ANTIOXIDANT POTENTIAL OF MALAYSIAN FRUIT EXTRACT (*MYRISTICA FRAGRANS*)

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Abstract

The aim of this experiment is to study the phytochemical content of Malaysian fruit *(Myristica fragrans),* commonly known as nutmeg. This study also includes the optimization of extraction conditions for both soxhlet and ultrasonic extraction to yield the highest total phenolic content, total flavonoid content and 2, 2-diphenyl picryl hydrazyl scavenging activity of nutmegs using Response surface methodology. Soxhlet extraction is carried out with different extraction time and type of solvent. However, ultrasonic-assisted extraction is carried out with different extraction time, concentration of solvent and temperature of ultrasonic water bath. It has been shown that the optimum value of total phenolic content, total flavonoid content and 2, 2-diphenyl picryl hydrazyl scavenging activity of soxhlet extraction on nutmegs are 12.290 mg, 17.09 mg and 95.837%, respectively with a desirability of 0.671. The optimum condition for soxhlet extraction of nutmegs to obtain optimum yield of total phenolic content, total flavonoid content and 2, 2-diphenyl picryl hydrazyl scavenging are by using methanol as solvent at 184 minutes extraction time. However, for the optimization value of total phenolic content, total flavonoid content and 2, 2-diphenyl picryl hydrazyl scavenging activity for ultrasonic- assisted extraction of nutmegs are 28.722 mg, 46.600 mg and 98.565%, respectively with a desirability of 0.977. The optimum condition for ultrasonic assisted extraction of nutmegs to obtain optimum yield of total phenolic content, total flavonoid content and 2, 2-diphenyl picryl hydrazyl scavenging are by extracting nutmeg at 40 minutes at 50 ℃ and at 40% ethanol concentration.

Keywords: *Myristica fragrans,* Phytochemical content, Response surface methodology, Soxhlet extraction, Ultrasonic-assisted extraction.

1. Introduction

Fruits and vegetables are known to be part of a healthy diet. A diet rich in vegetables and fruits can reduce blood pressure, risk of heart disease and stroke. Researchers have also proven that fruits and vegetables can prevent some types of cancer, reduce the risk of eye and digestive problems. It also has a positive effect upon blood glucose which can keep appetite in check [1]. These health benefits are studied to be associated with high antioxidant activity which inhibits the activity of reactive oxygen species (ROS) which can be found in fruits and vegetables [2]. Antioxidants protect cells against free radical damage, which damages cells by preventing or delaying oxidative damage of lipids, proteins and nucleic acid caused by ROS [3].

Poly-phenols are the most lavish antioxidant in the diet of fruits and vegetables compared to other classes of phyto-chemicals due to their ability to neutralize free radicals. Besides that, poly-phenols also delay or strengthen many enzymes. Phenolic compounds such as flavonoids and carotenoids from fruits and vegetables have major health benefits such as anti-bacterial and anti-inflammatory actions. Flavonoids are also known to reduce chances of cardiovascular diseases and to impede lipid per-oxidation and platelet aggregation [4, 5].

Extraction is defined as separation of active portions of plant from the inactive or inert components by using selective solvents in standard extraction methods. The extracts obtained are commonly used as medicinal agents. Therefore, it is important to standardize and optimize extraction procedures, as it contributes tremendously to the final quality of the outcome [6]. The most commonly used extraction method is maceration; however, it is time consuming. Modern extraction methods have been developed to increase the efficiency of extraction of organic compound such as ultrasonic assisted extraction (UAE) and soxhlet extraction (SE). UAE is a modified maceration method facilitated by the use of ultrasound [7]. It increases the permeability of cell walls and produces cavitation [6]. The cellular breakdown increases the solubization of metabolites in the solvent and improves extraction yields [7]. Soxhlet extraction is defined as a refluxing which solvent repeatedly washes the solid, extracting a desired compound into the flask [8]. It is a continuous process, which can be extracted, in a large amount with smaller quantity of solvent. It is economical in terms of time, energy and cost [6].

The parameters manipulated through the extraction methods are also important to standardize and optimize extraction procedures such as type of solvent used for extraction process. Alcoholic solvents are generally used to extract phenolics (phenolic compounds) from natural sources because although they are not highly selective to phenols, they give a high yield of total extract. A mixture of solvent and water is to be more competent in extracting phenolic constituents than the mono-component solvent system as it concerns its polarity. Antioxidant compound can be most effectively extracted in ethanol solvent [9]. Extraction time also plays a vital role in the optimization of extraction process. Extract increases with increasing time, however, due to the exposure to unfavourable environment aspects like temperature, light and oxygen, extended extraction time increases the chance of decomposition and oxidation of phenolic compound [10]. Lastly, increase in temperature increases the effectiveness of extraction process, the solvent viscosity decreases, an increase of solubility and diffusion coefficients of phenolics, also enhancing mass transfer and penetration of solvent into plant matrix. However, an increase in temperature can also weaken the phenol-protein and phenol-

polysaccharide interaction in plant material resulting in decomposition of antioxidants [10].

Studies have shown that Nutmegs (*Myristica fragrans*) (Fig. 1) is associated with various health benefits [11, 12]. It has many applications in culinary, pharmaceutical and cosmetic industries. This fruit can be easily found in the islands near Indonesia, Malaysia, Caribbean and tropical areas of the world such as Southern India. These fully grown trees can reach about 50 to 60 feet in height and is the source of nutmeg and mace. Nutmeg is a delicate, slightly sweet spice, which is used as flavouring agent in the food industry. This fruit consists of four parts, which are the skin, fruit, mace and seed. The fruit is a drupe, which upon ripening, splitsup to reveal ingle centrally situated oval-shaped kernel, known as nutmeg spice. The seed kernel enwrapped by crimson-red, lacy or thread-like arils known as mace [13]. The nutmeg tree is also popularly known for its essential oils that originated from the tree and leaves. These essential oils of nutmeg extract are very beneficial to health and are vastly used as an alternative herbal medicine.

Fig. 1. Myristica Fragrans (Nutmeg) [15].

As mentioned above, nutmeg due to its nutritive content of vitamins, minerals and organic compounds, has many health benefits. These benefits also include dietary fibre, manganese, vitamin B6, etc. Nutmeg has natural pain-relieving characteristics. It can reduce associated pain from wounds, injuries, strains and chronic inflammation such as arthritis. Other than that, nutmeg stimulates the digestive process by promoting peristaltic motion in the smooth muscle of intestine. It also reduces the discomfort of constipation and other intestinal issues such as fibre content in nutmeg can bulk up the bowel movements. Nutmeg is very well known as the king of spices when it comes to oral health because it helps conditions such as halitosis, known as bad breath. This is due to the active antibacterial components of nutmeg, which kills the bacteria that causes this condition and boosts the immunity of gums and teeth. Among the health benefits attained by nutmeg is the potential use against cancerous cells. Research has shown that a methanolic compound in nutmeg and its essential oil can induce cell death in leukaemia cells, which stops the spread of cancer. Lastly, the mineral content of nutmeg is known to maintain organ function. Among them is potassium, which relaxes blood vessels, therefore reducing blood pressure and lowering cardiovascular diseases [14]. The objectives of this research are to compare efficiencies of extraction of bioactive components of nutmeg by using

different extraction processes with varying parameters of soxhlet extraction i.e. type of solvent and time of extraction and ultrasonic extraction, i.e., concentration of solvent, temperature of extraction bath and time of extraction. Other than, this research is to optimize the extraction of bioactive components of nutmeg by using response surface methodology (RSM). As per authors' knowledge comparison between soxhlet and ultrasonic extraction of fruit nutmeg is lack in scientific literature. From this study, economical value of ultrasonic extraction of nutmeg fruit could be justified in terms of high antioxidant potential when compared to soxhlet.

2. Materials and Method

2.1. Fruit material

Nutmeg (*Myristica Fragrans*) was purchased from a fruit supplier in Penang, Malaysia. 10kg of nutmegs were purchased for this experimental work.

2.2. Chemicals

Organic solvent that were used for extraction methods were methanol, ethanol and ethyl acetate. For folin ciocalteau assay, to determine the total phenolic content, folin ciocalteau reagent, sodium carbonate and gallic acid were used. For the determination of total flavonoid content, ozsoy method was used, and the chemicals used were sodium nitrite solution, aluminum chloride, sodium hydroxide and quercetin. Lastly, to determine DPPH scavenging activity, 2, 2-diphenyl picryl hydrazyl (DPPH) assay, the chemical used was ethanolic 2, 2-diphenyl picryl hydrazyl. All solvents were purchased from Syntertec Enterprise.

2.3. Equipment

Equipment used during this research was water bath (WNB45L0), soxhlet extractor, ultrasonic cleaner (Elmasonic P120H/1013777), centrifuge (Scanspeed 1236MG/1248), UV-Vis spectrophotometer (Genesys 10S) and oven dryer (FAC-350). The centrifuge was used to separate mixture of sample with solvent into pellet and supernatant. The maximum capacity is 6 x 50ml with a maximum speed up to 12000 rpm, respectively. Ultra-Violet Visible Spectrometer was used to quantify antioxidant activity, total phenolic content and total flavonoid content of the extracted sample. Soxhlet extractor and ultrasonic cleaner were used to extract lipid from the solid material.

2.4. Experimental work

2.4.1. Sample preparation

Nutmegs were washed with tap water followed by distilled water and then peeled. The fruits were cut into small pieces and the seed was disposed. Then the fruits were dried in a hot air oven at 60 °C for 36 hours. A pulveriser was used to pulverize the fruit before being placed in a plastic container. Nutmeg raw powder to solvent ratio (1:20) was applied in soxhlet and ultrasonic assisted extraction. 7.5 g of nutmeg powder was used in 150 ml of solvent in both the experiments.

2.4.2. Extraction

The two different extraction processes were investigated to obtain the optimum extraction conditions for ultrasonic assisted extraction (UAE) and soxhlet extraction (SE). Extraction conditions were varied according to the respective process. Solid to solvent ratio used was 1:20 for both the extraction processes.

2.4.3. Soxhlet extraction (SE)

7.5g of fruits were ground, placed into the thimble and then filled with 150 ml of solvent. The soxhlet extractor was placed in a water bath and the variables varied for this process by RSM were extraction times, which were 180, 210 and 240 minutes and type of solvents, were methanol, ethanol and ethyl acetate. The temperature of water bath was maintained at boiling point of each solvent for the respective sample.

2.4.4. Ultrasonic assisted extraction (UAE)

7.5g of fruit were ground and placed into beakers labelled with respective condition and then filled with 150 ml of solvent. Ethanol was used as solvent for this extraction process. The beakers were then placed in the ultrasonic cleaner. The variables varied for this process by RSM were extraction times, which were 40, 60 and 80 minutes, temperatures, were 30, 40 and 50°C and solvent concentrations were 40%, 50% and 60%, which were prepared by dilution of solvent with distilled water. Frequency was maintained at 100% for the optimum extraction of all samples.

2.4.5. Filtration of extracts

After the ultrasonic extraction process, the extracts were filtered by separating them to obtain clear liquid by solid liquid separation such as centrifugation and filtration.

2.4.6. Centrifugation

After ultrasonic extraction process, the extracts were centrifuged at 5000 rpm, 25°C for 15 minutes. The liquid was separated to clear liquid and supernatant was obtain and was subjected to filtration.

2.4.7. Filtration

The centrifuged extract was then filtered by using whatman filter paper no. 1 to obtain clear liquid. Supernatant was disposed off.

2.5. Phytochemical content

2.5.1. Total phenolic content

The total phenolic content (TPC) in the extract was determined by using the folin ciocalteau method. 0.1 ml of sample after extraction process was mixed with 0.2ml folin ciocalteau reagent and 2 ml of distilled water was added. This mixture was left to rest for 3 minutes at room temperature (RT). After that, 1 ml of 20% sodium carbonate was added to the mixture and then left for an hour to be incubated at RT. Then absorbance was measured at 765 nm using a UV spectrophotometer. Blank sample was prepared as explained above by replacing plant extract with distilled

water. Gallic acid was used to produce a calibration curve. The total phenolic content in the extract was calculated and expressed as Gallic acid equivalents (GAE; mg/ g dry weight) using Eq. (1):

$$\text{GAE (mg)}/100(g)\, \text{DE} = \text{GAE} \times \frac{V}{M} \times 100 \tag{1}$$

where GAE = Gallic acid equivalent (mg/ml) obtained from calibration curve, DE = Dry weight, V = Volume of solvent used during the assay (ml), and M = Mass of plant used during the assay.

Preparation of standard curve for total phenolic content

Different dilutions of gallic acid with different concentrations (0.2, 0.4, 0.6, 0.8 and 1.0 mg/ml) were prepared. 0.2 ml of folin ciocalteu reagent was added to 0.1 ml of gallic acid solution. Then 2 ml of distilled water was added to the mixture. After that, 1 ml of 20% sodium carbonate was added to the mixture. The mixture was left to be incubated at RT for an hour. The absorbance of the mixture was measured at 765 nm using an UV Spectrophotometer. Blank sample was prepared with distilled water, by same procedure as explained above. The standard calibration curve was plotted with y-axis represents the absorbance values of gallic acid and x-axis represents the concentration of the gallic acid shown in Fig. 2. The calibrated equation obtained from standard curve was y = 0.286x + 0.606
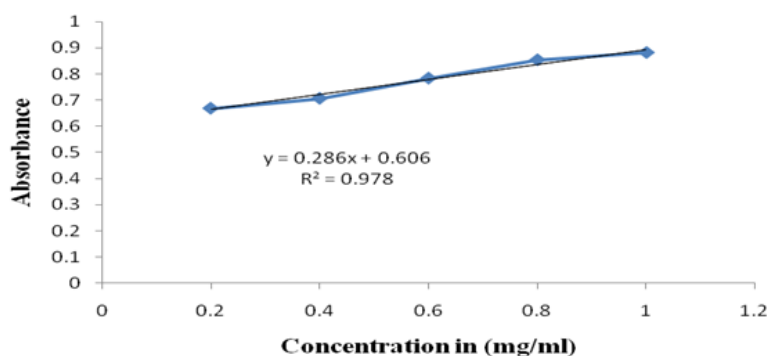

Fig. 2. Calibration curve of gallic acid.

2.5.2. Total flavonoid content

The total flavonoid content was determined by using the ozsoy method. 0.25 ml of plant extracts were mixed with 1.25 ml distilled water and adds 0.075 ml of 5% (w/v) of sodium nitrite solution was added. The mixture was incubated for 6 minutes at RT. After that 0.15ml of 10% (w/v) aluminium chloride was added to the above mixture. Then sodium hydroxide of 1M (0.5 ml) was added to the mixture followed by 0.275 ml of distilled water. The absorbance was measured at 510 nm using UV spectrophotometer. Blank sample was prepared as explained above by replacing plant extract with distilled water. Quercetin was used to produce a calibration curve. The total flavonoid content in the extract was calculated and expressed as Quercetin (QE; mg/g dried weight) using Eq. (2):

$$\text{QE (mg)}/100(g)\, \text{dried weight} = \text{QE} \times \frac{V}{M} \times 100 \tag{1}$$

where QE = Quercetin equivalent (mg/ml) obtained from calibration curve, $V =$ Volume of solvent used during the assay (ml), and $M =$ Mass of plant used during the assay.

Preparation of standard curve for total flavonoid content

Different dilutions of quercetin with different concentrations (0.1, 0.3, 0.5 and 0.7 mg/ml) were prepared. 0.25 ml of quercetin solution was mixed with 1.25 ml distilled water. Then the mixture was added with 0.075 ml of 5% (w/v) of sodium nitrite solution and this mixture was incubated for 6 minutes at RT. Then the mixture was added with 0.15ml of 10% (w/v) aluminium chloride. After that, 0.5ml of 1M of sodium hydroxide was added to the mixture, followed by 0.275 ml of distilled water. The absorbance was measured at 510 nm using a UV-Vis Spectrophotometer. Blank sample was prepared. The standard calibration curve was plotted with y-axis representing the absorbance values of quercetin and x-axis representing the concentration of quercetin, shown in Fig. 3. The calibrated equation obtained from standard curve is y = $0.239x + 0.171$.
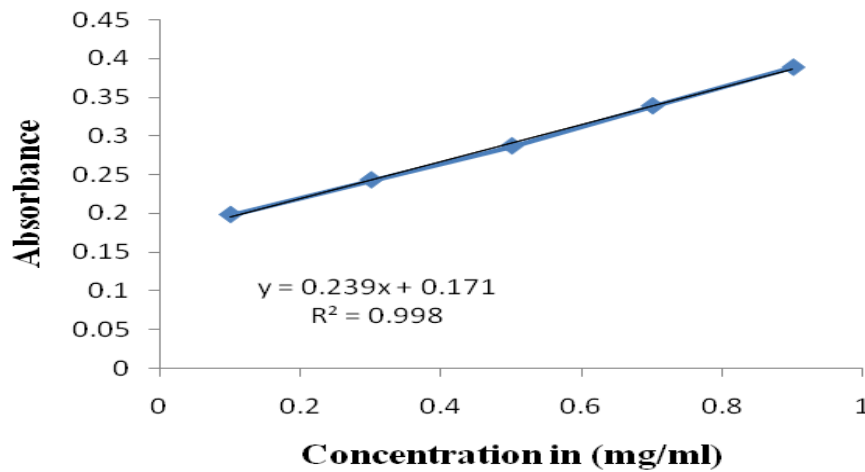

Fig. 3. Calibration curve of quercetin.

2.5.3. DPPH scavenging activity

DPPH radical scavenging activity of the extract was determined using 2, 2-diphenyl picryl hydrazyl (DPPH) assay. 0.1 ml of plant extract was added with 3.9 ml of ethanolic DPPH (0.025g/L). The mixture was left in a dark condition at RT for 30 minutes to block (UV light would trigger free radicals) UV light. This reaction would disrupt the results of the experiment. The absorbance was measured at 517 nm. The colour of the sample changed from deep violet to light yellow [16]:

DPPH scavenging activity (%) was calculated using the following formula:

$$\% \text{ inhibition of DPPH scavenging assay} = \frac{\text{Control}_{Abs} - \text{Sample}_{Abs}}{\text{Control}_{Abs}} \times 100 \qquad (3)$$

2.6. Experimental design by RSM

Response surface methodology (RSM) was used to determine the optimal conditions for both soxhlet and ultrasonic-assisted extraction processes. RSM was conducted using Design Expert Software (Version 10.0.3).

2.6.1. Optimization of response surface methodology (RSM)

The optimization was done on the basis of extracting conditions of both soxhlet and ultrasonic assisted extraction processes which yielded the highest total phenolic content, total flavonoid content and antioxidant activity of nutmegs.

The soxhlet extraction of *Myrisyristica fragrans* fruit was optimized through two independent variables - extraction time and type of solvent and the ultrasonic assisted extraction was optimized through three independent variables - extraction time, temperature and concentration of solvent on the dependent variables - TPC, TFC, and DPPH scavenging activity. A quadratic model was used to model the treatment effects and treatment interactions. The complete design using soxhlet extraction consisted of 14 experimental runs with 2 replications of the centre point and ultrasonic assisted extraction consisted of 17 experimental runs with 2 replications of the centre point.

2.6.2. Statistical analysis

Analysis of variance (ANOVA) procedure of the Design Expert Software (Version 10.0.3) was used to statistically analyze the means of triplicate experiments. ANOVA was used to determine the significance ($p < 0.05$) of the model and the interaction of the independent variables on the responses.

3. Results and Discussion

3.1. Soxhlet extraction

Table 1 shows the design matrix and the experimental results of the d-Optimal design for the total phenolic content (Y_1), total flavonoid content(Y_2) and DPPH scavenging activity (Y_3) of soxhlet extraction of Nutmegs with Design Expert 10.0.3.

For soxhlet extraction, total phenolic content was p=0.1587 (p>0.05) proving that the model has not significance. The p-values (Prob>F) of X_1 (extraction time) and X_2 (type of solvent) for TPC were 0.9785 and 0.0491, respectively as shown in Table 2. It proved that the extraction time was not a huge influencing factor of TPC. However, type of solvent proved to be an influence to the yield of TPC. Total flavonoid content was p=0.0386 (p<0.05) proving that the model has significance. The p-values (Prob>F) of X_1 (extraction time) and X_2 (type of solvent) for TFC were 0.5733 and 0.0063 respectively, as shown in Table 3.

It proved that the extraction time was not a huge influencing factor of TFC. However, type of solvent proved to be a great influence to the yield of TFC. Lastly, DPPH scavenging activity was p=0.1386 (p>0.05) concluding that the model has not significance. The p-values for DPPH scavenging activity were 0.2680 and 0.0949 respectively, as shown in Table 4. It is concluded that the extraction time and type of solvent were not a huge influencing factor of DPPH scavenging activity because of the high *p*-value (>0.05).

**Table 1. Experimental results of the d-Optimal
design for the total phenolic content, total flavonoid content
and antioxidant activity of soxhlet extraction of Nutmegs.**

Run	Extraction parameters		Responses		
	Extraction Time (min), X_1	Type of Solvent, X_2	TPC, Y_1 mg GAE/100g DW	TFC, Y_2 mg QE/100 g DW	DPPH scavenging activity, Y_3, %
1	195	Methanol	18.05	24.40	96.91
2	240	Ethyl Acetate	8.65	25.93	93.42
3	210	Ethyl Acetate	2.77	18.49	88.82
4	180	Ethyl Acetate	6.16	28.16	92.37
5	180	Ethyl Acetate	3.16	25.07	89.69
6	180	Methanol	9.34	10.92	95.10
7	225	Methanol	11.03	14.07	95.98
8	210	Ethanol	5.61	6.57	93.73
9	210	Methanol	13.04	14.07	96.14
10	240	Methanol	7.11	8.62	93.03
11	240	Ethanol	6.95	9.09	94.52
12	240	Ethanol	6.59	7.40	94.11
13	195	Ethanol	7.88	8.73	94.46
14	180	Ethanol	5.39	6.56	87.08

Table 2. ANOVA for TPC of SE of nutmegs.

Source	Sum of Squares	Degree of Freedom	Mean Square	F-value	p-value Prob >
Model	136.85	6	22.81	2.23	0.1587
X_1	7.983E-003	1	7.983E-003	7.812E-004	0.9785
X_2	96.52	2	48.26	4.72	0.0491
X_1X_2	20.43	2	10.21	1.00	0.4151
X_1	3.86	1	3.86	0.38	0.5581
Residual	71.53	7	10.22		
Lack of Fit	66.69	5	13.39	5.85	0.1523
Pure Error	4.58	2	2.29		
Cor Total	208.38	13			

Table 3. ANOVA for TFC of SE of nutmegs.

Source	Sum of Squares	Degree of Freedom	Mean Square	F-value	p-value Prob >
Model	651.38	6	108.56	4.31	0.0386
X_1	8.79	1	8.79	0.35	0.5733
X_2	573.30	2	286.65	11.37	0.0063
X_1X_2	18.04	2	9.02	0.36	0.7112
X_1	0.60	1	0.60	0.024	0.8815
Residual	176.46	7	25.20		
Lack of Fit	170.20	5	34.04	10.97	0.0856
Pure Error	6.21	2	3.10		
Cor Total	827.78	13			

Table 4. ANOVA for DPPH scavenging activity of SE of nutmegs.

Source	Sum of Squares	Degree of Freedom	Mean Square	F-value	p-value Prob >
Model	72.73	6	12.12	2.42	0.1368
X_1	7.26	1	7.26	1.45	0.2680
X_2	33.70	2	16.85	3.36	0.0949
X_1X_2	19.28	2	9.64	1.92	0.2162
X_1	4.48	1	4.48	0.89	0.3763
Residual	35.12	7	5.02		
Lack of Fit	31.43	5	6.29	3.41	0.2423
Pure Error	3.69	2	1.84		
Cor Total	107.84	13			

Mathematical equations were developed Eqs. (4) and (6) to explain the effects of the two independent variables, which are extraction time (X_1) and type of solvent (X_2) on the total phenolic content (Y_1), total flavonoid content (Y_2) and DPPH scavenging activity (Y_3) of soxhlet extraction of nutmegs respectively. The coefficients of regression equation were calculated using the Design Expert Software.

$$Y_1=8.70-0.084X_1+3.64X_2-1.45X_2^2-2.21X_1X_2+0.60X_1X_2^2-1.25X_1^2 \tag{4}$$

$$Y_2=15.71-1.15X_1-1.04X_2-7.77X_2^2 - 1.84X_1X_2+1.78X_1X_2^2-0.49X_1^2 \tag{5}$$

$$Y_3=93.93-0.82X_1+2.18X_2-0.54X_2^2-1.83X_1X_2+1.89X_1X_2^2-1.35X_1^2 \tag{6}$$

Extraction condition of soxhlet extraction was based on the TPC, TFC and DPPH scavenging activity of the nutmegs. The optimum values of the variables were obtained using Eqs. (4)-(6) using Design Expert Software. Figure 4 shows the desirability developed from optimum points through numerical optimization. The optimization values of TPC, TFC and DPPH scavenging activity were 12.58 mg, 17.40 mg and 95.837%, respectively with a desirability of 0.671. Out of 14 batches, 9 batches were selected, as shown in Figs. 5, 6 and 7 and describe the effects of interaction of the independent variables time of extraction and type of solvent on TPC, TFC and DPPH scavenging activity of soxhlet extraction of nutmegs.

From the present research work, results showed that the optimum condition for soxhlet extraction of nutmegs to obtain optimum yield of TPC, TFC and DPPH scavenging were by using methanol as the extracting solvent at 184 minutes extraction time.
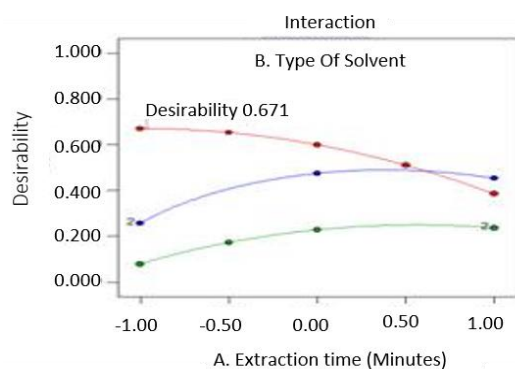
Fig. 4. Desirability of soxhlet extraction model.
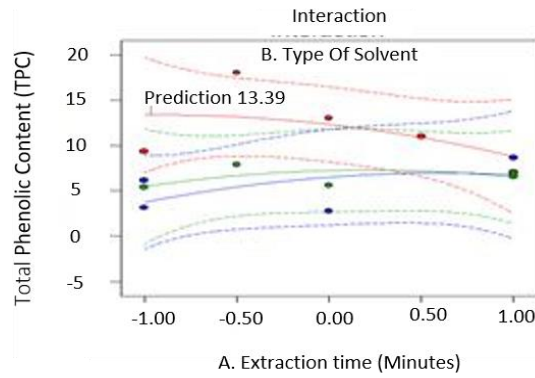*Red line for methanol, Blue line ethanol and Green line for ethyl acetate.

Fig. 5. Effect of the interaction on TPC.

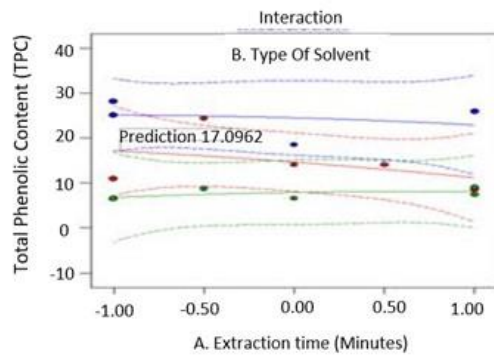***Dotted line shows different variations in solvents and Shaded line shows type of solvent.**

Fig. 6. Effect of the interaction on TFC.

*Dotted line shows different variations in solvents and Shaded line shows type of solvent.
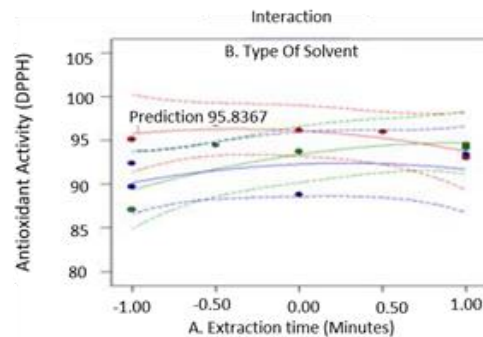

Fig. 7. Effect of the interaction on scavenging activity.

*Dotted line shows different variations in solvents and Shaded line shows type of solvent.

Effect of Type of Solvent on soxhlet extraction

The polarity of solvent increased as the extraction of TPC, TFC and Antioxidant Activity increased. Antioxidants consist of hydrophilic and lipophilic antioxidants. Phenolic compounds are mainly responsible for the antioxidant properties of fruits and vegetables, and most of these compounds are classified as hydrophilic

antioxidants [17]. Hydrophilic has affinity to interact with polar molecules such as methanol since polar molecule dissolved in polar solvent. Therefore, the solvent that has higher polarity will have greater tendency to interact with hydrophilic antioxidant. In addition, high polarity increased the solubility of phenolic and antioxidant contents in the solvent. With the increase in solubility of phenolic content and antioxidant content, the antioxidant activity would also increase.

In soxhlet extraction process, ethanol was less efficient in the extraction of antioxidant compounds than methanol, although their polarities were similar. This was due to the low solvation provided by ethanol because of the presence of the ethyl radical that is longer than the methyl radical present in methanol, which resulted in a lower solvation of antioxidant molecules. However, researchers have identified that in the food application industry [18-20], the use of ethanol may be preferable to methanol as the solvent of extraction as ethanol was less poisonous compared to methanol. Ethyl acetate yielded the lowest antioxidant from the extracted nutmegs, as it was a weak polar solvent. Therefore, it has a low solubility to phenolic content and antioxidant content.

Ethanol was used as the solvent for ultrasonic assisted extraction as ethanolic mixtures have acceptability for human consumption models. The mixtures of alcohols and water have been proven to be more efficient in extracting phenolic compound and antioxidant activity than the mono component solvent system. This is because as the polarity of the solvent system increases, the polar interactions between antioxidant activity and phenolic content with solvent system increases. The solubility of antioxidant activity and phenolic content in the solvent system is higher with aqueous ethanol mixture compared with the absolute solvent.

Therefore, extraction of phenolic content and antioxidant activity increased as the ratio of ethanol content increased. 100% ethanol (mono-component solvent) does not contribute to extract water soluble compound such as phenolic compounds. However, the addition of water to ethanol improves extraction rate, but too high water content brought an increased extraction of other compounds, and, then to lower phenol concentrations in the extracts [21, 22]. The maximum deviation between predicted and actual value for DPPH (other responses are not shown) using soxhlet method is only 8% and hence the above equations are valid to determine the response variables.
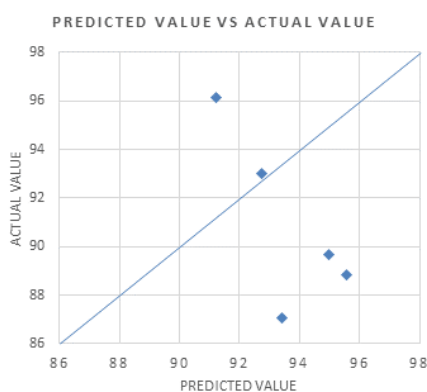

Fig. 8. Actual vs. predicted value for DPPH using soxhlet method.

3.2. Ultrasonic assisted extraction

Table 5 shows the design matrix and the experimental results of the d-Optimal design for the total phenolic content (Y_1), total flavonoid content (Y_2) and DPPH scavenging activity (Y_3) of soxhlet extraction of Nutmegs with Design Expert 10.0.3.

Table 5. Experimental results of the d-Optimal design for the total phenolic content (Y1), total flavonoid content (Y2) and antioxidant activity (Y3) of ultrasonic assisted extraction of Nutmegs.

Run	Extraction parameters		Responses			
	Extraction Time (min), X_1	Temperature of Extraction, °C ,X_2	Concentration of Ethanolic Solvent of Extraction(%) X_3	TPC, Y_1 mg GAE/100g DW	TFC, Y_2 mg QE/100 g DW	DPPH scavenging activity, Y_3, %
1	40.00	30.00	55.70	7.57	13.63	94.46
2	80.00	50.00	40.00	12.54	23.84	98.45
3	40.00	30.00	55.70	15.10	25.46	96.75
4	55.00	40.73	60.00	9.40	14.76	96.80
5	80.00	50.00	40.00	16.70	23.94	98.61
6	58.44	40.86	48.13	11.01	15.06	96.71
7	59.37	50.00	53.53	15.50	27.16	98.48
8	55.56	30.00	47.50	9.56	13.39	94.43
9	40.00	30.00	40.00	6.17	9.78	93.01
10	80.00	50.00	56.16	17.13	30.04	98.66
11	80.00	41.63	47.11	14.82	25.36	91.39
12	80.00	30.00	45.69	8.92	13.24	97.19
13	40.00	50.00	60.00	25.90	45.43	99.07
14	40.00	50.00	40.00	28.54	46.59	96.73
15	40.00	40.00	41.87	18.56	28.67	99.06
16	80.00	38.55	60.00	13.85	24.82	98.04
17	62.69	30.00	60.00	11.77	17.12	97.69

For ultrasonic assisted extraction on total phenolic content was p=0.0184 (p<0.05) proving that the model has significance. The p-values (Prob>*F*) of X_1 (extraction time), X_2 (extraction temperature) and X_3 (solvent concentration) for TPC were 0.0491, 0.0017 and 0.2754, respectively, as shown in Table 6. It proved that the solvent concentration was not a huge influencing factor of TPC. However, time and temperature of extraction proved to have influences on yield of TPC. Total flavonoid content was p=0.0062 (p<0.05) proving that model has significance. The p-values (Prob>*F*) of X_1 (extraction time), X_2 (extraction temperature) and X_3 (solvent concentration) for TFC were 0.0315, 0.0005 and 0.0666, respectively, as shown in Table 7. It proved that the extraction time, extraction temperature and solvent concentration had great influence on yield of TFC. Lastly, DPPH scavenging activity was p=0.7941 (p>0.05) concluding that the model has not significance. The p-values (Prob>*F*) for DPPH scavenging activity were 0.8433, 0.2004 and 0.4387, respectively, as shown in Table 8. It is concluded that the extraction time, extraction temperature and solvent concentration did not have huge influencing factors of DPPH scavenging activity because of the high *p*-value (>0.05)

Mathematical equations were developed [Eqs. (7), (8) and (9)] to explain the effects of the three independent variables, which were time of extraction (X_1), temperature of extraction (X_2) and concentration of solvent of extraction(X_3) on the total phenolic content (Y_1), total flavonoid content(Y_2) and DPPH scavenging

activity(Y_3) of ultrasonic assisted extraction of nutmegs. The coefficients of regression equation were calculated using the design expert software.

$$Y1 = 8.70 - 0.084X_1 + 3.64X_2 - 1.45X_2^2 - 2.21X_1X_2 + 0.60X_1X_2^2 - 1.25X_1^2 \tag{7}$$

$$Y2 = 15.71 - 1.15X_1 - 1.04X_2 - 7.77X_2^2 - 1.84X_1X_2 + 1.78X_1X_2^2 - 0.49X_1^2 \tag{8}$$

$$Y3 = 93.93 - 0.82X_1 + 2.18X_2 - 0.54X_2^2 - 1.83X_1X_2 + 1.89X_1X_2^2 - 1.35X_1 \tag{9}$$

Extraction condition of ultrasonic assisted extraction was based on TPC, TFC and DPPH scavenging activity of the nutmegs. The optimum values of the variables were obtained using Eqs. (7)-(9) using Design Expert Software. Figure 9 shows the desirability developed from optimum points through numerical optimization. The optimization values of TPC, TFC and DPPH scavenging activity were 28.39 mg, 46.86 mg and 98.5645% respectively with a desirability of 0.977. Figures 10, 11 and 12 show the effects of interaction of the independent variables time of extraction, temperature of extraction and concentration of solvent on TPC, TFC and DPPH scavenging activity of ultrasonic assisted extraction of nutmegs.

Table 6. Analysis of variance (ANOVA) for TPC
of ultrasonic assisted extraction of nutmegs.

| Source	Sum of Squares	Degree of Freedom	Mean Square	*F*-value	*p*-value Prob >*F*
Mode	506.04	9	56.23	5.41	0.0184
X_1	58.73	1	58.73	5.65	0.0491
X_2	253.35	1	253.35	24.37	0.0017
X_3	14.55	1	14.55	1.40	0.2754
X_1X_2	89.76	1	89.76	8.64	0.0218
X_1X_3	12.41	1	12.41	1.19	0.3107
X_2X_3	34.43	1	34.43	3.31	0.1116
X_1^2	60.80	1	60.80	5.85	0.0462
X_2^2	8.13	1	8.13	0.78	0.4058
X_3^2	8.44	1	8.44	0.81	0.3974
Residual	72.76	7	10.39		
Lack of Fit	35.72	5	7.14	0.39	0.8311
Pure Error	37.04	2	18.52		
Cor Total	578.80	16			

Table 7. Analysis of variance (ANOVA) for
TFC of ultrasonic assisted extraction of nutmegs.

Source	Sum of Squares	Degree of Freedom	Mean Square	*F*-value	*p*-value Prob >*F*
Model	1627.86	9	180.87	7.92	0.0062
X_1	164.06	1	164.06	7.18	0.0315
X_2	853.66	1	853.66	37.39	0.0005
X_3	107.50	1	107.50	4.71	0.0666
X_1X_2	214.96	1	214.96	9.41	0.0181
X_1X_3	35.86	1	35.86	1.57	0.2504
X_2X_3	74.72	1	74.72	3.27	0.1134
X_1^2	273.18	1	273.18	11.96	0.0106
X_2^2	32.76	1	32.76	1.43	0.2700
X_3^2	28.49	1	28.49	1.25	0.3009
Residual	159.84	7	22.83		
Lack of Fit	89.80	5	17.96	0.51	0.7634
Pure Error	70.04	2	35.02		
Cor Total	1787.70	16			

**Table 8. Analysis of variance (ANOVA) for DDPH scavenging
activity of ultrasonic assisted extraction of nutmegs.**

Source	Sum of Squares	Degree of Freedom	Mean Square	*F*-value	*p*-value Prob >*F*
Model	33.41	9	3.71	0.56	0.7941
X_1	0.28	1	0.28	0.042	0.8433
X_2	13.23	1	13.23	2.00	0.2004
X_3	4.46	1	4.46	0.67	0.4387
X_1X_2	1.52	1	1.52	0.23	0.6468
X_1X_3	7.396E-003	1	7.396×10^3	1.117×10^3	0.9743
X_2X_3	0.72	1	0.72	0.11	0.7507
X_1^2	0.18	1	0.18	0.027	0.8747
X_2^2	1.98	1	1.98	0.30	0.6012
X_3^2	2.60	1	2.60	0.39	0.5510
Residual	46.34	7	6.62		
Lack of Fit	43.68	5	8.74	6.59	0.1371
Pure Error	2.65	2	1.33		
Cor Total	79.75	16			

From the present research, it was found that the optimum condition for ultrasonic assisted extraction of nutmegs to obtain optimum yield of TPC, TFC and DPPH scavenging were by extracting nutmeg at 40.512 minutes at 49.99 ℃ and at 40% ethanol concentration.
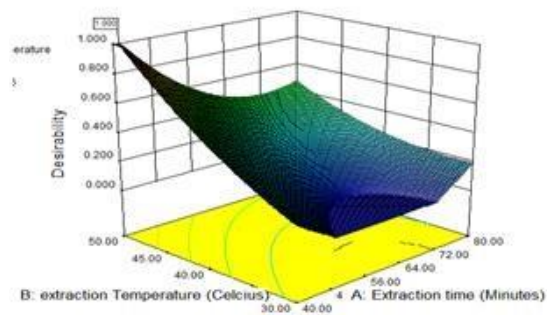
Fig. 9. Desirability of UAE model.
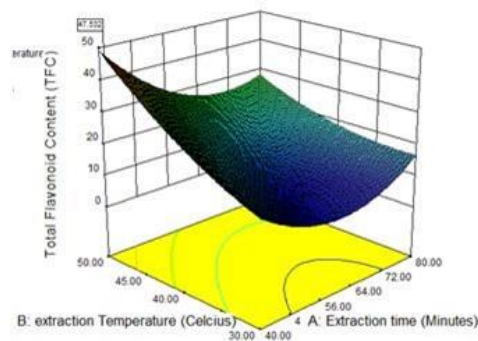
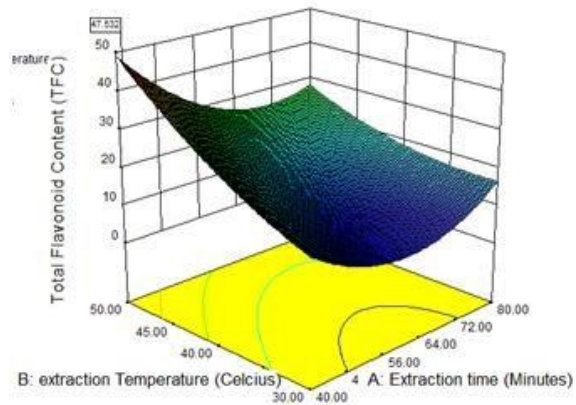**Fig. 10. Effect of the interaction on TPC.**

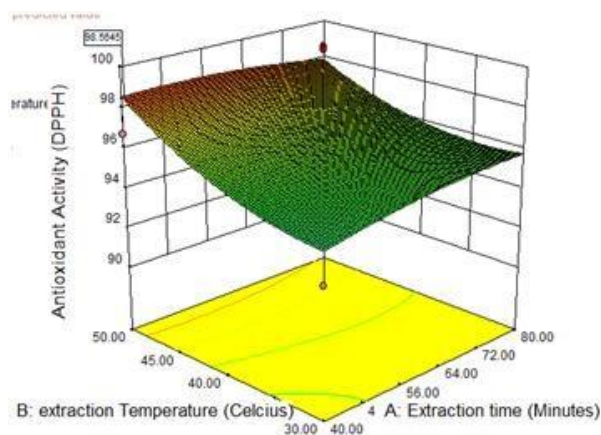**Fig. 11. Effect of the interaction on TFC.**

Fig. 12. Effect of the interaction on scavenging activity.

From all the above results, it can be seen that sample from the ultrasonic assisted extraction shows a higher total phenolic content, total flavanoid content when compared to the sample from the soxhlet extraction. DPPH scavenging activity from the ultrasonic assisted extraction is higher compared to soxhlet extraction. The results of phytochemical content from soxhlet extraction (effect of extraction time and effect of solvent) and from ultrasonic assisted extraction (effect of temperature on extraction process, effect of concentration of solvent used for extraction and effect of time of extraction) shows that the ultrasonic assisted extraction yields higher values for phytochemical content. This is due to the cavitation from the ultrasonic waves created in the water medium collapses near the beaker sending shock waves to the cell. The phenomena ruptures the cell wall of the fruit sample which releases the phytochemical content of cell more than fruit sample from soxhlet extraction. However, soxhlet extraction is less effective because cell wall, which is mainly made of cellulose, is tough to decompose as cellulose decomposes at 500℃. The temperature used during soxhlet extraction is not high enough to rupture the cell wall.

4. Conclusions

From this study, the optimum condition for soxhlet extraction of nutmegs for obtaining optimum yield of TPC, TFC and DPPH scavenging is at 184 minutes extraction time by using methanol as the extracting solvent. However, the optimum condition for ultrasonic assisted extraction of nutmegs to obtain optimum yield of TPC, TFC and DPPH scavenging are by extracting nutmeg at 40.512 minutes at 49.99 ℃ and at 40% ethanol concentration. It is also concluded that ultrasonic assisted extraction process yields a consistent value of TPC, TFC and DPPH scavenging activity of nutmegs compared to soxhlet extraction process.

Abbreviations	
Abs	Absorbance
ANOVA	Analysis of variance
DE	Dry weight
DPPH	2, 2-diphenyl picryl hydrazyl
GAE	Gallic acid equivalent
QE	Quercetin equivalent
ROS	Reactive oxygen species
RSM	Response surface methodology
RT	Room temperature
SE	Soxhlet extraction
TFC	Total flavonoid content
TPC	Total phenolic content
UAE	Ultrasonic assisted extraction
UV	Ultraviolet

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