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Research article

Effect of ethanol percentage upon various extraction methods of peanut based on antioxidant activity with *trans*-resveratrol and total phenolic contents

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ABSTRACT

The aim of this study was to determine the effect of ethanol percentage in the extraction solvent on the constituents of peanut extracts prepared from three extraction methods including Solid-Liquid Extraction (SLE), Ultrasound-Assisted Extraction (UAE) and Microwave-Assisted Extraction (MAE). Evaluation was performed through antioxidant tests together with *trans*-resveratrol and total phenolic contents. The ethanol content was set at 20 and 80%v/v. Although extraction with 80%v/v ethanol gave higher percentage yield of the extract, it provided lower resveratrol amount and total phenolic content in all extraction techniques. Lower ethanol content mostly showed positive influence on radical scavenging antioxidant capacity of the extract (DPPH and ABTS assay). Moreover, with DPPH and ABTS assay, *trans*-resveratrol and total phenolic contents demonstrated strong and moderate correlation with antioxidant activity, respectively. On the contrary, solvent with higher percentage of ethanol in SLE and UAE caused higher reducing power extracts with Ferric Reducing Antioxidant Power (FRAP) assay. This implied that, different ethanol composition in SLE and UAE extracted different types of compounds that possessed different mechanisms of antioxidant activity. Concerning extraction methods, UAE was the most effective extraction method which provided the highest antioxidant activity of the extract. According to this study, the percentage of ethanol and extraction methods had significant influence on the composition of the extracts assessed through antioxidant activities as well as *trans*-resveratrol and total phenolic contents.

1. INTRODUCTION

Extraction is a crucial role on bioactivity of the extract. The objective of extraction is to isolate the interesting compounds and discard the insoluble cellular matrix behind[1]. There are many extraction techniques to extract phytochemicals from medicinal plants. Maceration, a traditional method of Solid-Liquid Extraction (SLE), is commonly used in extraction because it is easiest and simplest method. However, it consumes a lot of time and solvent to finish extraction[1,2]. Soxhlet or hot continuous extraction, the sample is placed in a Soxhlet thimble

and the extraction solvent is heated and vaporized through the sample thimble and condense back continuously. This method requires smaller solvent volume but has higher risk in explosion of flammable liquid organic solvents[1,3]. The next ultrasound-assisted extraction (UAE) or sonication method is a green technique that has been reported to extract phytochemicals from plant[4]. UAE does not require a complicated instrument and is relatively low experimental cost[5]. The extraction processes start by initiating cell disruption and increasing mass transfer in the boundary layer surrounding the solid matrix[4]. UAE shows advantages over conventional extraction in terms of high recovery yield and extraction rate[6]. Another green extraction technique, Microwave-Assisted Extraction (MAE), radiates microwave energy to accelerate the partition of analytes from sample matrix to the medium[1]. Microwave radiation damages the cell structure by making severe thermal stress and localizing internal high pressure toward the cell wall which results in the rupture of the cell walls[7]. Finally, the mass of phytochemicals is transferred outside the cell which could increase extraction efficiency and shorten extraction time subsequently[8,9].

Apart from extraction techniques, solvent types and strength was the most influential parameters in almost all extraction methods[10,11]. The common solvents usually use for extraction including water, methanol, ethanol or their mixtures but ethanol is preferable since it is eatable and popularly used in food industry. However, changing solvent composition results in changing solvent polarity which consequently affects the solubility of bioactive compounds and the bioactivities of the extract[6]. Normally, higher ethanol content in the extraction solvent of SLE, UAE and MAE would provide higher extraction yield but not total phenolic content and antioxidant activity[6,12–14].

The extraction parameters for SLE, UAE and MAE, except ethanol content, were selected according to our previous screening results. In this study, the effect of ethanol content on antioxidant activity of peanut extract was determined. Antioxidant activity was evaluated through DPPH, ABTS and FRAP assay. Moreover, total phenolic and *trans*-resveratrol contents were also quantified and figured out the relationship to antioxidant activity.

2. MATERIALS AND METHODS

2.1. Chemicals

2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) or ABTS, 2,2-diphenyl-1-picrylhydrazyl or DPPH, Trolox® and *trans*-resveratrol were purchased from Sigma-Aldrich, China or US. Gallic acid, Folin-Ciocalteu's reagent and 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) were procured from Sisco Research Laboratories (SRL, India). Other chemicals such as Iron(III) chloride hexahydrate, sodium acetate trihydrate and sodium carbonate were used at least analytical reagent grade.

2.2. Preparation of samples

Raw peanuts were bought from a local market at Putamonthon Sai 2 Rd, Bangkok, Thailand. It was cleaned, unpeeled and blended by household blender. The coarse powder was dried at 60°C and then crushed into fine powder and stored at 2-8°C until extraction.

2.3. Extraction Methods

2.3.1. Solid-Liquid Extraction (SLE)

One gram of dried ground peanut kernel was transferred to a 50-mL conical tube. Fifty millilitres of either 20% or 80% (v/v) ethanol was added and incubated for 2 hours in water bath that previously set to 70°C. The sample was filtered through Whatmann No.1 and the filtrate was dried under evaporator at 38°C. Dry extract was kept at -20°C until analysis. The sample was performed six replicates for each condition.

2.3.2. Ultrasound-Assisted Extraction (UAE)

One gram of dried ground peanut kernel was transferred to a 50-mL conical tube. Fifty millilitres of either 20% or 80% (v/v) ethanol was added and sonicated in ultrasonic bath (Crest®, 230D) at 70°C for 2 hours. The sample was filtered through Whatmann No.1 and the filtrate was dried under evaporator at 38°C. Dry extract was kept at -20°C until analysis. The sample was extracted six replicated for each condition.

2.3.3. Microwave-Assisted Extraction (MAE)

One gram of dried ground peanut kernel was extracted with 15 mL of either 20% or 80%

(v/v) ethanol in 100-mL round bottom flask. It was connected to a condenser and extracted by CEM microwave system. Six replications of extraction were operated at 70°C, 300W for one minute. After that, the sample was filtered through Whatmann No.1 and the filtrate was dried under evaporator at 38°C. Dry extract was kept at -20°C until analysis.

2.3.4. Determination of Extraction Yield (%)

The percentage yield of the extract was calculated using the formula:

Extraction yield (%) = a/b × 100

When, a = dry weight extract

and b = dry peanut sample weight

2.4. HPLC method for determination of trans-resveratrol

Content of *trans*-resveratrol was determined by Poroshell 120 EC-C18, 4.6 x 150 mm, 2.7 µm particle size UHPLC column. Mobile phase consisted of 0.1% formic acid in water (A) and 0.1% formic acid in methanol (B). The gradient elution program was 5 min; 22%B, 12 min; 23%B, 28 min; 63%B, 29 min; 80%B, 30 min; 5%B and 36 min; 5%B. Flow rate was 0.3 mL/min and injection volume was 5 µL. Column temperature was set to 40°C. *trans*-Resveratrol was detected at 306 nm and reported as µg/g dry sample. The range of *trans*-resveratrol standard was 0.2 – 10 µg/mL.

2.5. Determination of total phenolic content

The total phenolic content (TPC) method was adapted from Chuenchom et al[15]. The dry extract was dissolved in 10 mL of methanol and 50 µL of this solution was diluted to 1 mL with ultrapure water (UPW). Then, 100 µL of sample solution was mixed with 50 µL of 50% (v/v) Folin-Ciocalteu's reagent and stand for 5 minutes. Finally, 250 µL of 20% (w/v) sodium carbonate was added and the solution was incubated in the darkness. After 2 hours at room temperature, the solution was centrifuged at 9500 rpm for 5 minutes and measured absorbance at 700 nm using microplate reader (ASYS340, USA). Calibration was performed using gallic acid (0.01 – 0.10 mg/mL) as a standard. Total phenolic content was expressed as mg Gallic Acid Equivalents (GAE) per g of dried peanut sample.

2.6. Determination of Antioxidant activity

2.6.1. DPPH radical scavenging activity

DPPH method was adapted from Chuenchom et al[15]. A 50 µL of methanolic extract was diluted to 1 mL with methanol. Then, 100 µL of diluted solution was reacted with 100 µL of 0.2 mM DPPH solution in 96-well plate and kept in dark place. After 30 minutes, the absorbance was read at 515 nm by microplate reader (ASYS340, USA). DPPH radical scavenging activity was compared to Trolox® and reported as milligram Trolox® equivalent per gram dry peanut sample (mgTE/g). The concentrations of Trolox® for calibration curve ranged from 1.25 – 25 µg/mL.

2.6.2. Ferric Reducing Antioxidant Power (FRAP) determination

FRAP was determined using colorimetric method which was adapted from Rebaya et al (16). The FRAP reagent was made by mixing 2 mL of 20 mM $FeCl_3$, 2 mL of 10 mM TPTZ, 2.2 mL of UPW and 20 mL of 300 mM Acetate buffer pH 3.6 and kept at 37°C until analysis. The 50 µL of methanolic extract was diluted to 1 mL with UPW. Then, 50 µL of sample solution was reacted with 150 µL of FRAP reagent in 96-well plate and left for 30 minutes in dark place. The absorbance was measured at 593 nm by microplate reader (ASYS340, USA) and the results were expressed as milligram Trolox® equivalent per gram dry peanut sample (mgTE/g). The concentrations of Trolox® for calibration curve ranged from 5 – 25 µg/mL.

2.6.3. ABTS radical scavenging activity

ABTS antioxidant test was followed Chuenchom et al[15] method with slightly modification. The $ABTS^{+\cdot}$ was generated by mixing 1 mL of 7 mM ABTS solution with 1 mL of 2.4 mM $K_2S_2O_8$ solution and leaving for 14 hours. Then, the radical solution was diluted with methanol until the absorbance value at 734 nm was 0.8±0.02AU. The sample methanolic extract was diluted to concentration of 50 µg/mL. Then, 50 µL of sample solution was reacted with 100 µL of $ABTS^{+\cdot}$ working solution in 96-well plate and left for 30 minutes in dark place. The absorbance was measured at 734 nm by ASYS340 microplate reader, USA. The results were calculated as milligram Trolox®

equivalent per gram dry peanut sample (mgTE/g) by using Trolox® calibration curve equation range of 1.25 – 25 µg/mL.

2.7. Statistical analysis

Data was statistically analysed by Microsoft Excel 2013 software. The descriptive statistic, mean and standard deviation (SD), was used to express a number of quantitated responses. Paired t-test was utilized to compare means between two samples. The differences among group means were analysed by one-way ANOVA and then post hoc analysis by Turkey's HSD test. The relationships between *trans*-resveratrol, total phenolic content and antioxidant capacity were determined statistically by Pearson's correlation analysis. The level of significance for all statistical analysis was 95% confidence interval.

3. RESULTS

Total phenolic and *trans*-resveratrol contents were analysed quantitatively and shown in table 1. TPC and *trans*-resveratrol amount extracted from 20%v/v ethanol (2.62 – 4.18 mgGAE/g and 1.31 – 3.34 µg/g, respectively) were statistically larger than those extracted from 80%v/v ethanol extraction (1.68 – 2.41 mgGAE/g for TPC and 1.11 – 2.41 µg/g for *trans*-resveratrol). On the contrary, higher extraction yield was the only response from 80%v/v ethanol extract.

Table 1. %yield, *trans*-resveratrol and total phenolic of SLE, UAE and MAE extracts

	%yield		*trans*-resveratrol (µg/g)		TPC (mgGAE/g)	
EtOH	20%v/v	80%v/v	20%v/v	80%v/v	20%v/v	80%v/v
SLE	$8.67\pm0.95^{**,b}$	$13.01\pm0.52^{**,b}$	$2.73\pm0.29^{*,b}$	$1.72\pm0.07^{*,b}$	$4.18\pm0.37^{*,a}$	$1.68\pm0.14^{*,b}$
UAE	$10.43\pm0.65^{**,a}$	$24.87\pm0.82^{**,a}$	$3.34\pm0.35^{*,a}$	$2.41\pm0.08^{*,a}$	2.62 ± 0.14^{b}	2.41 ± 0.57^{a}
MAE	$4.32\pm0.72^{**,c}$	$8.32\pm0.39^{**,c}$	$1.31\pm0.14^{*,c}$	$1.11\pm0.03^{*,c}$	$2.62\pm0.45^{*,b}$	$1.76\pm0.12^{*,b}$

mean±SD, n=6

*,**within the row showed significant different using paired t-test ($^{*}p<0.01$, $^{**}p<0.001$)

^{a,b,c} within the column with the different letter were significantly different ($p<0.05$)

The antioxidant values from FRAP, DPPH and ABTS assay were shown in table 2 and were compared by bar graph in figure 1. Antioxidant activity was highest at 80%v/v ethanol peanut extract using FRAP assay (3.54±0.11 mgTE/g). DPPH and ABTS, other antioxidant assays which related to radical scavenging antioxidation, mostly showed greater activities with 20%v/v ethanol extraction and UAE was the most efficient extraction method since its extract possessed better antiradical activity than SLE and MAE.

The relationship between TPC, *trans*-resveratrol and antioxidant activity was demonstrated by Pearson's correlation value and shown in table 3. The strong correlation between *trans*-resveratrol and antiradical activity was observed (r^2=0.784, 0.867 for DPPH and ABTS, respectively). The moderate correlation was found between TPC and DPPH (r^2=0.387) and ABTS assay (r^2=0.437). Additionally, DPPH assay showed strong correlation with ABTS assay (r^2=0.844) while showed moderate correlation to FRAP assay (r^2=0.491). Negative correlation was found between both total phenolic and *trans*-resveratrol contents and FRAP assay.

Table 2. Antioxidant values of peanut extract by FRAP, DPPH and ABTS assay comparing three extraction methods

Extraction method	EtOH mgTE/g	FRAP	DPPH	ABTS
SLE	20%v/v	2.71±0.25	1.61±0.16	0.80±0.02
	80%v/v	3.30±0.14	1.45±0.03	0.66±0.07
UAE	20%v/v	2.59±0.23	1.71±0.12	0.96±0.05
	80%v/v	3.54±0.11	2.05±0.03	0.87±0.06
MAE	20%v/v	2.97±0.41	1.23±0.12	0.66±0.04
	80%v/v	2.72±0.22	0.98±0.04	0.53±0.07

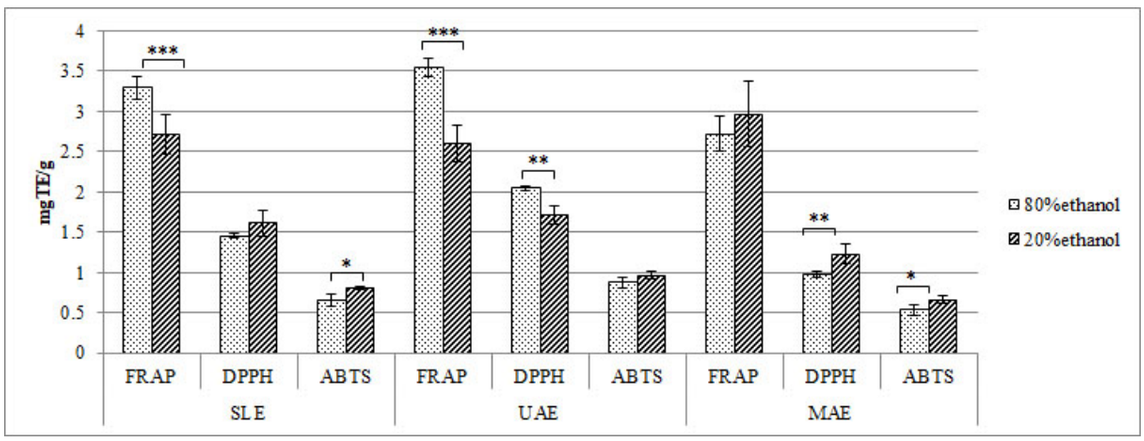

Figure 1. Antioxidant capacities of SLE, UAE and MAE extracts
*,**,*** showed significant different using paired T-test (*p<0.05, **p<0.01, ***p<0.001)

Table 3. Pearson's correlation of *trans*-resveratrol, total polyphenols, and antioxidant capacity (FRAP, DPPH and ABTS)

	trans-resveratrol	TPC	FRAP	DPPH
TPC	0.559[d]			
FRAP	-0.029	-0.149		
DPPH	0.784[d]	0.387[d]	0.491[d]	
ABTS	0.867[d]	0.437[d]	0.089	0.844[d]

n = 32, [d]Correlation is significant at p<0.05.

4. DISCUSSION

The %yield, TPC and *trans*-resveratrol amount in table 1 showed that higher %yield did not relate to the levels of *trans*-resveratrol and total phenolic content. *trans*-Resveratrol and TPC were greater extracted by 20%v/v ethanol than 80%v/v ethanol ($p<0.010$). This finding was in agreement with Karacabey E and Mazza G's work which revealed that *trans*-resveratrol diffusivity decreased with an increase in ethanol concentration beyond 55%v/v[17]. Focusing on extraction techniques, UAE could extract *trans*-resveratrol better than SLE and MAE ($p<0.010$). These might be from the superior fundamental theory of UAE that can cause a disruption of plant cells and facilitate mass transfer of cellular components into an extraction media[4]. This result was in accordance with the work of Garcia et al which extracted *trans*-resveratrol from peanut grass with three extraction techniques and concluded that the best extraction method was obtained by UAE[18]. Additionally, the amount of *trans*-resveratrol in our study by UAE at 20%v/v ethanol (3.34±0.35 µg/g) was higher than the previous studies[19–22]. The highest total phenolic content (4.18±0.37 mgGAE/g dry sample) was obtained when extracted with SLE at 20%v/v ethanol content and it was higher than previous publications[23–25] but lesser than the studies of Attree et al and Khang et al[26,27]. The reason might be from difference cultivars of peanut[25] and germination period[27,28]. The highest TPC value was disclosed at 20%v/v ethanol extraction and it was consistent with the study of Ballard et al who reported that 30%v/v ethanol content produced the highest yields of phenolic compounds and it ecreased with increasing ethanol concentration[29]. This phenomenon was explained by the higher amount of water in extraction solvent creating a more polar medium that can facilitate the extraction of polyphenols[30]. Comparing extraction methods, SLE with 20%v/v ethanol gave the highest TPC value. This finding contradicted the study of Jovanović et al who concluded that UAE provided highest TPC extract[30] and Ballard et al who reported that MAE was the most efficient method to extract TPC[8]. The reason might be from a high extraction temperature of UAE (70°C) in our study which could lead to a degradation of some polyphenols[31].

Antioxidant activity of peanut extract was determined by three analytical methods; FRAP, DPPH and ABTS. Comparison of antioxidant capacity among 3 extraction techniques in Figure 1 found that peanut extracts showed good antioxidant activity to FRAP assay which corresponded to the study of Chai et al[28.] The compounds can act as an antioxidant through the reduction mechanism of oxidized intermediate in the chain reaction[32]. The higher percentage of ethanol content in SLE and UAE gave the higher reducing power extract by FRAP assay. This suggested that different types of compounds which acquired antioxidant activity through reduction mechanism were also extracted. Although higher reducing power extract in this study had found mostly in 80%v/v ethanol extraction of SLE and UAE, it has been reported that the major anti-oxidation mechanism of phenolic compounds in peanut was from radical scavenging property[15] which could refer to DPPH and ABTS assay. Anti-oxidant capacity of peanut extracts measuring by DPPH and ABTS assay found that 20%v/v ethanol extraction provided better activities than 80%v/v ethanol extraction, except UAE. This might be due to the greater total phenolic content found in 20%v/v ethanol extraction samples, and antioxidant activity mostly attributes to those phenolic compounds[33]. Among different extraction methods, UAE mostly exhibited higher antioxidant activity of the extract than SLE and MAE. The privilege of UAE might be caused by the large amount of extraction yield (Table 1). The dry extract likely contained a wide range of compounds that could also act as antioxidant such as flavonoids, triterpenes, alkaloids or phytosterols[33]. The result was supported by the study of Jovanović et al and Trusheva et al that UAE provided higher total flavonoid content than SLE[30] and MAE[2].

To explain the relationship between phenolics and antioxidant activity of the extract, correlation analysis was carried out and shown in table 3. The result found that there was strong correlation between *trans*-resveratrol and antiradical activity, DPPH and ABTS (r^2=0.784, 0.867 respectively). Moreover, the TPC showed weak to moderate correlation with DPPH and ABTS assay (r^2=0.387, 0.437 respectively) but had no relationship with FRAP assay. The correlation analysis might imply that the radical scavenging

activity of the peanut extract was mainly from *trans*-resveratrol and other phenolic compounds while the reducing power of the extract may contribute by other types of chemical components. This finding was supported by the study of Fidrianny et al who claimed that there was a strong correlation between total flavonoid content and FRAP value ($p<0.01$)[24]. However, higher activity in DPPH assay of UAE with 80% v/v ethanol than with 20% v/v ethanol might result from much more % yield of in UAE with 80% ethanol. Additionally, DPPH assay revealed positive correlation with ABTS assay ($r^2=0.844$). This was because DPPH and ABTS have the same radical scavenging antioxidant mechanism so samples showed high antioxidant capacity with DPPH assay, usually showed high antioxidant capacity with ABTS assay as well.

5. CONCLUSIONS

The extractability of antioxidants from peanut kernels revealed that ethanol concentration and extraction methods had a significant effect on composition of the extracts and the antioxidant property of the extracts. Lower ethanol content in extraction medium mostly had positive influence on *trans*-resveratrol amount, TPC and radical scavenging antioxidant activity by DPPH and ABTS assay. Additionally, TPC showed weak to moderate while *trans*-resveratrol showed strong positive correlation with radical scavenging activity, DPPH and ABTS test. Furthermore, UAE provided higher antioxidant capacity of the extract than SLE and MAE. It can be concluded that using of UAE together with lower ethanol concentration extraction solvent seemingly be more appropriate extraction condition. However, other factors such as optimum extraction temperature and time can also affect the antioxidant property of the extract and should be investigated to find the best extraction condition.

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Conflict of interest (If any)

All contributing authors declare no conflicts of interest

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