Antioxidant activity and the effect of different parts of *areca catechu* extracts on Glutathione-S-Transferase activity *in vitro*

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ABSTRACT

Areca nut (*Areca catechu L.*) or Pinang is one of the most widely used psychoactive substance with several hundred million users worldwide, predominantly in Southern Asia. This study evaluates the antioxidant activity and the total phenolic compound of methanolic and aqueous extract of seeds (ripe and unripe seeds), root and adventitious root. The antioxidant activity was determined using the 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay and total phenolic using the *Folin-Ciocalteu* method. The results from this study showed that the antioxidant activities of the water and methanolic extracts of the seeds as determined by the 1, 1-Diphenyl-2-picrylhydrazyl (DPPH) presented higher percentage inhibition than the root and adventitious root. The methanol extracts gave higher antioxidant activities than the aqueous extracts and comparable to that BHT and Vitamin C. The results also showed that unripe seeds methanolic extract possessed higher content of phenolic (186.2 ± 0.04 mg GAE/g) and total flavonoid (18.13 ± 0.007 mg/g) than other parts of *A. catechu*. We also evaluated the *in vitro* effect of various *A.catechu* methanolic and aqueous extract on the activity of Phase II metabolizing enzyme, glutathione-S-transferase (GST) in rat liver. Unripe seeds methanolic showed the effective GST specific activity inhibition with an IC_{50} of 115.05 µg/mL with maximum inhibition >70%. These results suggest that areca nut extracts have the potential to prevent oxidative damage in normal cells due to their antioxidant characteristics.

Keywords: *Areca catechu,* Antioxidant Activity, GST, DPPH, Total phenolic compounds

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INTRODUCTION

Plants contain a diverse group of phenolic compounds including simple phenolics, phenolic acids, anthocyanins, hydroxycinnamic acid derivatives, and flavonoids. All the phenol classes have the structural requirements of free radical scavengers and have potential as food antioxidants. [1-2] Although many other plant species have been investigated in the search for natural antioxidants but generally there is still a demand to find more information concerning the antioxidant potential of plant species. [3]

Areca-nut is the hard, edible, endosperm of the palm *Areca catechu* Linn. (Palmaceae) which grows throughout South, South-east Asia and several Pacific Ocean islands. It is commonly consumed by Asian populations and Asian communities living in Europe and North America and one of the most widely used psychoactive substances, with several hundred million users worldwide, predominantly in southern Asia. [4, 6-7]

Areca catechu (*A. catechu*) is the most commonly used drug in the world after tobacco, ethanol and caffeine. [8] It is usually used in betel chewing common among the Indians and Malays as a breath freshener, digestive aid, worm expellant, aphrodisiac and to maintain stamina. It is claimed to possessed effects such as euphoria, cooling, cold protection, a sense of well-being, palpitation, heightened alertness and resistance to anger. [9-11] In India and China, areca nut has been used as an anthelmintic in man and animals for long time ago. Together with the juice from betel leaves it has a stimulating effect on the central nervous system. It is effective against tapeworm and roundworm. [12-13]

Areca nut contains a number of chemical components such as alkaloids (arecoline, arecaidine, guvacine and guvacoline). [13-17] It also contains phenolic compounds such as hydroxychavicol and safrole and presence of tannins, gallic acid, catechin, beta-sitoserol, gum and amino acids. [18-19] The purpose of this study was to evaluate the contents of total phenolics and antioxidant activity of methanolic and aqueous extracts of fruits (unripe and ripe), root and adventitious root of *A. catechu*. Our study also explore a possible relationship between

phenolic content and antioxidant activity and to find out that which extracting solvents and which part of the plant give extracts that have the highest antioxidant activities. The effects of *A. catechu* extracts on glutathione-S-transferase (GST) activities were also investigated. The glutathione-s-transferase family of enzymes are proteins which are widely distributed in the body, particularly in liver cytosol, and which catalyse the conjugation of a variety of compounds with the endogenous tripeptide glutathione (glutamylcycteinylglycine, abbreviated to GSH).

MATERIALS AND METHODS

Chemicals and drugs used were as follows:

Folin–Ciocalteu's phenol reagent, gallic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), anhydrous sodium carbonate, 1-chloro-2,4-dinitrobenzene (CDNB), reduced glutathione (GSH), potassium sodium tartrate, were obtained from Sigma Chemical Co., Ltd (St. Louis, MO, USA). Potassium chloride was purchased from BDH Chemicals (Poole, England). Propylene glycol (propane-1,2-diol) and polysorbate-80 (Tween-80) were from Fisher Scientific (Loughborough, UK). Potassium dihydrogen orthophosphate were purchased from Ajax Chemicals (New South Wales, Australia). Dipotassium hydrogen phosphate was from Riedel de-Haen (Seelze, Germany) and Tannic acid from R & M Chemicals (Canada).

Plant materials

Different parts of *A. catechu* such as seeds (ripe and unripe), roots, and adventitious roots were collected from Balik Pulau, Penang, Malaysia, in November 2008 and identified by Herbarium Unit, School of Biology, Universiti Sains Malaysia, Penang, Malaysia. The voucher specimen number of the plant is 11085. The voucher specimen has been deposited at the Universiti Sains Malaysia Herbarium.

Plants were dried in the oven at room temperature for 2 weeks to get consistent weight. The dried plants were later ground to powder. 200g of ground plant material was soak in methanol and distilled water for 72 hrs at room temperature, respectively. Extracts were filtered and concentrated to dryness under reduced pressure at 40°C using a rotary evaporator and freeze dryer. The % yield of each crude extract was calculated. All crude extracts were kept in refrigerator at 4°C. [20]

Male Sprague–Dawley rats were obtained from the Animal House, Universiti Sains Malaysia, Penang, Malaysia. Animals were kept at 25±1 °C under a 12 h light/dark cycle. All experiments were performed according to the approved protocols of the Animal Ethics Committee, Universiti Sains Malaysia, Malaysia. Animals had free access to water and food *ad libitium* and were fasted for 10 h before each experiment.

Twelve male Sprague-Dawley rats with weight range of 150-200g were used to prepare cytosolic and microsomal fraction. The rats were sacrificed by exposing them to overdose diethyl ether in a sealed jar. The animal was confirmed dead once the animal does not respond to pain induced by the pinching of its hind paws. The rats were dissected by cutting the abdomen and the whole liver is taken out. Livers from rats of each experimental group were removed and were washed with 67 mM ice-cold potassium-sodium phosphate buffer with 1.15 % KCl, pH 7.4 to flush out the blood, to maintain the osmotic pressure and to preserve the optimum physiologic pH. Then each liver was blotted dry and weighed. The livers were homogenated and spun twice using standard procedure to yield the cytosolic fraction and microsomal fraction. The protein concentration for each fraction was determined using the Lowry method. For microsomal fraction, cytochrome P-450 concentration was determined by using standard procedure.

Determination of rat liver GST's activity toward 1-chloro-2, 4-dinitrobenzene (CDNB) method was adopted from the method described by Habig *et. al* (1974) with slight modification. Glutathione-s-transferase (GST) activity was assayed using cytosolic fraction incubated with reduced glutathione (GSH), (CDNB) and various concentrations of *A.catechu* extracts at the wavelength of 340 nm for 5 minutes. The change of the absorbance was observed for 5 minutes once the reaction started. The GST activity was calculated from the obtained result. Tannic acid was used as a positive control (data not shown).

The total flavonoid content was determined using a colorimetric method. 0.25 ml of the sample (catechin for standard or extracts) was mixed with 1.25 ml of distilled water in a test tube, followed by the addition of 0.075 ml of 5% sodium nitrate solution. After 6 minutes, 0.15 ml of 10% aluminium chloride solution was added and the mixture was allowed to stand for 5 minutes before the addition of 0.5 ml of 1 M sodium hydroxide solution. 2.5 ml of distilled water was added and the absorbance was measured immediately at 510 nm.

Total phenolic content (TPC) of plant extracts was determined using the *Folin Ciocalteu* assay by Kahkonen *et al* (1999) with some modifications. *Folin–Ciocalteu*

reagent (1.5 ml; diluted 10 times) and sodium carbonate (1.2 ml; 7.5% w/v) were added to the extracts (300 µl; triplicate). The tubes were vortexed and allowed to stand for 30 min at 40°C for color development. Absorbance was then measured at 765 nm using UV-VS spectrophotometer. Total phenolic content were expressed as mg gallic acid equivalents/g dry weight (GAE). [21]

The effect of the extracts on DPPH radical was estimated with some modifications. [22] Different dilutions of the extract (1 ml; triplicate) were added to 2 ml of DPPH (5.9 mg/100 ml methanol). The reaction mixture was left in the dark at room temperature for 30 min. The absorbance of the mixture was measured spectrophotometrically at 517 nm. Ascorbic acid and BHT were used as references. The ability to scavenge DPPH radical was calculated by the following equation:

$$\text{DPPH radical scavenging activity (\%)} = \left[\frac{(Abs_{control} - Abs_{sample})}{(Abs_{control})}\right] \times 100$$

Where; $Abs_{control}$ is the absorbance of DPPH radical + methanol;

Abs_{sample} is the absorbance of DPPH radical + sample extract /standard.

The experimental results were expressed as mean ± standard deviation (SD) and level of significance was assessed using ANOVA test. Significant difference was statistically considered at the level of $P<0.05$.

RESULTS AND DISCUSSION

The present study determines the antioxidant activity of different parts of *A. catechu* and their effect on GST activity *in vitro* in male Sprague Dawley rats. Two different extracts of *A. catechu*, namely methanolic and aqueous were evaluated for their antioxidant activity and effect on GST. The calculated % yields of extracts from dry weight of various part of areca plants in various solvents are shown in Table 1. The % yields of methanol extracts were higher than water extracts. Unripe seeds methanol extract had the highest yield, 4.204 % and root aqueous extract had the lowest yield 0.094 %. The yield extract ranged from 0.094 % to 4.204. These indicated that substances in *A. catechu* were quite polar and lot of polar compounds dissolved in polar solvents.

The assay of the scavenging of DPPH radical is widely used to evaluate the antioxidant capacity of extracts from different plant materials. DPPH is one of the compounds that possess a proton free radical and shows a maximum absorption at 517 nm. The percentage of inhibition of

Table 1. The % yield of sample extracts of *A. catechu*

Sample	Methanol Extract	Aqueous Extract
	Yield (% w/w)[a]	
Seeds (ripe)	1.496	0.982
Seeds (unripe)	4.204	2.522
Adventitious root	0.738	0.116
Root	0.734	0.094

[a] calculated from dry weight

Table 2. Comparison of 50% inhibitory concentration for % DPPH radical scavenging activity, total flavonoid, total phenolics and their effects on GST activity.

Extracts	DPPH[a] scavenging IC_{50} (µg/mL)	Total flavonoid (CE) mg/g	Total Phenolic (mg GAE/g extract)	GST Inhibition IC_{50} (µg/mL)
Ripe seeds methanolic	0.021	16.67 ± 0.004	163.9 ± 0.12	413.12
Unripe seeds methanolic	1.87	18.13 ± 0.007	186.2 ± 0.04	115.05
Root Methanolic	2.83	9.93 ± 0.004	56.0 ± 0.02	2373.45
Adventitious root methanolic	2.71	9.40 ± 0.003	58.0 ± 0.01	1410.32
Ripe seeds aqueous	2.49	13.03 ± 0.004	112.2 ± 0.10	189.14
Unripe seeds aqueous	2.07	17.27 ± 0.004	163.6 ± 0.05	178.53
Root aqueous	3.35	8.70 ± 0.004	54.5 ± 0.01	7699.46
Adventitious root aqueous	3.35	8.30 ± 0.004	49.0 ± 0.04	3552.73
Ascorbic acid	2.69	NA	NA	NA
BHT	2.58	NA	NA	NA

NA: Not Applicable, BHT= Butylated hydroxytoulene,
[a]*Lower* IC_{50} value indicates higher antioxidant activity

DPPH within the assay time will reflect the antioxidant capacity of the extract assessed. The proton radical scavenging action is known to be one of the various mechanisms for measuring antioxidant activity. The IC_{50} of the standard compounds, BHT and vitamin C were 2.58 μg/ml and 2.69 μg/ml, respectively. For *A. catechu* extracts, methanol extracts displayed the highest scavenging effect than water extracts. The scavenging effect of ripe seeds extracts on DPPH radicals showed the highest value than unripe seeds, roots and adventitious roots. Ripe seeds methanol exhibit the highest percentage of inhibition with 94.3 ± 0.007 (IC_{50} = 0.021 μg/ml), which is higher than BHT and ascorbic acid followed by the adventitious root methanol (93.9 ± 0.007) and unripe seeds methanol (92.9 ± 0.004). The extracts of root aqueous and adventitious root aqueous exhibited the lowest DPPH radical scavenging activity. The results were in agreement with past literature. The antioxidant activity of *A. catechu* was found to be among the highest DPPH radical scavenging activity of nine medicinal plants traditionally used in Chinese medicine, compared to *Glycyrrhiza uralensis, Solvia miltiorrhiza, Paeonia suffruticosa, Spirodela polyrrhiza, Cornus officinalis, Alpinia officinarum, Nelumbo nucifera* and *Cinnamomun cassia*. [23] The IC_{50} value of the extracts ranged from 0.021-3.35 μg/ml. The study showed that the extracts have the proton-donating ability and could serve as free radical inhibitors or scavengers, acting possibly as primary antioxidant.

Flavonoids are polyphenolic compounds that are ubiquitous in nature and are categorized according to chemical structures into flavonols, flavones, flavonones, isoflavanones, catechins, anthocyanidins and chalcones. [24] Over 4000 flavonoids have been identified, many of which occur in fruits, vegetables and beverages (tea, coffee, beer, wine and fruit drinks). Epidemiology studies have shown that flavonoid intake is inversely related to mortality from coronary heart disease and to the incidence of heart attacks. The mechanisms of flavonoids are through scavenging or chelating process. Results obtained in the present study revealed that the level of these total phenolic contents and total flavonoid in the methanol and aqueous extracts of the different parts of *A. catechu* was shown (Table 1). The unripe fruit methanol extract shows the highest total flavonoid content followed by the ripe fruit methanol extract and adventitious root methanol extract. Generally, methanol extract of *A. catechu* had the highest phenolic content than the aqueous extract. Meanwhile, unripe seeds methanol showed the highest phenolic content (186.2 ± 0.04 mg GAE/g) than the other parts of *A. catechu* and adventitious root aqueous (49.0 ± 0.04 mg GAE/g) had the lowest phenolic content. The results showed that for *A. catechu*, the phenolic content was in the order of the unripe seeds methanolic > ripe seeds methanolic > unripe seeds aqueous > ripe seeds aqueous > adv. root methanolic > root methanolic > root aqueous > adv. root aqueous.

Glutathione S-transferase (GST) constitute a multigene superfamily of cytosolic enzyme that catalyse the nucleophilic addition of glutathione to electrophilic centres of a wide variety of compounds, thereby playing an important role in protecting cells from xenobiotics. GST activities were determined spectrophotometrically by monitoring the thioether formation at 340 nm using 1-chloro-2,4-dinitrobenzene (CDNB) as the substrate. [25] The extracts were used within 0.001-1 mg/mL final concentrations in order to calculate the percent inhibition of GST activity and respective IC_{50} values. The IC_{50} values were calculated by plotting the percentage inhibition of GSTs specific activity versus log concentration of *A. catechu* extracts. Plant extracts with high polyphenols are known to have important inhibitory effects on glutathione-S-transferase in the literature. In addition, flavonoids have been shown to inhibit GST activity in human blood platelets, rat liver and rat kidney. The results showed that, unripe seeds methanolic extracts which have higher phenolics content and total flavonoids content were more effective in GST inhibition as given in Table 2.

Based on our results, a maximum inhibition (>70% inhibition) occur at higher dose. Seeds parts of *A. catechu* showed a maximum inhibition >70% than other parts of *A. catechu*. Unripe seeds methanolic showed (93% inhibition), ripe seeds methanolic (92.4% inhibition), ripe seeds aqueous (86% inhibition) and unripe seeds aqueous, (85.1% inhibition). It was in the following order: unripe seeds methanolic > ripe seeds methanolic > ripe seeds aqueous > unripe seeds aqueous. At a concentration higher than 50 μg/mL, methanolic and aqueous extracts of *A. catechu* significantly decreased GST activity in a dose-dependent manner ($P<0.05$). It is reported that the reaction of glutathione conjugation with a large number of foreign compounds with electrophilic centers is catalyzed by GST. At concentrations of 1000 μg/mL, ripe seeds methanolic and unripe seeds methanolic reduced GST activity about 92.4% and 93%, respectively. Aqueous extracts of *A. catechu* also shown inhibition at concentrations of 1000 μg/mL, unripe seeds aqueous, (85.1% inhibition) and ripe seeds aqueous, (86% inhibition). It has been suggested that the presence of polyhydroxylations in plant polyphenols is important for GST inhibition. [26] Previous report from Taiwan, reported that phenolics in *A. catechu*

were mainly distributed in root followed by fresh unripe seeds, leaf, spike, and vein, while the contents of alkaloids in *A. catechu* were in the order of root > fresh unripe seeds > spike > leaf > vein. Total amounts of phenolics in areca seeds were well correlated with the length and maturation, but those of alkaloids were only correlated with the maturation. Upside-down areca seeds, areca seeds growing up ward (opposite to normal seeds, growing downward), contained a much higher amount of arecaidine (4 mg/g of fresh wt) than normal fresh unripe areca seeds (1.5 mg/g of fresh wt). [27]

In addition, flavonoids have been shown to inhibit GST activity in human blood platelets, rat liver and rat kidney. Besides, plant phenolic compounds such as quercetion, curcumin, ellagic acid, caffeic acid,and chlorogenic acid have been reported to be responsible for the inhibition of the GST *in vitro*. [28]

CONCLUSION

The screening of antioxidant, content of total phenolics, total flavonoid and GST inhibitory activities was performed on various extracts of *Areca catechu*. Methanol extracts of *A. catechu*, especially the seeds extracts, have considerable activities compared with the other parts of *A. catechu*. Further research should be followed to isolate and identify the active ingredients with strong antioxidationability in *A. catechu*.

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