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Full Length Research Paper

Antioxidant activities of extracts from areca (*Areca catectu* L.) flower, husk and seed

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The antioxidant activities of areca (*Areca catectu* L.) flower, husk and seed extracts were evaluated using 3 complementary *in vitro* assays, inhibition of DPPH (1,1-diphenyl-2- picrylhydrazyl) radicals, inhibition of hydroxyl radicals and reducing power system. The EC_{50} values were calculated for all the methods in order to evaluate the antioxidant efficiency of areca extracts. The phenol and flavonoid contents were also analyzed. Areca seed has the best antioxidant properties, presenting much lower EC_{50} values for DPPH radicals scavenging activity, hydroxyl radicals scavenging activity and reducing power. Furthermore, the highest polyphenols and flavonoids contents were found from areca seed extracts. It is suggested that areca seed is an excellent food material with a potential nutrition and antioxidation.

Key words: *Areca catectu* L. extracts, antioxidant activity.

INTRODUCTION

Free radicals were a major interest for early physicists and radiologists and much later found to be a product of normal metabolism. Although oxygen is essential for aerobic forms of life, oxygen metabolites are highly toxic. As a conse quence, reactive oxygen species (ROS) are known to be implicated in many cell disorders and in the development of many diseases including cardiovascular diseases, atherosclerosis, cataracts, chronic inflammation and neurodegenerative diseases (Gutteridge, 1993; Knight, 1995). ROS and free radicals are also considered as inducers of lipid peroxidation and cause the deterioration of foods (Rechner et al., 2002). Although organisms have endogenous antioxidant defences produced during normal cell aerobic respiration against ROS, other antioxidants are taken from the diet, both from natural and synthetic origin (Rechner et al., 2002). Antioxidants, which can inhibit or delay the oxidation of an oxidizable substrate in a chain reaction, therefore, appear to be very important in the prevention of many diseases (Halliwell et al., 1992). Thus, synthetic antioxidants are widely used in the food industry. However, because of their toxic and carcinogenic effects, their use is being restricted. Thereby, interest in finding natural antioxidants, without undesirable side effects, has increased greatly (Rechner et al., 2002).

The number of antioxidant compounds synthesized by plants as secondary products, mainly phenolics, serving in plant defence mechanisms to counteract ROS in order to survive, is currently estimated to be between 4000 and 6000 (Havsteen, 2002; Robards et al., 1999; Wollgast and Anklam, 2000). A direct relationship has been found between the content of total phenolics and antioxidant capacity of plants (Ferreira et al., 2007; Robards et al., 1999). In fact, to counteract deleterious action of ROS, phenolic compounds, naturally distributed in plants are effective (Ferreira et al., 2007; Pereira et al., 2006).

Because purified phenolic compounds are difficult to obtain and because extracts sometimes have better antioxidant activities than those of pure molecules, there is a growing interest for the use of plant extracts (Calliste et al., 2005). To find new natural sources of active compounds, we studied the antioxidant potential of different extracts of *Areca catectu* L. The use of betel nut, as a masticatory by humans has been known since the 4th century A. D. in different parts of the world. It is estimated that over 600 million individuals consume areca nut (also called areca nut) in one form or another world-wide. In old Indian scripts, such as Vagbhata (4th century) and Bhavamista

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(13th century), betel nut has been described as a therapeutic agent. Its use was recommended in many diseases, such as leucoderma, leprosy, anaemia and obesity. It was also reported to have de-worming properties. In China, it has been used as a vermifuge since the 6th century and is still employed as such in some parts (Sharan, 1996). In the Philippines the flowers are sometimes added to salads. The nuts, husks, young shoots, buds, leaves and roots are used in various medicinal preparations (Staples and Bevacqua, 2006).

Although it has already been demonstrated that areca fruit contain total phenolics and tannin (Zhang et al., 2008), little is known about the antioxidant potential of areca fruit and other areca extracts, such as husk and flower.

In this work, the antioxidant properties of areca extracts were evaluated through several biochemical assays, DPPH (1,1-diphenyl-2-picrylhydrazyl) radical–scavenging activity, reducing power and inhibition of hydroxyl radicals.

MATERIALS AND METHODS

Materials

Areca flower, husk and seed were obtained from areca (*Areca catechu* L.) plant grown in Hainan, China. Areca flower and fruit were picked in April and June, 2008 respectively. The samples were cleaned, washed with distilled water, cut into small pieces, dried overnight in an air dryer at 40°C, ground to a particle size of 25 mesh using a grinder. Rutin and DPPH (1, 1-diphenyl-2-picrylhydrazyl) was purchased from Sigma Chemical Company (St. Louis, MO) and vitamin C (ascorbic acid) was obtained from Guangzhou chemical reagent factory, China. All other reagents were analytical grade.

Extraction of antioxidative compounds

For antioxidant compounds extraction, a fine dried powder (25 mesh) of sample (areca flower, husk and seed, 5 g for all extracts) was extracted using 50 ml of 70% ethanol at 75°C for 2.5 h by reflux. The extracts were filtered through Whatman No.4 paper under reduced pressure, frozen and then lyophilized (Ly-8-FMULE, Snijders). All the samples were redissolved in 70% ethanol at a concentration of 5.0 mg/ml and analysed for their contents of polyphenols and flavonoids and DPPH radical-scavenging activity, reducing power and inhibition of hydroxyl radicals.

Determination of antioxidant contents

Contents of total phenolics in the extracts were estimated by a colorimetric assay based on procedures described by Singleton and Rossi (1965) with some modifications. Basically, 1 ml of sample was mixed with 1 ml of Folin and Ciocalteu's phenol reagent. After 3 min, 1 ml of saturated sodium carbonate solution was added to the mixture and it was adjusted to 10 ml with distilled water. The reaction was kept in the dark for 90 min, after which the absorbance was read at 725 nm (UV-2450 spectrophotometer). Catechin was used for constructing the standard curve (2.0 - 12.0 µg/ml; $y = 0.0631 x - 0.0611$; $R^2 = 0.9992$) and and the results were expressed as mg of catechin equivalents/g of extract.

Flavonoid contents in the extracts were estimated by a colorimetric assay based on procedures described by Hertog et al. (1992) with some modifications. Basically, 0.5 ml of the sample added to a test tube which contained 4.5 ml of distilled water and then added 0.3 ml of 5% sodium nitrite solution and allowed to stand for 5 min. 0.6 ml of 10% aluminium chloride was added, and after 6 min, 2.0 ml of 1 mol/l sodium hydroxide was added and the mixture was diluted with another 2.1 ml of distilled water. The absorbance of the mixture at 510 nm was measured immediately. Rutin was used for constructing the standard curve (8.0 - 40 µg/ml; $y = 0.022 x + 0.0109$, $R^2 = 0.9994$) and flavonoid contents were calculated using a standard calibration curve, prepared from rutin. The flavonoid content was expressed as mg/g DW.

DPPH radical scavenging activity

To evaluate the free radical scavenging activity, the extracts were allowed to react with a stable free radical, DPPH (Brand-Williams et al., 1995). Various concentrations of areca extracts (0.3 ml) were mixed with 2.7 ml of 70% ethanol solution containing DPPH radicals (40 µg/ml). The mixture was shaken vigorously and left to stand for 60 min in the dark (until stable absorbance values were obtained). The reduction of the DPPH radical was determined by reading the absorbance at 517 nm. The radical-scavenging activity (RSA) was calculated as a percentage of DPPH discoloration, using the equation

$$\% \text{ RSA} = [(A_{DPPH} - A_S)/ A_{DPPH}] \times 100$$

where A_S is the absorbance of the solution when the sample extract is added at a particular level and A_{DPPH} is the absorbance of the DPPH˙solution (Barros et al., 2007). The extract concentration providing 50% of radical-scavenging activity (EC_{50}) was calculated from the graph of RSA percentage against extract concentration. Ascorbic acid was used as standards.

Hydroxyl radical scavenging activity

The scavenging activity for the hydroxyl radical was evaluated using the method of Halliwell et al. (1987) at a wavelength of 510 nm with a UV-visible spectrophotometer. The radical scavenging activity was calculated using the following equation,

$$\text{Hydroxyl radical scavenging activity (\%)} = [(A_0 - A_i)/A_0] \times 100$$

where A_0 is the absorbance of the control at 510 nm and A_i is the absorbance of the sample at 510 nm.

Reducing power

The reducing power of the extracts was assessed by the method of Oyaizu (1986). Various concentrations of the extracts (2.5 ml) were mixed with 2.5 ml of 200 mM sodium phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixture was incubated at 50°C for 30 min. After 2.5 ml of 10% trichloroacetic acid (w/v) was added, the mixture was centrifuged at 1000 rpm for 10 min. The upper layer (5 ml) was mixed with 5 ml of deionised water and 1 ml of 0.1% of ferric chloride and the absorbance was measured spectrophotometrically at 700 nm (Barros et al., 2007). Blank sample was prepared using distilled water instead of extract. The values are presented as the means of triplicate analyses. The extract concentration providing 0.5 of absorbance (EC_{50}) was calculated from the graph of absorbance at 700 nm against extract concentration. Ascorbic acid was used as standards.

Table 1. Extraction yields and contents of total phenolics and flavonoids in areca extracts.

Fraction	Extraction yield (%)	Total phenolics (mg/g)	Flavonoids (mg/g)
Flower	1.76 ± 0.19c	20.09 ± 1.21c	6.12 ± 0.24c
Husk	3.24 ± 0.11b	59.22 ± 1.48b	52.57 ± 3.02b
Seed	8.75 ± 0.95a	114.14 ± 3.41a	77.36 ± 5.06a

SD: standard deviation (means ± standard error of means of the 3 cultivars).
Data followed by different letters in the same column are significantly different at 0.05 probability level.

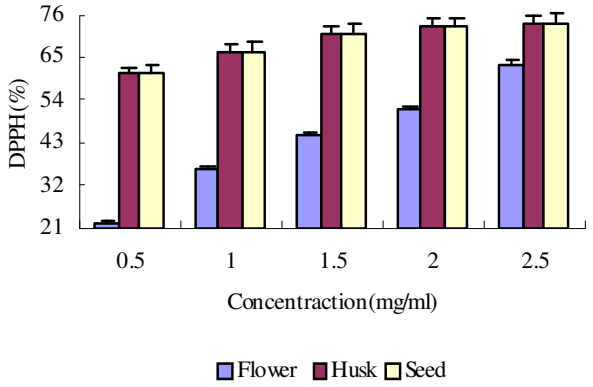

Figure 1. Radical-scavenging activity (RSA) of different concentrations areca flower, husk and seed extracts.

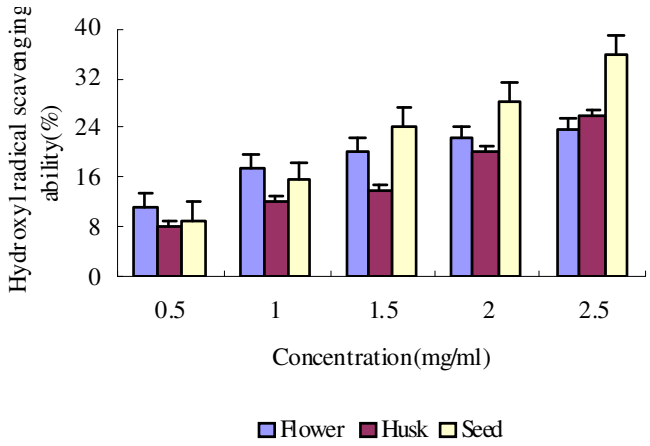

Figure 2. Radical-scavenging activity (hydroxyl radical) of different concentrations areca flower, husk and seed extracts.

Statistical analysis

All experiments were conducted in triplicate and statistical analyses was done according to the software DPS3.01user's guides. The data were presented as mean ± SD. Determination of significant differences of the means between various treatments of areca flower, husk and seed were performed by t-test.

RESULTS AND DISCUSSION

Extraction yields and contents of antioxidative compounds

Water with ethanol was selected as the extraction solvent since both are commonly used in the food industry in a variety of ways and are more highly stable in the human body than any other solvents. The extraction yield is highly valued because a low extraction yield means a lower productivity despite high antioxidation. Therefore, 70% ethanol was used as the extraction solvent for this study. The extraction yields were expressed in terms of the solid content in the dried product per soluble solid content in areca husk, flower and seed used on a dry basis. Table 1 showed the extraction yields of the 70% ethanol extracts from areca flower, husk and seed, the extraction yields of areca flower, husk and seed were 1.76, 8.75 and 3.24%, respectively. Despite the low values obtained for the extraction yields, the antioxidant contents found were good, indicating that the extraction was efficient. Nevertheless, a relationship between the extracted mass and the corresponding total phenolics and flavonoids were not observed in all cases. Most of the phenolic or polyphenolic compounds in nature have antioxidative activities, e.g. tocopherols, flavonoids and derivatives of cinnamic acid, phosphatidic and other organic acids. Flavonoids were the major components of the total phenolic content of areca flower, husk and seed (Table 1). The significant difference was observed in the total phenolics and flavonoids of 3 fractions of areca. And areca seed contained the most total phenolics and flavonoids, followed by husk and flower.

Antioxidant activities in areca flower, husk and seed

Figures 1 - 3 showed the antioxidant activity of areca flower, husk and seed extracts examined as a function of their concentration. Several biochemical assays were used to screen the antioxidant properties, scavenging activity on DPPH radicals (measuring the decrease in DPPH radical absorption after exposure to radical scavengers), scavenging activity on hydroxyl radicals (measuring the decrease in hydroxyl radical absorption after exposure to

Table 2. EC_{50} values (mg/ml) and anti-radical power obtained in the antioxidant assays for areca extracts.

Assay		Flower	Husk	Seed
DPPH radical scavenging activity	EC_{50}	1.838a	1.489b	0.409d
Hydroxyl radical scavenging activity	EC_{50}	6.754 a	5.380b	3.575c
Reducing power	EC_{50}	2.685a	1.466b	0.188d

Data followed by different letters in the same line are significantly different at 0.05 probability level.

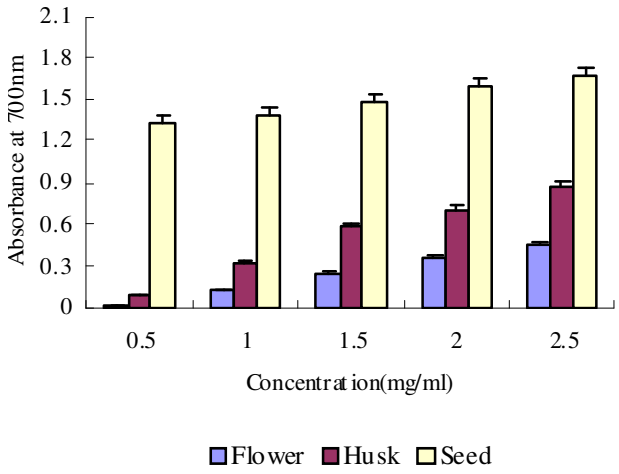

Figure 3. Reducing power of different concentrations area flower, husk and seed extracts concentration.

radical scavengers) and reducing power (measuring the conversion of a Fe^{3+}/ferricyanide complex to he ferrous form). The assays were per-formed for each extract separately. Nevertheless, those assays were carried out using whole extracts instead of individual compounds. Additive and synergistic effects of phytochemicals in fruits and vegetables are responsible for their potent bioactive properties and the benefit of a diet rich in fruits and vegetables is attributed to the complex mixture of phytochemicals present in whole foods (Liu, 2003). This explains why no single antioxidant can replace the combination of natural phytochemicals to achieve the health benefits. Analysis of Figures 1 - 3 revealed that antioxidant activity increased with the concentration, good results being obtained, even at low extract concentrations.

DPPH, a stable free radical with a characteristic absorption at 517 nm, was used to study the radical scavenging effects of ethanol extracts. The decrease in absorption is taken as a measure of the extent of radical scavenging (Suja et al., 2005). The radical-scavenging activity (RSA) values were expressed as the ratio percentage of sample absorbance decrease and the absorbance of DPPH solution in the absence of extract at 517 nm. From the analysis of Figure 1, we can conclude that the scavenging effects of all extracts on DPPH radicals increased with the concentration increase and were excellent, especially in the case of areca seed. The RSA values were also good for husk, but areca flower revealed a very low value.

The hydroxyl radical is an extremely reactive free radical formed in biological systems and has been implicated as a highly damaging species in free radical pathology, capable of damaging the biomolecules of living cells. We investigated the hydroxyl radical scavenging activity of ethanol extracts using the Fenton reaction (Figure 2), the hydroxyl radical scavenging activity also increased with concentration. All extracts revealed a good scavenging activity on hydroxyl radicals, especially in the case of areca seed. The scavenging activity was also good for husk, but areca flower revealed a very low value (23.8% at 2.5 mg/ml).

The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. A higher absorbance indicates a higher ferric reducing power (Meir et al., 1995; Shimada et al., 1992; Duh et al., 1998). Figure 3 showed the reducing powers of the ethanol extracts as a function of their concentration. The reducing power also increased with concentration and the values obtained for all the extracts were good. Areca seed had the highest reducing power in 3 fractions of areca. With regards to reducing power, higher reducing activities can be attributed to higher amounts of polyphenolics and the reducing capacity of a compound may reflect its antioxidant potential (Lee et al., 2007). It has been reported that the reducing properties are generally associated with the presence of reductones, which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom (Shimada et al., 1992). Hence, areca seed may have the highest amounts of reductones and polyphenolics in 3 fractions of areca.

Table 2 showed antioxidant activity with EC_{50} values of areca flower, husk and seed measured by different biochemical assays. Areca seed revealed the best antioxidant properties in 3 fractions of areca by the DPPH assay, hydroxyl radical assay and reducing power assay, followed by husk and flower. The obtained results are in agreement with the phenol and flavonoid contents determined for each sample and shown in Table 2. In DPPH radicals scavenging activity, the EC_{50} of areca seed, husk and flower were 0.409, 1.489 and 1.838 mg/ml,

Table 3. Correlations established between the concentration of extracts with antioxidant activity EC_{50} values (df = 3).

Extract		DPPH radical scavenging activity	Hydroxyl radical scavenging activity	Reducing power
Flower	Equation Linearity	Y = 19.326x + 14.487 R^2 = 0.9855	Y = 5.866x + 10.379 R^2 = 9069	Y = 0.2242x - 0.1019 R^2 = 0.9983
Husk	Equation Linearity	Y = 20.078x + 20.111 R^2 = 0.9363	Y = 8.754x + 2.901 R^2 = 0.9654	Y = 0.3938x - 0.0775 R^2 = 9846
Seed	Equation Linearity	Y = 6.576x + 58.99 R^2 = 0.9159	Y = 13.202x + 2.805 R^2 = 9928	Y = 0.1742x + 1.2279 R^2 = 0.9890

respectively. In hydroxyl radicals scavenging activity, the EC_{50} of areca seed, husk and flower was 3.575, 5.380 and 6.754 mg/ml, respectively. In reducing power system, the EC_{50} of areca seed, husk and flower was 0.188, 1.466 and 2.685 mg/ml, respectively. The EC_{50} values of areca seed obtained for reducing power were better than those for DPPH radicals, scavenging effects on hydroxyl radicals. These results showed that areca seed exhibited significantly ($p < 0.05$) the highest antioxidative activity than areca husk and flower.

In complex systems, such as food and food preparations, various different mechanisms may contribute to oxidative processes, such as in Fenton reactions, where transition metal ions play a vital role, different reactive oxygen species might be generated and various target structures such as lipids, proteins and carbohydrates, can be affected. Therefore, it is important to characterize the extracts by a variety of antioxidant assays (Halliwell, 1997). In reducing power assay, the general ability of the extracts to donate electrons is tested whereas, in the DPPH assay, hydrogen atoms are involved as well. The results from the antioxidant assays show that areca flower, husk and seed can act as radical scavengers to a certain extent. Areca seed exhibited the highest antioxidative activities.

In the present study, despite the high coefficient of correlation values (R^2) obtained, proving the existence of correlation, the only results that showed statistical significance were those gathered for EC_{50} radical-scavenging activity and concentrations, as can be seen in Table 3. It is regrettable that the correlation of EC_{50} radical-scavenging activity and total phenolics and flavonoids was not studied. It was possible that the relationship between the antioxidant activity and total phenolics and flavonoids of areca extracts was complex and difficult to describe by statistical tools. The flavonoid contents also correlated with EC_{50} scavenging capacity values, although with less good correlation coefficient values (Barros et al., 2007).

Conclusion

Overall, areca seed extracts revealed the highest DPPH radical scavenging activity and reducing power, while areca flower showed the best radical-scavenging activity of hydroxyl radicals. It is interesting that the ethanol extracts of areca seed exhibited the highest antioxidative activity in 3 fractions of areca by the DPPH assay and reducing power, but it was the lowest antioxidative activity in 3 fractions of areca by hydroxyl radical assay.

The present study suggests that areca seed extracts are useful nutritional antioxidants for the nutraceutical industry. Total phenolics and flavonoids of areca extracts should be the most activity substances, but the components responsible for the antioxidative activity of areca seed extracts are currently unclear. Therefore, further study is required for isolation and identification of antioxidant active compounds from ethanol extracts of areca seed. After adequate treatment they can, for
example, be included in foods with remarkable benefits for human or animal health.

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