In vitro Anti-oxidative Activity and Tyrosinase Inhibition of Inca Peanut (*Plukenetia volubilis* L.) Shell Extracts from Different Preparation Methods

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Received: December 4, 2019; Accepted: December 20, 2019

Abstract

In the present investigation, the bioactivities of the inca peanut (*Plukenetia volubilis* L.) shell extracts prepared by various methods were determined for food and cosmetic applications. The inca peanut shells were extracted and determined total phenolic content, anti-oxidation by DPPH assay, tyrosinase inhibition and phytochemical analysis. The results showed that the extract from non-cooked shell before extracting by the hot mixture of water and ethanol (NM2) gave the highest total phenolic content at 129.95±7.58 mg GAE/g and the highest free radical scavenging activity at SC_{50} of 0.12±0.03 mg/mL. The highest inhibition of tyrosinase activity was presented by the extract from roasted shell before extracting by cold ethanol (RE1) at IC_{50} of 6.90±1.40 mg/mL. In addition, the selected extracts contained the phytochemical contents of flavonoids and triterpenoids. This study has demonstrated the anti-oxidation and tyrosinase inhibition potential of the inca peanut shell extracts.

Keywords: *Plukenetia volubilis*; total phenolic content; free radical scavenging; tyrosinase inhibition; phytochemical analysis

1. Introduction

Plukenetia volubilis L., a plant in the Euphorbiaceae family commonly called inca peanut, is a climbing shrub and oleaginous plant that grows mostly in the rain forest of South America (Nascimento *et al.*, 2013). The star-shaped green fruits contains edible dark brown seeds (Fanali *et al., 2011)*. The seeds are composed of kernel and shell at 66 and 34 %, respectively. The oil from inca peanut seeds contains a rich source of fatty acid with 34 % of linoleic acid and 51 % of linolenic acid (Chirinos *et al.*, 2016). The seeds and leaves reduce the lipid profile in postprandial lipemia and hypercholesterolemia (Nascimento *et al.*, 2013), while tocopherols are found in the nut gave health benefits for cancer, type 2 diabetes and heart disease (de Souza *et al.*, 2013). Several

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DOI: 10.14456/tjst.2020.30

investigations supporting the biological effects of inca peanut oil have been studied. However, there are few available researches about the bioactivities of shell extracts prepared by various methods. Preparation with various methods to inca peanut shell may affect their biological activity. This study was aimed to investigate total phenolic contents, anti- oxidative activity, tyrosinase inhibition and phytochemical analysis of inca peanut shell extracts prepared by different preparations. The results of this work provide the proper preparation of inca peanut shell extracts for further development as a novel bioactive compounds for food and cosmetic applications.

2. Materials and methods

2.1. Materials

All chemicals were of analytical grade. Folin-Ciocalteu reagent, L-(+)-ascorbic acid (vitamin C), kojic acid, L-DOPA tyrosinase from mushroom (2687 units/mg solid), dimethyl sulfoxide (DMSO), 2,2-diphenyl-1-picryhydrazyl radical (DPPH) and butylated hydroxytoluene (BHT) were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). Inca peanut shells were obtained from Mea Tang province, Chiang Mai, Thailand.

2.2. Inca peanut shell extraction

Inca peanut shells were ground by a grinder. The shells were processed as non-cooking (N), heating by oven (O) and roasting (R). For heating by hot air oven, the shells were heated for overnight at 50 °C. For roasting, the shells were heated for 5 min in an iron pan at 150 °C with continuous stirring. Before extraction by cold or hot process, 20 g of the processed inca peanut shell was soaked for 24 h in 200 L of water (W), 95 % ethanol mixed with water at 1 : 1 (v/v) (M) or 95 % ethanol (E). For the extraction by cold process (1), the samples were shaken by an orbital shaker at room temperature for 2 h at 90 rpm. The samples extracted by hot process (2) containing 95 % ethanol were refluxed for 2 h at 70±2 °C, while the samples extracted using water were boiled at 100±2 °C for 2 h by hot plate. The filtrates were concentrated and lyophilized by a rotary evaporator (Eyela N-1001, Tokyo, Japan) and freeze dryer (Eyela FDU-2110, Tokyo, Japan), respectively. The various processes of inca peanut shell extraction were abbreviated and shown in Table 1.

2. 3. Determination of total phenolic content

Total phenolic content of inca peanut extracts was measured by Folin- Ciocalteu colorimatric assay using 96-well microplate with slightly modification (Zhang *et al.*, 2006). Initially, the extracts at 10 mg/mL were prepared and dissolved in 10 % DMSO. 25 µL of the extracts solution was mixed in a 96-well plate with 50 µL of 20% Na_2CO_3 and 25 µL of 1 N Folin-Ciocalteu reagent, covered the plate and incubated at 25±2 °C for 2 h in the dark. The absorbance at 730 nm was measured by the microplate reader (BioTek Synergy H1, VT, USA). All the samples were assayed in triplicate. The calibration curve of gallic acid was used and calculated the total phenolic contents as following equation:

Absorbance = 8.9936 x gallic acid (mg/mL) + 0.0028; (R^2 = 0.99994). The total phenolic content was presented as mg/g gallic acid equivalents (GAE).

Table 1 Abbreviations of the inca peanut shell extracts

System	Abbreviations	Description
Solvent	E	Ethanol
	M	Mixture of ethanol and water (1 : 1)
	W	Distilled water
Process before solvent extraction	N	Non cooking
	O	Heating by oven
	R	Roasting
Extraction	1	Cold extraction
	2	Hot extraction

2.4. Free radical scavenging assay

Free radical scavenging activity of inca peanut extracts was evaluated by a modified DPPH method in 96-well plate (Tachibana *et al*., 2001). Each well contained 50 µL of the samples dissolved in 10 % DMSO at 0.001-10 mg/mL and an equal volume of DPPH solution. The absorbance at 515 nm was measured after incubation at 25±2 °C for 30 min. The positive controls at 0.001-10 mg/mL were vitamin C, kojic acid and BHT. All the samples were assayed in triplicate. The samples were calculated the antioxidant activity as follows: % scavenging activity = [($AB_{control}$ - AB_{sample}) ÷ $AB_{control}$] x 100, where $AB_{control}$ = the absorbance of the control and AB_{sample} = the absorbance of the sample. SC_{50} value was obtained from the sample concentration at 50 % scavenging activity.

2.5. Tyrosinase inhibition assay

Tyrosinase inhibition activity of inca peanut extracts was evaluated by using L-DOPA and mushroom tyrosinase (Masuda *et al*., 2005). In 96-well plate, the samples dissolved in 10 % DMSO, 20 µL of the samples at 0.001-10 mg/mL, 40 µL of 0.1 M phosphate buffer at pH 6.8 and 20 µL of tyrosinase solution in phosphate buffer (46 units/ml) were added. The plate was allowed to react at 25±2 °C for 10 min and then added 20 µL of 2.5 mM L-DOPA in phosphate buffer. The absorbance at 475 nm was measured after incubation at 25±2 °C for 10 min. The positive controls at 0.001–10 mg/ml were vitamin C, kojic acid and BHT. All the samples were assayed in triplicate. The samples were calculated tyrosinase inhibition activity as follows: % tyrosinase inhibition = [($AB_{control}$ - AB_{sample}) ÷ $AB_{control}$] x 100, where $AB_{control}$ = the absorbance of the control and AB_{sample} = the absorbance of the sample. IC_{50} value of tyrosinase inhibition was obtained from the sample concentration at 50 % inhibition activity.

2.6. Preliminary phytochemical analysis

The inca peanut shell extracts were investigated phytochemical analysis of carotenoid, tannin, flavonoid, steroid and terpenoids. 0.05 g of the extract was added 100 µL of the concentrated H_2SO_4 and 2 mL of chloroform for carotenoid investigation. The presence of carotenoid indicated the blue or green solution (Trease and Evans, 2002). 2 mL of the extract solution was added magnesium ribbon and 1 mL of the concentrated HCl for flavonoids investigation. The presence of flavonoids indicated the pink-red color (Allen *et al*., 1974). 2 ml of the extract dissolved in ethanol was added 100 µl of $Ca(OH)_2$ solution for tannin investigation. The presence of tannin indicated the formation of marine to grey precipitate (Evans, 1991). 0.05 g of the extract was added 2 mL of dichloromethane, sonicated for 15 minutes and filtrated for steroids and triterpenoids investigation. The filtrate was dried in an evaporating dish, allowed to cool to room temperature, mixed with 20 µL of acetic anhydride and then added 20 µL of concentrated sulphuric acid. The presence of steroids and triterpenoids showed green and deep red color, respectively (Bhandary *et al*., 2012).

2.7. Statistical analysis

Results were evaluated in three replicates and reported as means ± standard deviations (SD). Significant difference was considered at $p < 0.05$.

3. Results

3. 1. Percentage yields and physical characteristics

The percentage yields and physical characteristics of the extracts prepared by different solvents, temperatures and processes were summarized in Tables 2. Most extracts were brown color and solid form, whereas the extracts from cold extraction by ethanol, OE1 and RE1, were oily semisolid form. The extracts obtained from ethanolic extraction may contained both polar and non-polar compounds. The extracts gave percentage yields ranging from 2.35 to 24.60 %. The lowest and highest percentage yield were from the extract from the shell roasted before extracting by cold ethanol (RE1) and the extract from the non-cooked shell before hot water extraction (NW2), respectively.

3.2. Total phenolic content

The inca peanut shell extracts were presented total phenolic content in Table 2. The extracts were shown total phenolic content varied from 43.44 to 129.95 mg of GAE/g. The extract from the non-cooked shell before hot extraction by the mixture of water and ethanol (NM2) presented the highest total phenolic content with GAE value of 129.95±7.58 mg/g. The extraction by alcoholic solvents has been provided non-selective phenolic constituents from plant sources. The lowest total phenolic content was observed in the extract from the shell roasted before extracting by cold ethanol (RE1) with GAE value of 43.44 ± 1.45 mg/g. The extracts by hot solvents extraction gave significantly higher total phenolic content than the extracts by cold solvents extraction (NM2, OE2, OM2, RE2 and RM2) at $p < 0.05$ as shown in Table 2.

Table 2 The percentage yield and total phenolic content of the inca peanut shell extracts

Processes	Solvents	Samples	Appearance and color	Percentage yield (%)	Total phenolic content (mg GAE/g ± SD)
Non cooking (N)	Ethanol (E)	NE1	Solid in light brown	2.75	53.31±0.87
		NE2	Solid in dark brown	5.00	49.12±0.63
	Mixture (M)	NM1	Solid in brown	9.40	90.09±2.63
		NM2	Solid in brown	13.10	129.95±7.58*
	Water (W)	NW1	Solid in dark brown	11.40	114.16±3.72
		NW2	Solid in brown	24.60	111.90±6.65
Heating by oven (O)	Ethanol (E)	OE1	Oily liquid in dark green	2.40	44.03±0.82
		OE2	Solid in dark brown	5.20	49.83±2.24*
	Mixture (M)	OM1	Solid in brown	10.45	80.31±3.12
		OM2	Solid in brown	12.60	125.85±2.11*
	Water (W)	OW1	Solid in dark brown	12.70	111.34±1.78
		OW2	Solid in dark brown	13.20	114.94±2.53
Roasting (R)	Ethanol (E)	RE1	Oily liquid in brown	2.35	43.44±1.45
		RE2	Solid in dark brown	3.95	64.68±0.65*
	Mixture (M)	RM1	Solid in dark brown	10.30	79.90±0.96
		RM2	Solid in brown	14.00	113.14±4.17*
	Water (W)	RW1	Solid in dark brown	12.30	103.10±2.39
		RW2	Solid in brown	12.05	102.74±1.53

Percentage yield (%) = [Dried extract weight (g)/dried inca peanut shell weight (g)] x 100; GAE = gallic acid equivalents; The asterisk (*) indicated significant difference compared with the cold extraction ($p < 0.05$).

3.3. Free radical scavenging activity

Table 3 presented the free radical scavenging activity of the inca peanut shell extracts. The highest activity was from the extract of non-cooked shell before extracted by the hot mixture of water and ethanol solvent (NM2) at SC_{50} values of 0.12±0.03 mg/mL, which was 0.43 folds of ascorbic acid (0.05±0.01 mg/mL). The previous study investigated the effects of kernel maturity of inca peanut cultivated in Thailand on antioxidant activity and chemical constituents. The effect of temperature on the anti-oxidative activity of the extracts from the mixture of ethanol and water showed that the extracts by hot solvents exhibited higher free radical scavenging activity than the extracts by

cold solvents. The hot extraction gave the high contents of heat stable total phenolic compounds as previous described. These results implied that the hot extraction by the mixture of water and ethanol was suitable for prepared the inca shell peanut extracts with high content of total phenolic compound and free radical scavenging activity. The percentages of free radical scavenging activity of inca peanut shell extracts and the standard agents with the high concentration at 1.00 mg/mL were presented in Figure 1. Ascorbic acid, kojic acid and BHT gave the free scavenging activity of 88.87±1.03, 37.47±4.60 and 70.71±5.12 %, respectively. The ethanolic extracts at the concentration of 1.00 mg/mL showed free radical scavenging activity significantly greater than the mixture of water and ethanol extracts (M) and water extracts (W) with $p < 0.05$.

Table 3 Free radical scavenging and tyrosinase inhibition activity of the inca peanut shell extracts

Processes	Samples	Free radical scavenging				Tyrosinase inhibition		
		SC_{50} ± SD (mg/mL)	Fold of vitamin C	Fold of kojic acid	Fold of BHT	IC_{50} ± SD (mg/mL)	Fold of kojic acid	Fold of vitamin C
Non cooking (N)	NE1	0.38±0.03	0.14	9.65	3.43	ND	-	-
	NE2	0.33±0.07	0.16	11.09	3.94	ND	-	-
	NM1	0.24±0.02	0.22	15.31	5.44	ND	-	-
	NM2	0.12±0.03*	0.43	29.68	10.54	>1000	-	-
	NW1	0.24±0.08	0.22	15.29	5.43	61.64±6.65	0.0019	0.0039
	NW2	0.20±0.02	0.26	18.28	6.49	>1000	-	-
Heating by oven (O)	OE1	0.16±0.04	0.33	23.05	8.19	>1000	-	-
	OE2	0.19±0.01	0.28	19.06	6.77	ND	-	-
	OM1	0.30±0.09	0.18	12.20	4.33	ND	-	-
	OM2	0.19±0.03*	0.27	18.95	6.73	ND	-	-
	OW1	0.15±0.07	0.36	24.91	8.85	7.68±2.03	0.00156	0.0313
	OW2	0.21±0.03	0.25	17.46	6.20	>1000	-	-
Roasting (R)	RE1	0.30±0.05	0.18	12.19	4.33	6.90±1.40	0.0174	0.0348
	RE2	0.21±0.04	0.26	17.76	6.31	ND	-	-
	RM1	0.38±0.07	0.14	9.65	3.43	19.52±0.84	0.0061	0.0123
	RM2	0.21±0.04*	0.25	17.54	6.23	ND	-	-
	RW1	0.43±0.05	0.12	8.51	3.02	>1000	-	-
	RW2	0.30±0.06*	0.18	12.12	4.30	>1000	-	-
	Vitamin C	0.05±0.01	-	-	-	0.24±0.03	-	-
	BHT	1.30±0.59	-	-	-	ND	-	-
	Kojic acid	3.66±1.02	-	-	-	0.12±0.02	-	-

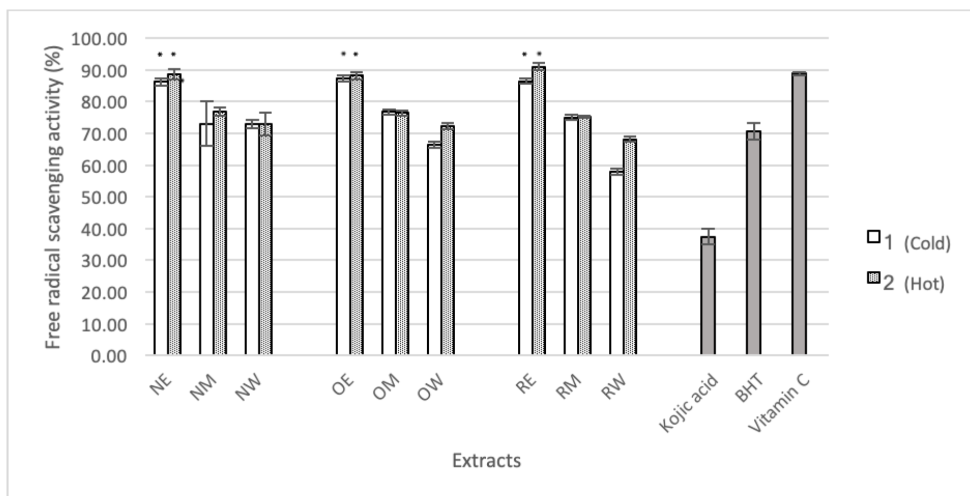

Figure 1 Comparison of the percentages of free radical scavenging activity of the inca peanut shell extracts and the standard agents at 1.00 mg/mL (kojic acid, BHT and vitamin C). The asterisk (*) indicated significant difference compared with the mixture of ethanol and water extracts (M) and the water extracts (W) ($p < 0.05$)

Table 4 Total phenolic content, free radical scavenging activity, tyrosinase inhibition activity and phytochemical analysis of the selected inca peanut shell extracts

Samples	mg GAE/g±SD	SC_{50} ± SD (mg/mL)	IC_{50} ± SD (mg/mL)	Phytochemical analyses				
				Flavonoids	Tannins	Triterpenoids	Steroids	Carotenoids
NW1	114.16±3.72	0.24±0.08	61.64±6.65	+	-	+	-	-
OW1	111.34±1.78	0.15±0.07	7.68±2.03	+	-	+	-	-
RM1	79.90±0.96	0.38±0.07	19.52±0.84	++	-	+	-	-
RE1	43.44±1.45	0.30±0.05	6.90±1.40	+++	-	++	-	-

3+ to 1+ represented the intensity content of the phytochemical constituents in the selected extracts; GAE = gallic acid equivalents; SC_{50} = the sample concentration providing 50 % of free radical scavenging activity; IC_{50} = the sample concentration providing 50 % of tyrosinase inhibition activity.

3.4. Tyrosinase inhibition

The tyrosinase inhibition of the inca peanut shell extracts was shown in Table 3. The highest tyrosinase inhibition activity was from kojic acid at IC_{50} value of 0.12±0.02 mg/mL. NW1, OW1, RE1 and RM1 extracts gave the IC_{50} values of 61.64±6.65, 7.68±2.03, 6.90±1.40 and 19.52±0.84 mg/mL, respectively. In Table 3, all extracts indicated no correlation between free radical scavenging and tyrosinase inhibition activity. RE1 extract exhibited the highest tyrosinase inhibition, which was 0.0348 and 0.0174 folds comparing with ascorbic acid and kojic acid, respectively. RE1 extract prepared by cold

extraction may contained high contents of the heat labile components which involved in tyrosinase inhibition activity.

3.5. Phytochemical analysis

In Table 4, the selected extracts were presented total phenolic content, free radical scavenging activity, tyrosinase inhibition activity and phytochemical analysis. All extracts contained flavonoids and triterpenoids. The intense amounts of flavonoids and triterpenoids were observed in the extracts of RE1 at 3+ and 2+ level, respectively.

4. Discussion

In this study, we examined the bioactivities of the inca peanut shell extracts prepared by various methods. The results of this study demonstrated that the high percentage yields were obtained by the heated solvent extraction because of releasing the cellular constituents by breaking down the plant cell wall (Manosroi *et al.*, 2016). From previous studies, the kernels were measured the total phenolic content in a range of 0.06-1.41 mg GAE/g (Štěrbová *et al.*, 2017), while the shell contained at 74.56 mg GAE/g (Wang *et al.*, 2018). Phenolic compounds containing aromatic ring with hydroxyl groups, which were accumulated in the polar soluble part of plant vacuoles could be easily extracted by the polar solvents (Rispail *et al.*, 2005). The extraction of phenolic compounds by using the mixtures of alcohols and water has more effective than the corresponding mono-solvent system (Spigno and de Faveri, 2007). In general, polyphenolic compounds were more effectively extracted by using ethanol at 40-50 % (Jayaprakasha *et al.*, 2008). Furthermore, the samples yield and total phenolic content were increased because of using high temperature extraction, which can affect the cell wall structure of plant cells and released the phenolic-matrix bonds (Prasad *et al.*, 2009). An important role of temperature on the extraction process may explain that high extraction temperature enhances the diffusion coefficient and the phenolics solubility (Corrales *et al.*, 2009). The radical scavenging activity of Inca peanut extracts against DPPH radical was also assessed. According to many reports, the DPPH assay presented free radical scavenging activity at 9.36-13.72 % (Singanusong and Jiamyang yuen, 2018). The inca peanut shell extracts by various solvent were demonstrated the antioxidant capacities by ORAC, FRAP and ABTS assay at the values of 92.5-192.6, 45.0-114.0 and 34.1-93.9 µmol/TE g, respectively (Wang *et al.*, 2018). The anti-oxidant mechanism of these phenolic compounds was scavenging activity by transferring the single electron (Choi *et al.*, 2002). Some polyphenol constituents found in the inca peanut shell extracts were also the groups of tyrosinase inhibitors by the roles of both direct inhibition and competed substrates of tyrosinase (Chang, 2009). The presence of phytocompounds as terpenoids and flavonoids could explain the results which they were found in inca peanut seeds (Arroyo-Acevedo *et al.*, 2018). Interestingly, flavonoid and triterpenoids which found in the extracts of inca peanut shell may involve tyrosinase inhibition activity. When

tyrosine was used as a substrate by tyrosinase, flavonoids which chelated copper in the active site of tyrosinase showed the tyrosinase inhibitory activity (Cuellar *et al.*, 2001; Tazawa, 2001).

5. Conclusion

This research presented the preparation and biological activities assay of inca peanut shell extracts. The extract from the non-cooked shell before hot extraction by the mixture of water and ethanol (NM2) gave the highest total phenolic content and free radical scavenging activity, while the extract from the shell roasted before extracting by cold ethanol (RE1) showed the highest of tyrosinase inhibition activity. These extracts exhibited the potential of the new bioactive extracts from inca peanut shells that can be used as a natural antioxidant and whitening agent for food and cosmetic applications.

6. Acknowledgement

This study was funded from the 2018 grant of the School of Pharmaceutical Sciences, University of Phayao, Phayao, Thailand.

7. References

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