Effects of Binary Solvent Extraction System and Extraction Time on Antioxidant Activity from Roselle (Hibiscus Sabdariffa L.) Seeds

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Abstract: Roselle (Hibiscus sabdariffa L.) has been broadly utilized in nourishment industry, particularly its petal part. Notwithstanding, the roselle seeds are considered as waste despite the fact that it was conceivably recognizable as cancer prevention agent sources. The point of this investigation was to decide the best parameter (term and dissolvable) for removing Roselle (Hibiscus sabdariffa L.) seeds by a beat ultrasonic-helped extraction. The cell reinforcement exercises of ultrasonic-helped Roselle (Hibiscus sabdariffa L.) seeds were assessed by a 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical rummaging limit test, 2,2'- azino-bis(3-ethylbenzothiazoline-6-sulphonic corrosive) (ABTS) radical searching limit examine, ferric diminishing cancer prevention agent control (FRAP) measure, and β-carotene fading hindrance test. Add up to phenolic content (TPC) and aggregate flavonoid content (TFC) assessments were done to decide the phenolic and flavonoid substance in Roselle (Hibiscus sabdariffa L.) seeds separate. The outcome displayed that the best extraction parameter utilized 80% ethanol for 10 minutes.

Keywords: binary; solvent; Roselle; extraction system; Antioxidant

I. INTRODUCTION

Natural antioxidant found in plants, including fruits and vegetables are rich in bioactive compounds[1]. Bioactive compounds have a major role in antioxidant activity that can decrease the effect of damage due to oxidative stress [2]. Synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), mono-tert-butyl hydroquinone (TBHQ) have been commonly used in food processing to prevent the oxidation[3]. On the other hand, use of synthetic antioxidant could lead to health risk and toxicity risk[4]. Hence, the usage of antioxidants from natural sources keeps increasing. Studies of natural antioxidant have been developed due to the increasing number of public awareness about the dangers of synthesis antioxidant[3]. Natural antioxidant is contained in many herbs, spices, and plants[5]. Antioxidant activity has been revealed in roselle plant extract, since the medicinal benefits have been widely developed[6]. The part of roselle petals is widely used in the process of jam, jellies, and beverage[7]. On the other hand, the use of roselle plants causes some of their seeds to be wasted[3]. Therefore, roselle seeds were solely become waste.

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However, the antioxidant properties of roselle seeds have also been reported due to their high phenolic acid content in roselle seed oil extract, such as vanillic, caffeic, gallic, ferulic, p-hydroxybenzoic, protocatechuic, p-coumaric acids[8].

The total phenolic compound of roselle seed show varying result due to difference in methods and solvents[9]. Ultrasonic-assisted, microwave extraction and supercritical fluid extraction are some of the new extraction methods[10]. Ultrasonic-assisted extraction is one of the most modern extraction techniques used in food processing since conventional solvent extractions has less efficiency and more time consuming[11]. Furthermore, ultrasonic extraction is more efficient and consume less energy[12]. Ultrasonic-assisted extraction will increase the extraction yield because its frequency can break down the sample matrix to improve diffusion[11].

This study investigated the antioxidant properties of roselle (*Hibiscus sabdariffa L.*) seeds extracted with pulsed ultrasonic-assisted extraction. The objective of this research were to determine the optimum parameters (ethanol concentration and extraction time) of roselle seed with pulsed ultrasonic-assisted extraction and to compare the antioxidant activity among the different antioxidant measurement methods.

II. METHODOLOGY

Sample

Dried roselle (Hibiscus sabdariffa L.) seed was attained from the Malaysian Agricultural Research and Development Institute (MARDI, Serdang, Malaysia) and was ground into powder with a processor (Panasonic, Japan). The roselle seed powder molecule estimate was ±1 mm. The extraction of roselle seed was performed utilizing Pulsed Ultrasonic-Assisted Extraction[13]. Fifty grams of roselle seed powder was added to the dissolvable that readied in various focus (60%, 80%, and 100%) ethanol. Ultrasonic extraction (Ultrasonic Homogeniser Labsonic P, 400W, Sartorius, AG) was embraced with a 5 min beat term period and 5 min beat interim period.

Antioxidant Activities 2,2-Diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Capacity Assay

The DPPH radical searching measure was executed as portrayed before as indicated by Thoo et al[14]. The examples remove (200µL) were blended with 1 mL of 0.004% DPPH arrangement and 2.8 mL of ethanol.

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The absorbance of the subsequent arrangements and the clear (with same synthetic concoctions, aside from test) were estimated following 30 minutes hatching. The vanishing of DPPH• was perused spectrophotometrically at 517 nm utilizing UV-Vis spectrophotometer (Model XTD 5, Secomam, Domont, France).

2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulphonicAcid) (ABTS) Radical Scavenging Assay

ABTS radical searching test was resolved utilizing technique for Chandel et al[15]. with slight adjustment. ABTS radical cations were created by blending the 7mM ABTS and 2.45mM potassium persulphate arrangement. The blend was permitted to remain in obscurity with room temperature for 12-16 hours before use (enactment). 20 µL of test was added to 1980 µL of ABTS and permitted to respond for 6 minutes. The absorbance was taken up at 734 nm which estimated by utilizing UV-Vis spectrophotometer (Model XTD 5, Secomam, Domont, France).

Ferric Reducing Antioxidant Power (FRAP)

Ferric lessening cell reinforcement control (FRAP) was resolved dependent on the technique portrayed by Wong et al[13]. The FRAP arrangement was set up by blending 25 mL of acetic acid derivation cradle (300 mM) with 2.5mL of 10mM 2,4,6-tripyridyl-striazine (TPTZ) and 2.5 mL of FeCl3· 6H2O (20mM). The blend was hatched at 37°C for 30 minutes before being use. Fifty milliliters of the example is added to 950 □L of FRAP reagent in a test tube. The blend was permitted to respond for 30 minutes in obscurity at room temperature. The absorbance was estimated at 593 nm by utilizing UV-Vis spectrophotometer (Model XTD 5, Secomam, Domont, France).

β-Carotene Bleaching (BCB) Inhibition Assay

β-Carotene Bleaching movement was resolved as portrayed by Mohd-Esa, et al[6].and Vancoselos, et al[16]. with marginally adjustment. One milliliter of a 0.2 mg/ml β-carotene arrangement in chloroform was added to round base jars containing 0.02 ml of linoleic corrosive and 0.2 ml of Tween-20. The chloroform was expelled at 40°C under vacuum. The resultant blend was weakened with 100 mL of refined water promptly and blended for 1– 2 min to shape an emulsion. A clear blend was arranged likewise yet without β-carotene and a control containing 0.2 ml of 80% (v/v) methanol rather than concentrate.

The aliquot (3 mL) of the emulsion was added to a cylinder containing 0.1 ml of the example separate, at that point put in water shower to be hatched and shaken at 50°C for 30 minutes. Absorbance was perused at 470 nm at 15 min interims, utilizing an UV– Vis spectrophotometer (Model XTD 5, Secomam, Domont, France). The cancer prevention agent movement of each example was determined as percent hindrance in respect to the control as indicated by Wong, et al[13].

Total Phenolic Content (TPC)

The aggregate phenolic content test was characterized by utilizing Folin-Ciocalteu measure as depicted by Wong, et al[13]. and Al-Okbi, et al[17]. Roselle seed concentrate and roselle seed oil extricate (300 □L) was added to 0.5 mL of refined water and 1.25 mL Folling-Ciocalteu reagent. The blend was shaken to be blended well and permitted to remain at room temperature for 6 minutes before including 1.2 mL of sodium carbonate (7.5%, w/v) arrangement. At that point, the blend was blended throughly and permitted to represent 30 minutes in obscurity. After hatching, the absorbance was estimated at 765 nm utilizing UV-Vis spectrophotometer (Model XTD 5, Secomam, Domont, France) which was perused versus the clear reagent.

III. RESULT AND DISCUSSION

Extraction Solvent Evaluation

Table 1 shows Antioxidant Activity of Roselle seed extract.According to the previous study, using binary solvent would improve the extraction of phenolic compounds[19]. A binary solvent that consists of ethanol and water would be necessary to enhance the extraction of bioactive compounds with different solubility and polarity[20]. Aqueous solvent showed a superior extraction ability than the pure solvent because the combination of polar and non-polar solvent might raise the polarity index of solvents and enhance the extraction efficiency[21]. Ethanol and water can be used together for increasing the polarity of solvent, considering that ethanol is a low polar solvent and water is strong polar solvent[22]. Durling et al[23]. declared that combination of ethanol and water may be more suitable for sage extraction because of the varies polarities of bioactive compounds.

Table. 1 Antioxidant Activity of Roselle seed extract by different ethanol concentration and extraction time

SAMPLE		Antioxidant Activity					
[C] (%)	t (mins)	DPPH (%)	ABTS (%)	FRAP (mg TEAC/ 100 g)	BCB (%)	TPC (mg GAE/ 100 g)	TFC (mg CE/ 100 g)
60	5	36.58 ± 3.59^{f}	60.81 ± 0.58^{e}	302.67 ± 8.70^{e}	21.63 ± 1.78^{e}	485.84 ± 11.45^{h}	537.17 ± 1.88^{g}
	10	46.13 ± 1.45^{cde}	69.95 ± 3.30^{d}	400.09 ± 3.47^{cd}	25.58 ± 0.92^{de}	623.45 ± 28.30 ^{g}	616.35 ± 1.48^{f}
	15	54.69 ± 4.71^{c}	78.61 ± 0.74^{c}	435.54 ± 35.64^{bc}	31.25 ± 0.58^{d}	766.60 ± 24.37^{f}	649.24 ± 3.76^{f}

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80	5	65.35 ± 2.86^{b}	78.13 ± 0.72^{c}	474.05 ± 17.52^{ab}	40.55 ± 0.63^{c}	1094.74 ± 39.05 ^{d}	710.92 ± 0.71^{e}
	10	81.76 ± 1.71^{a}	97.54 ± 1.09^{a}	493.30 ± 11.57^{a}	42.77 ± 3.02^{c}	1629.50 ± 43.93 ^{b}	837.64 ± 3.35^{cd}
	15	71.67 ± 3.13^{b}	86.15 ± 0.61^{b}	416.08 ± 12.51^{cd}	42.59 ± 2.85^{c}	1392.98 ± 2.98 ^{c}	864.85 ± 3.69^{c}
100	5	38.71 ± 4.21^{def}	45.10 ± 1.63^{f}	200.27 ± 18.59^{d}	63.58 ± 5.16^{a}	2017.80 ± 74.95^{a}	1040.69 ± 41.36^{a}
	10	44.81 ± 2.99^{ef}	60.79 ± 2.78^{e}	231.95 ± 16.61^{f}	53.90 ± 4.35^{b}	1024.56 ± 32.99^{de}	925.89 ± 33.70^{b}
	15	51.64 ± 1.34^{cd}	64.02 ± 1.53^{e}	374.05 ± 32.53^{f}	59.13 ± 0.99^{ab}	947.69 ± 47.71^{e}	778.64 ± 15.04^{d}

Value are presented in means ± standard deviation (n=3); mean values at the same column with different superscripts are significantly different ($P < 0.05$). [C] : Ethanol Concentration, t: Extraction Time , GAE : Galic Acid Equivalent, CE : Catechin Equivalent, TEAC : Trolox Equivalent.

Ethanol (C_2H_5OH) or ethyl liquor was connected in the extraction since it has been broadly used to extricate cell reinforcement and phenol mixes from different plants and plant-based nourishments [24]. Ethanol has been delegated GRAS (Generally Recognized as Safe) as per Food and Drug Administration[25], so that using ethanol for extracting the plant-based sample would be safer. Ethanol has been frequently employed for the recovery of polyphenols from a plant matrix. Moreover, ethanol is one of semi-polar solvents that has ability to extract hydrophilic compounds such as flavonols, alkaloids, polyphenols and saponins[26] Ethanol is more effective to extract sterol, flavonoid, phenolic and alkaloid while pure water may dissolve alkaloid and glycoside compounds[27]. The number of polyphenols, flavonoids and other bioactive compounds extracted from the seeds might be affected by the concentration of solvent[28]. Ethanol 60% was chosen as the lowest concentration solvent, while ethanol 100% as the highest concentration solvent in this study. The previous study showed that ethanol with 60%-80% concentration was effective for extracting antioxidant from plants[24,29]. On the other hand, 95% ethanol extraction will also extract some lipid components which may diminish the phenolics extraction in samples[29].

The concentrations of ethanol which were used in this study are 60%, 80%, and 100%. The results showed that roselle seed extracted by ethanol 80% exhibited the highest value of DPPH radical scavenging assay, ABTS radical scavenging assay, and FRAP, while showed the second highest for the TPC (Table 1). This is an agreement with the findings of Sultana et al[24]. who investigated that aqueous (80%) ethanol had a better result for extracting phenolic compounds. According to Mohd-Esa et al[6]. roselle seed extracted by distilled water showed higher DPPH radical scavenging activity value than 80% methanol extract. In the present study, DPPH assay value of 80% ethanol extract also exhibited a better result compared to the water extract of roselle seed. Furthermore, increasing the concentration of ethanol possibly to extract more impurities due to the change of solvent polarity[30]. The crude extract of roselle seed may contain impurities that could interrupt antioxidant activity assessment, as roselle seed contains 33.5% protein and 22.1% fat in whole seeds[31]. As reported at Prior et al[32]. protein fraction in the plant sample may contribute to antioxidant capacity measurement.

Hence, all of the individual phenolic compounds of roselle seeds were not able to be recovered by a single of ethanol concentration[31]. Moreover, the increasing concentration of ethanol more than 70% will raise the extraction yields of total flavonoids due to the relative polarity and the increase in effective swelling of the plant by water, which helped increase the surface area for solute-solvent contact[30]. Using the suitable solvent could offer the better physical characteristics such as vapor pressure, viscosity and surface tension of extraction, which may enhance the efficiency of sonication activity for the flavonoid extract[33]. The present study showed that total phenolic content of roselle seeds extracted by ethanol exhibited better values than the water extract and 80% methanol[6]. This may be assumed that phenolic compounds of roselle seed extract have more nonpolar properties and suitable to be extracted with ethanol-based solvent.

Extraction Time evaluation

Extraction time is one of the essential parameters in the extraction procedure for phenolic mixes so as to enhance the recuperation of the part from the example. In any case, an abundance extraction time will cause the decreased yield of phenolic mixes[14]. In the present examination, results demonstrated that 10 minutes extraction of roselle seed remove with 80% ethanol deliver the most astounding cell reinforcement movement for DPPH radical searching test, ABTS radical rummaging test, FRAP. However, TFC, BCB inhibition assay and TPC for roselle seeds extracted with 100% ethanol show the highest value at 5 minutes extraction and kept decreasing as the extraction time increased. It was predicted that reduction of antioxidant activity might be caused by heat and oxygen exposure[31]. Extract of total phenolics might be decreased because of the oxidation that caused the polymerization to insoluble compounds[28]. Moreover, the flavonoid compounds were unstable in thermal process as the extraction time prolonged[30].

Ultrasonic Extraction-Assisted (UAE) could increase the extraction yield of targeted compound, in conjunction with other advantages such as low solvent consuming, more efficient, and reducing extraction time[11].

The lowest and highest extraction time that had been chosen in this study were 5 minutes and 15 minutes, respectively. Those durations had been used in the study as a consideration that using UAE might not require much time to extract the sample.

Chan et al[34]. also declared that increasing temperatures would cause undesired solvent evaporation and overheat which could arise poor yields of antioxidant activity especially for extracts that thermally sensitive. Thus, some of antioxidant compounds are rapidly degraded by high temperature, exactly at above 70°C. We ascribed that temperature of ethanol 100% extraction reached above 70°C. Hence, the antioxidant activity value of TPC, TFC and BCB inhibition assay have been decreasing as the prolonged time.

Long extraction duration would produce high temperature that increases the chance of oxidation and other degenerative reactions of phenolics[24,35]. This situation would lead the yield decreasing of phenolics compounds in the extracts. Furthermore, the unstable phenolic compounds inside the roselle extract could be disrupted during the extraction due to the longer exposure of time and vibration. Ideal extraction time will be changed for each measure because of the diverse degrees of phenolic polymerisation, connection of phenolics with different constituents and solvency of the phenolics which impact the balance time between strong example and arrangement[21].

Comparison between different antioxidants assays by correlations analysis

Antioxidant activity of roselle seeds were closely related with phenolic compounds that consist of them. Phenolic compounds have shown the potential antioxidant activity in both *in vitro* and *in vivo* assay[36]. Phenolic compounds are the most important groups of secondary metabolites in therapeutic herbs and dietary plants that characterized by one or more hydroxyl groups attached to the aromatic ring. Varied phenolic compounds such as simple phenols, phenolic acid, flavonoids, coumarin, etc. have various kind of advantageous biological capacities, including antioxidant activity[37].

Yield of polyphenolic extricate from plants was extensively influenced by the normal for dissolvable. Henceforth, extraordinary bioactive mixes might possibly be dissolvable specifically dissolvable, because of their extremity and substance properties[38]. They are more probable dissolvable in the dissolvable which has the indistinguishable qualities with them. This investigation demonstrated that Roselle seed extricates acquired utilizing diverse solvents had distinctive aggregate phenolic substance. The distinctions saw in yields could be identified with the extremity of specific dissolvable utilized in the extraction. Connection between's the cell reinforcement tests is shown in Table 2.

Table 2. Pearson's correlation coefficients of antioxidant activities

	DPPH	ABTS	FRAP	BCB	TPC	TFC
DPPH		0.899**	0.774**	$0{,}003^{ns}$	0.339^{ns}	0.125^{ns}
ABTS	0.899**		0.881**	-0.339^{ns}	0.013^{ns}	-0.216^{ns}
FRAP	0.774**	0.881**		-0.403*	0.245^{ns}	-0.430*
BCB	0.003^{ns}	-0.339^{ns}	-0.403*		0.700**	0.864**
TPC	0.339^{ns}	0.013^{ns}	-0.149^{ns}	0.700**		0.862**
TFC	0.125^{ns}	-0.216^{ns}	-0.430*	0.864**	0.862**	

^{ns}= not significance
* Significant level at P < 0.05
** Significant level at P < 0.01

DPPH radical scavenging assay, FRAP and ABTS radical scavenging assay displayed good correlations between each other as they exhibited the value 0.774 for DPPH-FRAP, 0.899 for DPPH-ABTS, and 0.881 for FRAP-ABTS. The significant correlations might be assumed that antioxidant compounds in roselle seeds have ability to scavenge free radicals by donating electron and parallel with the ferric reducing power. Be that as it may, DPPH radical rummaging examine, FRAP and ABTS radical searching measure indicated low and invert relationship with BCB hindrance test and TFC.These can be related due to BCB inhibition assay test determined antioxidant activity based on the reaction with radicals from linoleic acid oxidation[13], while DPPH radical scavenging assay, FRAP and ABTS radical scavenging assay determined the antioxidant activity by hydrogen or electron donating.

BCB inhibition assay and TPC exhibited the correlation value at 0.735 that denoted they have a good correlation between each other. These result indicated that phenolic compound possesses the roles of antioxidant activity in the extract. Past investigation likewise demonstrated a positive connection between's aggregate phenolic substance and BCB restraint examine[6]. In contrast, TPC showed low correlation with DPPH and ABTS, also reverse correlation with FRAP. This low correlation between total phenols with DPPH radical scavenging assay, ABTS radical scavenging assay and FRAP probably occured because some major antioxidant components consist in roselle seeds might not be only phenolics, they could be sterols, tocopherols, ascorbic acid, and carotenoids. This might be assumed in accordance to high tocopherol content in roselle seeds oil extract about 82%[8]. Furthermore, the differences correlation between antioxidant activity and total phenolic compound occurred because total phenolic does not present all of the antioxidant.

IV. CONCLUSION

This study showed that the highest DPPH radical scavenging assay, ABTS radical scavenging assay and FRAP assay of roselle seeds extract was employed 80% ethanol at 10 minutes extraction. However, the highest results of BCB inhibition assay, TFC, TPC showed in as roselle seed extracted by 100% ethanol at 5 minutes extraction. As such, 80% ethanol at 10 minutes extraction for roselle seed extract was ascribed the best parameters as DPPH radical scavenging assay, ABTS radical scavenging assay and FRAP were more suitable for determining the optimised parameter than TFC and TPC inhibition assay, which more portrayed the individual compounds. Moreover, extraction with pure solvent (100% ethanol) considered extracting more impurities. The results of antioxidant activities of roselle seed extract proved that the discarded-seed would be highly potential to be a source of natural antioxidant.

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