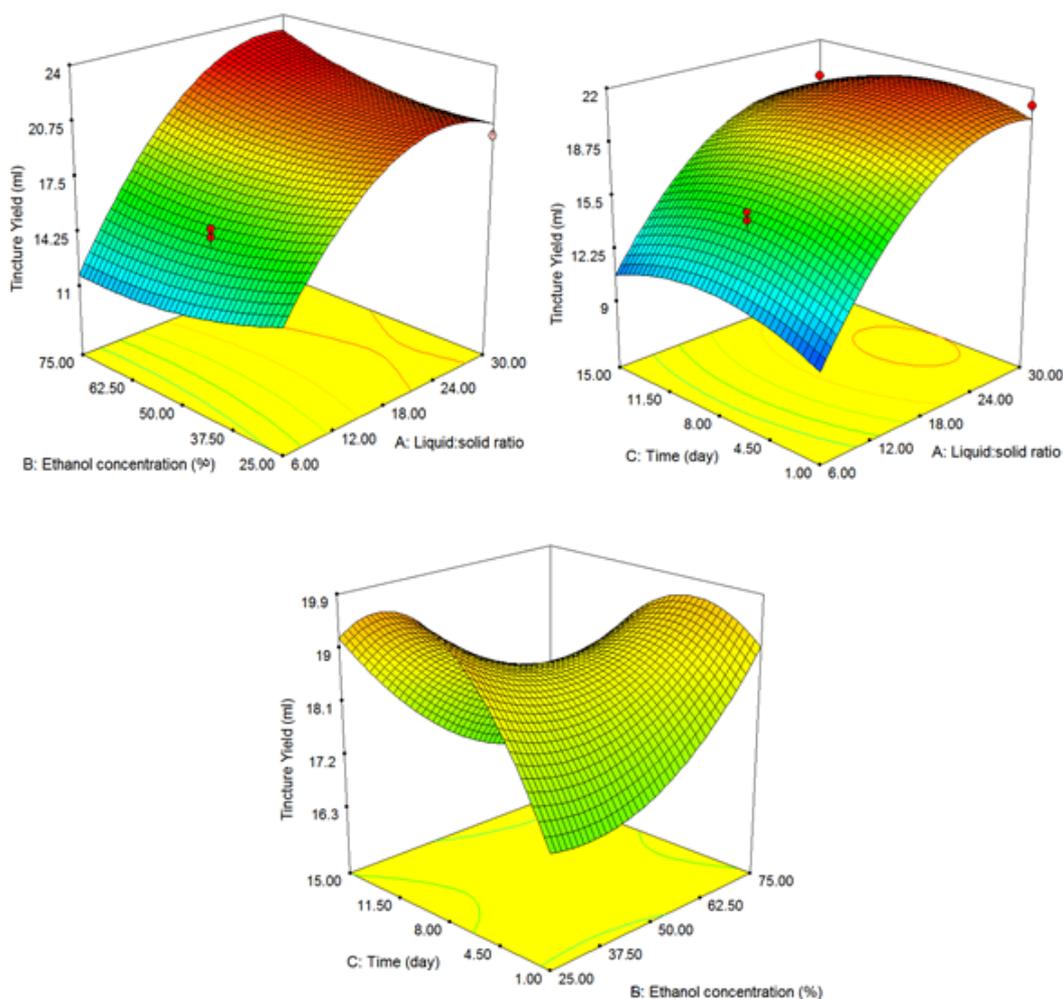


Fig. 5 Effect of processing variables on tincture yield values of *O. onites*

### Optimization for the parameters

Optimization of processing variables levels for the studied parameters was performed by using desirability function and the results were given in Table 4. As is seen in the table, maximum plant total phenolic content (56.11 mg GAE/g sample) would be at the level of 30 ml liquid level, 60% ethanol and 1.14 day while the minimum value (24.33 mg GAE/L) was for the sample prepared by the addition of 6 ml ethanol (25%) for 5 day storage. It could be said that to extract the maximum level of phenolic substance from the cell of the plants, the ethanol level should be high compared to plant level which means that the increment of the liquid level in the mixture will provide the better extraction of phenolic substance. For the tincture samples, the highest phenolic content would be at 6 ml liquid per one gram plant and 50% ethanol concentration stored for 15 days while the lowest phenolic content would be at 30 ml liquid and 73% ethanol concentration. It could be said that the optimized processing parameters for the best tincture formulation having high bioactivity were 50% ethanol and 1:5 liquid/solid ratio and 15 days storage. Antiradical activity of samples was determined to similar to the phenolic results and the highest antiradical activity was for the samples prepared by 50% ethanol and 1:5 liquid/solid ratio. In addition to that, the highest tincture yield would be at 30 ml liquid while the lowest yield was for 6 ml liquid.

Table 4. Response optimization for calculation of maximum (Max) and minimum (Min) response values of bioactive parameters of *O. onites* plant extract and tincture

Parameters	A	B	C	Max	Min	Desirability
PTPC	30	60	1.14	56.11		1.000
	6	25	5		24.33	1.000
EC <sub>50</sub>	5.75	25	1.7	95.49		1.000
	30	61.5	2.3		41.48	1.000
TY	30	67	7.75	22.12		1.000
	6	72.5	15		8.94	1.000
TTPC	6	50.0	15	6733		0.953
	30	73	14		945	1.000
ARA	6	50.5	1	41.6		0.927
	30	72.5	15		3.24	1.000

<sup>o</sup>A: Liquid/solid (ml), B: Ethanol concentration (%), C: Time (day)

<sup>§</sup> PTPC: Plant Total Phenolic Content (mg GAE/g sample), EC<sub>50</sub>: Efficient concentration, TY: Tincture yield (ml), TTPC: Tincture Total Phenolic Content (mg GAE/L), ARA: Antiradical activity (% inhibition for 1/40 diluted samples)

### Conclusion

Effects of three factors namely ethanol concentration, liquid/solid ratio and storage time on the bioactivity of final tincture sample were determined and it was concluded that these factors affected the bioactive parameters of samples significantly except storage time. *O. onites* tincture was determined to be effective in terms of the total phenolic content and antiradical activity. Increase of liquid level in the samples resulted in an increase of total phenolic content of plant materials and increase or decrease of ethanol concentration after 50% caused a decrement in the phenolic level of the tincture samples because of the solubility of the phenolic decrement. Tincture yield changed significantly depending on the liquid/solid ratio. It was concluded that the best extraction conditions for the plant sample would be at 60% ethanol concentration and 30 ml ethanol level. These parameters were determined to be 6 ml (1:5 solid/liquid) ethanol level, 50% ethanol concentration and 15 days storage for the tincture production. These results would be useful for the producers to manufacture more effective tincture samples having concentrated phenolic substance.

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