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Optimization of Extraction of Bioactive Compounds from Medicinal Herbs Using Response Surface Methodology

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Abstract. Antioxidants are bioactive components used to relieve the detrimental effects of oxidative stress caused by the presence of free radicals. These valuable compounds are naturally available in medicinal plants. The present study aims to investigate the influence of two independent variables, namely temperature (oC) and time (hours) on the extraction yield of phenolic compounds, flavonoids and antioxidant activity from garlic, oregano, and parsley. The optimized conditions for the extraction of bioactive components from medicinal plants were determined using two-factor central composite design (CCD) combined with response surface methodology (RSM). The order of experiments was completely randomized using central composite design with five (5) centre points. All experimental data was analyzed using "Design Expert" software (Design- Expert 7.0.0 Trial, State-Ease Inc., Minneapolis MN, USA). A second-order polynomial model proposed for predicting the responses. Major factors affecting the yield of bioactive components from the extracts of garlic, oregano, and parsley were determined using one-way analysis of variance (ANOVA). Results were analyzed using a significant level of 95%. The antioxidant activity decreases from: oregano > parsley > garlic. ANOVA analysis indicated that all experimental data were in close agreement with that of the predicted data hence indicating the reliability of the experimental data and the suitability of the proposed quadratic model. The optimum conditions proposed by ANOVA were 47.1oC, 6 hours for extraction using maceration method.

Keywords: medical plants, antioxidants, total antioxidant activity, optimization, response surface methodology (RSM)

1. Introduction

For centuries, medicinal plants have been proven to exhibit potential medicinal properties which include anti-fungal, anti-inflammatory, antioxidant, anti-carcinogenic, anti-diabetic, and anti-depressant [1]. Contemporary, science has acknowledged the active actions of medicinal plants; hence prompting a significant increase in the study on medicinal plants as a remedy for various forms of diseases and disorders [2], [3]. The synthetic drugs lead to various forms of side effects and due to their high toxicity level, the demand is on the rise for traditional medication for primary health care [4]. The medicinal plants chosen for the present study, which included garlic, oregano, and parsley, belong to the families of Allium, Lamiacea, and Apiaceae.

Garlic, oregano, and parsley, just like any other medicinal plants, are rich in antioxidant constituents such as phenolics and flavonoids [6], [7]. Antioxidants are substances use to relief disorders related to oxidative stress caused by large amounts of free radicals. Free radicals are highly reactive substances which are produced naturally in the human body as a by-product of cellular processes or as a result of unhealthy lifestyle [7]. Antioxidants function to interact with free radicals hence terminating the chain reaction before severe damages occur on vital organs [5]. On a separate note, there are reports indicating an increasing demand for natural antioxidants in the food industry as an alternative to synthetic preservatives [2], [5], [9].

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The effectiveness of anti-oxidative properties varies according to the chemical characteristics as well as its physical location of the plant [5].

Phenolic compounds are the most common phytochemical substances that are found in all parts of a plant, which includes bulb, leaves, flowers, and stems[8], [9]. These substances can be divided into two main categories namely; phenols and flavonoids [9]. The main active constituents of garlic is allicin [10], oregano are carvacrol and thymol [11], and parsley are apiin and malonyl-apiin [12].

The present study therefore was conducted to optimize the process parameters (temperature and time) for the extraction of bioactive compounds from garlic, oregano, and parsley using maceration method with the aid of response surface methodology. The effects of these parameters on the total anti-oxidative properties were evaluated. In this study, ethanol was chosen as the extraction solvent because it is a strong polar solvent [13], it has low toxicity level [14] and is commonly used in conventional extraction [15]-[19].

The extracts were analysed for their phenolic and flavonoid content using Folin-Ciocalteu assay and aluminium chloride colorimetric assay. The antioxidant activity was measured using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) scavenging assay. All in all, this paper demonstrates a different perspective on antioxidants in hopes to stimulate the interest of future researchers to indulge further into this field of study.

2. Methodology

2.1. Chemicals

Ethanol 95% (Denatured) (ChemSoln). Gallic acid (MERCK), Sodium carbonate anhydrous, analytical grade (Fisher Scientific), Sodium nitrite (R&M Chemicals), Aluminium chloride (Systerm), Folin & Ciocateu's Phenol Reagent, [AR Grade] (ChemSoln), Quercetin (Sigma-Aldrich), and 2,2-Diphenyl-1-Picrylhdrazyl (DPPH) (Riendemann Schidt), Sodium hydroxide, [AR Grade] (Riendemann Schidt).

2.2. Sample Preparation

For this study, fresh garlic, oregano and parsley leaves were purchased from Giant Hypermarket, Subang Jaya, Selangor. Prior to extraction, all medicinal plants were oven-dried (Memmert model UN75) at 50^{o}C until constant weight (72 hours). The dried plant materials were then grind using a blender (PENTEC model TAC-383E), sieved and stored in a cool dry place till analyses.

2.3. Maceration

Dried plants weighing about 5 g was placed into a 250ml conical flask along with the ethanol solvent. The mouth of the flask was sealed with aluminum foil to prevent the evaporation of solvent. The flask were placed into an orbital incubator shaker (LM-400D) and left for the extraction process to take place according to the experimental design proposed by central composite design (CCD). The solid-to-solvent ratio of 1:30g/ml was selected and remained constant for all the experiments. This parameter was fixed based on the findings reported by Bancha *et al*. [14], which states that the relationship between the extractions yields of phenolic compound to solid-to-solvent ratios are inversely proportional. The maximum yield of phenolic compound reported was by using 1:30g/ml solid-to-solvent ratio. Extracts were filtered using Whatman No.1 filter paper. All analyses were performed on the same day of extraction.

2.4. Determination of Total Phenolic Content

Total phenolic content of each medicinal plant were determined by spectrometry using Folin-Ciocalteu reagent assay suggested by Kamtekar et al. [17] with some modifications. A calibration curve was developed at gallic acid concentrations of (10, 20, 40, 60, 80, 100 µg/ml). Gallic acid was used as a standard for the calibration curve. For the determination of phenolic content, 0.5ml of Folin Ciocalteu's reagent was added into 5ml of distilled water, shaken, and left to rest for 5 minutes. Then 1.5ml of 20% (w/v) sodium carbonate was added into the prepared solution and the volume was made up to 10ml by adding distilled water. The mixture was left to incubate in a cool dry place for 2 hours. An intense blue colour solution was formed. Sample absorbance was measured at 750 nm against a blank using UV-visible spectrophotometer (Model Genesys 10S) instrument. All extracts were performed in triplicates. The concentration of total phenolic

content was calculated using a calibration plot (y = 0.0141x −0.0094, R2 = 0.9996) and expressed as gallic acid equivalent (GAE) by using the following equation:

$$\text{Total Phenolic content (mg GAE/g)} = \frac{P \times V \times D}{W \times (100\text{-}M) \times 10} \quad (1)$$

Where:

P = Total phenolic content calculated from calibration curve (mg GAE/l)

V = Volume of extraction solvent (ml)

D = Dilution factor

W = Fresh weight of sample (g)

M = Moisture content of sample (%)

2.5. Determination of Total Flavonoid Content

Total flavonoid content was measured spectrometrically using aluminum chloride colorimetric assay proposed by Kamtekar *et al*. [17] with slight modifications. A calibration curve was developed at quercetin concentrations of (100, 200, 400, 600, 800, 1000 µg/ml). Quercetin was used as a standard for the calibration curve. For flavonoid determination, 0.3ml of 5% (w/v) of sodium nitrite solution was added into 4ml of distilled water. The solution was left to rest for 5 minutes. Then, 0.3ml of 10% (w/v) of aluminum chloride was added and the mixture was set to rest for a minute. 2ml of 1M sodium hydroxide solution was added into the mixture. An orange solution was form and the mixture is made to 10ml by adding distilled water. Sample absorbance was measured at 510 nm against a blank using UV-visible spectrophotometer (Model Genesys 10S) instrument. All extracts were performed in triplicates. The concentration of total flavonoid content was calculated from the calibration plot ($y = 0.0005x - 0.0139$, $R^2 = 0.995$ and expressed as quercetin equivalent (QE) by using the following equation:

$$\text{Total flavonoid content (mg QE/g)} = \frac{F \times V \times D}{W \times (100\text{-}M) \times 10} \quad (2)$$

Where:

F = Total flavonoid content calculated from calibration

curve (mg QE/l)

V = Volume of extraction solvent (ml)

D = Dilution factor

W = Fresh weight of sample (g)

M = Moisture content of sample (%)

2.6. Determination of DPPH Radical Scavenging Activity

The antioxidant activity of each extract was measured using the method proposed by Himaja et al [18] with slight modifications. (0.1mM) DPPH solution was prepared by dissolving 1.9mg of DPPH into 100ml of ethanol solution. The mixture was prepared in an amber bottle wrapped with aluminum foil to minimize light exposure. The DPPH solution was left to react for 30 minutes before used for analysis. Briefly, 1ml of extract was added into 3ml of crude extract and the sample was observed to change from purple to yellowish. The sample was prepared and left in a dark room for 30 minutes before the sample absorbance value at 517nm was measured against a blank using UV-visible spectrophotometer (Genesys 10S) instrument. The radical scavenging activity was measure using the following formula:

$$\text{DPPH scavenging activity (\%)} = \frac{Ao - As}{Ao} \times 100 \quad (3)$$

Where:

Ao = Absorbance reading of the control

As = Absorbance reading of the sample.

3. Results and Discussion

3.1. Model Analysis

The effects of extraction parameters such as temperature (30°C – 50°C) and time (4 hours – 6 hours) on polyphenolic compounds (phenolics and flavonoids), as well as the antioxidant potential from garlic, oregano, and parsley were investigated using response surface methodology (RSM). All experimental runs were conducted according to the central composite design (CCD) as listed in Table 1. All results were statistically analysed by analysis of variance (ANOVA). Results are as listed in Table 2. Statistical significance was based on the confidence level of 95%. Hence, ($p<0.05$) indicates that the model terms are significant on the response variable. ANOVA analysis suggested quadratic models to represent all experimental data. On the whole, the coefficients of determination are reliable, with R^2 values generally above 80%. Based on previous studies, R^2 value less than 80% indicates that the model does not very well explain the relationship between the experimental variables [19]. On contrary, an R^2 value above 80% indicates that the model closely fit the regression line. For clear demonstration of the effects of extraction parameters on the extract of antioxidant compounds and potential, response surface plot was generated for all responses.

Table 1: Experimental values for the extraction yield of bioactive compounds from garlic, oregano and parsley.

Run	Extraction temperature, °C	Extraction time, hours	Garlic		Oregano		Parsley	
			TPC (mg GAE/g)	TFC (mg QE/g)	TPC (mg GAE/g)	TFC (mg QE/g)	TPC (mg GAE/g)	TFC (mg QE/g)
12	40	4	0.140 ± 0.001	0.612 ± 0.010	17.755 ± 0.009	25.222 ± 0.226	4.079 ± 0.009	13.426 ± 0.000
10	47.1	4	0.245 ± 0.002	0.931 ± 0.026	19.615 ± 0.049	32.361 ± 0.237	6.270 ± 0.165	12.835 ± 0.000
11	32.9	4	0.094 ± 0.001	0.925 ± 0.029	17.711 ± 0.024	27.223 ± 3.002	4.376 ± 0.006	10.923 ± 0.049
1	30	5	0.161 ± 0.002	1.216 ± 0.019	10.770 ± 0.033	22.235 ± 0.237	3.311 ± 0.008	9.038 ± 0.000
2	40	5	0.149 ± 0.001	2.784 ± 0.019	15.499 ± 0.018	23.967 ± 0.052	4.003 ± 0.005	11.823 ± 0.000
4	40	5	0.154 ± 0.001	2.621 ± 0.017	15.018 ± 0.097	24.266 ± 0.288	4.219 ± 0.002	12.639 ± 0.049
6	50	5	0.151 ± 0.003	1.278 ± 0.017	26.523 ±3.448	45.804 ± 0.091	8.321 ± 0.005	11.795 ± 0.049
8	40	5	0.143 ± 0.001	0.533 ± 0.010	15.230 ± 5.981	26.476 ± 0.315	4.994 ± 0.002	12.470 ± 0.049
7	40	5	0.187 ± 0.001	0.959 ± 0.061	14.753 ± 0.009	31.883 ± 0.103	4.121 ± 0.005	15.142 ± 0.049
9	40	5	0.145 ± 0.001	1.284 ± 0.042	15.224 ± 0.016	28.986 ± 0.274	4.325 ± 0.015	13.032 ± 0.049
3	32.9	6	0.135 ± 0.001	0.948 ± 0.019	15.722 ± 0.024	26.476 ± 0.226	4.326 ± 0.073	10.219 ± 0.000
5	47.1	6	0.243 ± 0.001	0.987 ± 0.048	27.057 ± 0.018	46.551 ± 0.288	8.501 ± 0.089	15.400 ± 0.049
13	40	6	0.136 ± 0.001	1.121 ± 0.001	17.824 ± 0.057	21.368 ± 0.186	4.467 ± 0.003	13.257 ± 0.000

3.2. Model Fitting

Response surface methodology was applied to optimize the extraction of bioactive components from garlic, oregano, and parsley. The summarized ANOVA results from each medicinal plant are listed in Table 2. Using the experimental data, the coefficients of the quadratic equation were calculated. The predicted responses as a function of independent variables are expressed using the second-order polynomial equation. The general mathematical expression of the equation is expressed as follow:

$$Y = \beta_0 + \sum_{i=1}^{2} \beta_i X_i + \sum_{i=1}^{2} \beta_{ii} {X_i}^2 + \sum \sum_{i<j=1}^{2} \beta_{ij} X_i X_j \quad (4)$$

Table 2: Summarized variance of analysis (ANOVA) for total phenolic content, total flavonoid content, and antioxidant activity with confidence level of 95%.

	Total Phenolic Content					
	Garlic		Oregano		Parsley	
R^2/ Adj R^2	0.9281/0.8768		0.9187/0.8606		0.9255/0.8724	
	COD	P value prob > f	COD	P value prob > f	COD	P value prob > f
Model		0.0007		0.0011		0.0008
Intercept	0.14		15.14		4.33	
A: Temp (^{o}C)	0.045	<0.0001	4.44	0.0002	1.64	<0.0001
B: Time (h)	8.36E-03	0.1559	0.69	0.2907	0.34	0.1449
AB	0.016	0.0708	2.36	0.0287	0.57	0.0938
A^2	0.017	0.0206	2.2	0.0117	0.95	0.0038
B^2	0.011	0.1041	1.77	0.0295	0.18	0.456
	Total Flavonoid Content					
	Garlic		Oregano		Parsley	
R^2/ Adj R^2	0.8842/0.8015		0.8252/0.7004		0.7716/0.6085	
	COD	P value prob > f	COD	P value prob > f	COD	P value prob > f
Model		0.0036		0.0139		0.033
Intercept	0.98		27.12		13.02	
A: Temp (^{o}C)	0.63	0.0006	7.32	0.0023	1.37	0.01
B: Time (h)	0.21	0.0945	1.00	0.5447	0.2	0.6224
AB	0.38	0.0413	3.73	0.1364	0.82	0.1853
A^2	0.35	0.0183	4.58	0.0298	-1.19	0.0259
B^2	0.073	0.5496	-0.79	0.6545	0.28	0.5326
	DPPH Scavenging Activity					
	Garlic		Oregano		Parsley	
R^2/ Adj R^2	0.8687/0.775		0.8683/0.7742		0.8627/0.7646	
	COD	P value prob > f	COD	P value prob > f	COD	P value prob > f
Model		0.0054		0.0055		0.0063
Intercept	5.74		93.59		19.27	
A: Temp (^{o}C)	1.38	0.0006	0.22	0.0119	4.23	0.001
B: Time (h)	0.32	0.2180	-8.03E-03	0.9035	1.93	0.0143
AB	0.42	0.2425	0.081	0.3989	0.69	0.5475
A^2	0.68	0.0299	0.03	0.6787	2.29	0.0286
B^2	0.26	0.3344	0.40	0.0007	0.044	0.9593
Note: COD coefficient of determination						

Where Y is the response of extraction yield of bioactive components or antioxidant activity, β_0, β_i, β_{ii}, β_{ij} are constant coefficients of intercept, linear, quadratic, and interaction terms, respectively while X_i and X_j are the independent variable; temperature and time. The developed mathematical expressions for all experiments are listed in Table 3 with all insignificant terms being eliminated from equation.

3.3. Effect of Extraction Temperature and Time on Total Phenolic Content

The yield of phenolic extract as a function of extraction temperature and time were graphically represented in three-dimensional surface plots as shown in Fig. 1. Results demonstrate that temperature has a greater impact on the extraction yield of phenolic compounds compared to that of time. It is observed that,

for a given time, the yield of phenolic content gradually increases with increasing temperature. This is because raising the extraction temperature leads to a decrease in solvent viscosity hence improving the efficiency of mass transfer of polyphenolic compounds and breaking the cellular constituents of the plant cells [20]–[24]. The results are also in agreement with Shi *et al*. [23] who reported that an increase in temperature aids in the phenolic interactions by softening the plant tissues. Although increasing the extraction temperature generally increases the response of extraction yields of phenolic content, elevated temperatures may result to the decomposition of thermal liable components hence influencing the quantification of bioactive compounds [24].

Table 3: Model equations in terms of coded factors (Maceration extraction).

	Garlic
TPC	$0.16 + 0.045\ X_1$
TFC	$1.03 + 0.63\ X_1 + 0.38\ X_1 X_2 + 0.34\ X_1^2$
DPPH	$5.92 + 1.38\ X_1 + 0.64\ X_1^2$
	Oregano
TPC	$15.14 + 4.44\ X_1 + 2.36\ X_1 X_2 + 2.20\ X_1^2 + 1.77\ X_2^2$
TFC	$26.57 + 7.32\ X_1 + 4.68\ X_1^2$
DPPH	$93.62 + 0.22\ X_1 + 0.39\ X_2^2$
	Parsley
TPC	$4.45 + 1.64\ X_1 + 0.92\ X_1^2$
TFC	$13.21 + 1.37\ X_1 - 1.22\ X_1^2$
DPPH	$19.30 + 4.23\ X_1 + 1.93\ X_2 + 2.28\ X_1^2$

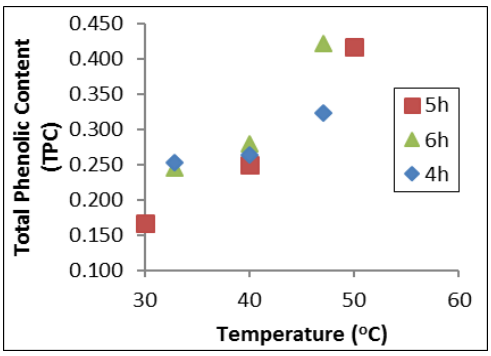
Noted:
X_1: extraction temperature
X2: extraction time

The results represented in colour coded three dimensional plots with cool blue indicting low desirability towards the response and warm yellow indicates a higher desirability. The shape of the three dimensional graphs concaves upwards indicating that the yield of phenolic contents displays an increasing trend with increasing extraction temperature and time. The effect of extraction temperature and time can be seen to be most significant in parsley and it is represented by a sharper bend in the upward direction compared to that of oregano and garlic. The optimized conditions proposed for the highest extraction of phenolic compounds were given at 47.1^{o}C, 6hours with the extraction yield of (0.243 ± 0.001 mg GAE/ g DW), (27.057 ± 0.018 mg GAE/ g DW), and (8.501 ± 0.089 mg GAE/ g DW) for garlic, oregano, and parsley respectively. The extraction of total phenolic content did not deviate much from the two suggested operating conditions. Results obtained through experimental conduct were slightly higher than the predicted values.

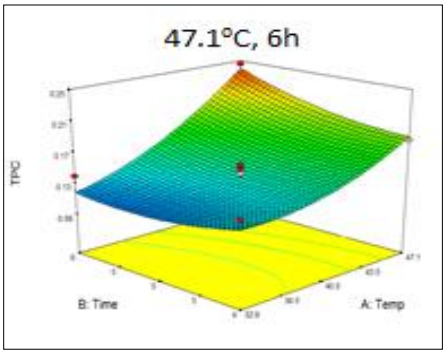
3.4. Effect of Extraction Temperature and Time on Total Flavonoid Content

The effect of extraction temperature and time on the total flavonoid content (TFC) of the three different plant extracts, using maceration method is shown in Fig. 2. It can be observed that the amount of TFC obtained increases with increasing temperature at a given time. This can be due to the findings that suggest increasing extraction temperature decreases solvent viscosity subsequently improving the efficiency of mass transfer of polyphenolic compounds [21]−[24]. However, it is interesting to note that in Fig. 2(c) the curve concaves downwards indicating that the yield of flavonoid content decreases beyond the stated temperature and time. This could be due to the reason that the flavonoid content in parsley has reached an equilibrium state with the extracting solvent. Further extracting will only leave the phenolic compounds exposed to light and oxygen which enhances the degradation process of antioxidants [25].

The proposed optimized conditions for the highest extraction of flavonoid compounds were 47.1℃, 6hours with the extraction yield of (0.987 ± 0.048 mg QE/ g DW), (46.551 mg QE/ g DW), and (15.4 ± 0.049 mg QE/ g DW) for garlic, oregano, and parsley respectively. The extraction of total flavonoid content did not deviate much from the two suggested operating conditions. Results obtained through experimental conduct were slightly higher than the predicted values.



(a)



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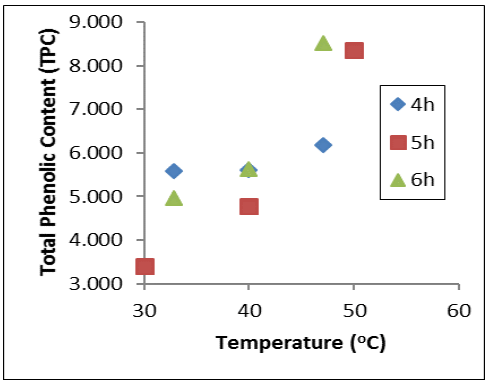
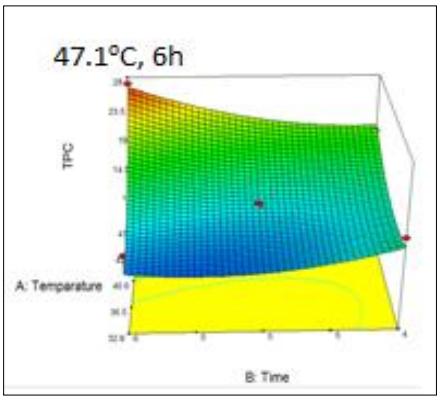


(c)



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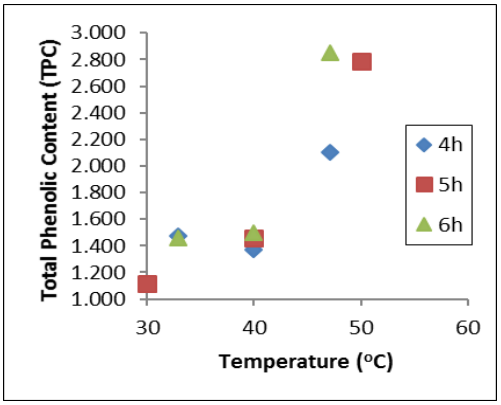

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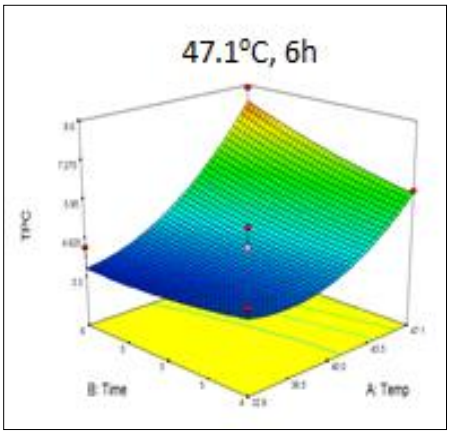
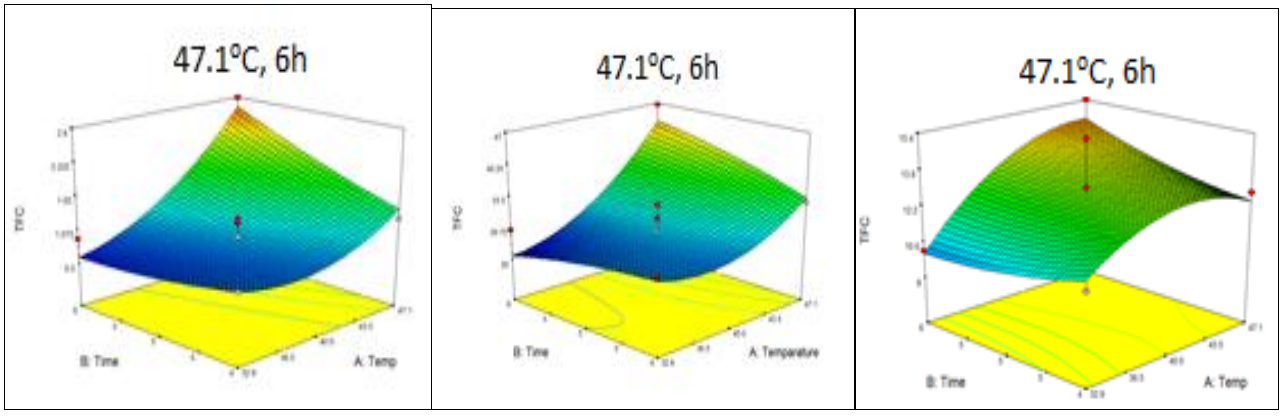


(f)

Fig. 1: Effects of extraction temperature and time on total phenolic contents (TPC) of (a-b) garlic, (c-d) oregano, and (e-f) parsley.

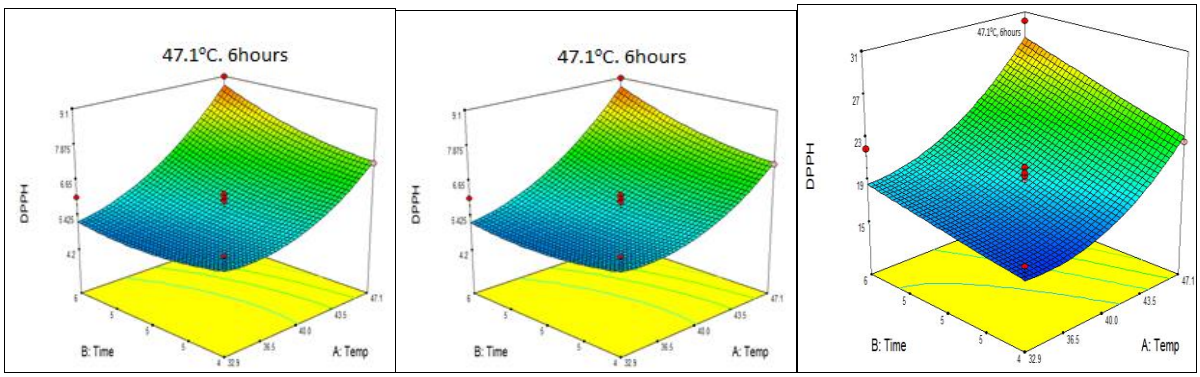


(a) (b) (c)

Fig. 2: Effects of extraction temperature and time on total flavonoid contents (TFC) of (a) garlic, (b) oregano, and (c) parsley.

3.5. Effect of Extraction Temperature and Time on DPPH Scavenging Activity

DPPH scavenging activity of different plant extracts using maceration method is shown in Fig. 3. The scavenging activity was spectrometrically measured using UV-visible spectrophotometer (Genesys 10S) instrument base on the absorbance value at wavelength 517 nm, which is indicated by the change in color of the solution from purple to yellow. The degree of decolourisation indicates the free radical scavenging activity [26]. Radical scavenging activity increases with increasing percentage of free radical scavenging inhibition. Present results depicts the scavenging activity for oregano is the highest followed by parsley and garlic. These variations can be attributed to the fact that different plant extracts have different chemical compositions [25]. There is no reported result comparing the three plants under a single experiment and at certain conditions. However, collecting data from various research data show agreement regarding the trend rather than specific numbers [12], [13], [27]. The present results demonstrate that the scavenging activity is significantly higher for the extraction of oregano compared to parsley and garlic which is the lowest. To understand this behaviour, Fig. 3 shows the effect of scavenging activity as temperature changes with time. The curvature of the Temperature-Time curve along with temperature rate (dT/dt) is highest for oregano and lowest is garlic. The results explain how the T-t is approaching their optimized values.



(a) (b) (c)

Fig. 3: Effects of extraction temperature and time on the scavenging activity of (a) garlic, (b) oregano, and (c) parsley.

The proposed optimized conditions were 47.1^{o}C, 6hours with 8.78%, 94.3%, and 28.46% of DPPH scavenging activity for garlic, oregano, and parsley respectively. The DPPH scavenging activity did not deviate much from the two suggested operating conditions. Results obtained through experimental conduct were slightly higher than the predicted values.

4. Conclusion

Antioxidants are substances used in medical field to relief disorders caused by large amounts of free radicals which are highly reactive substances produced as a by-product naturally in the human body. In this study, an optimization process of the extraction of bioactive compounds was achieved with the aid of mathematical software for optimization assisted by ANOVA statistics. The high correlation obtained indicates that the second-order polynomial model was suitable for the optimization of temperature and time on the extraction of bioactive components from garlic, oregano, and parsley. From the graphical representations, temperature and time significantly influence the phenolic compounds, flavonoids and antioxidant activity from the extracts of the three plants. The best suggested operating conditions to obtain the highest yield of bioactive components are at 47.1^{o}C for 6 hours using maceration. The model is said to be reliable based on the ANOVA analysis.

5. Acknowledgements

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