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Research Article

A COMPARITIVE STUDY OF IN-VITRO ANTIOXIDANT ACTIVITY OF DIFFERENT EXTRACTS OF ARECA SEED COLLECTED FROM *ARECA CATECHU* PLANT GROWN IN ASSAM

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ABSTRACT

Plant polyphenolic compounds have been recognised for their ability to prevent oxidation of susceptible substances by virtue of their electron donating property due to presence of large number of phenolic hydroxyl groups. The seed of *Areca catechu* contains higher proportions of polyphenolic compounds mainly tannins. Our objective involves quantification of tannins, determination of total phenolic content and evaluation of oxidative potential of different extracts obtained from the seed. Results shows that the methanol extract (ME) obtained from successive extraction has highest tannin content (TC), total phenolic content (TPC) and exhibited higher reducing power and hydrogen peroxide scavenging ability in comparison to petroleum ether, ethyl acetate and water extract (WE). But the methanol-water extract (MWE) obtained from Soxhlet extraction gave higher yield and found to have higher TC, TPC and also exhibited highest reducing power and hydrogen peroxide scavenging ability. However the MWE exhibited comparable antioxidant activity to that of ME obtained from successive extraction by maceration. A positive relationship was observed to be exists in between the antioxidant activity and the TPC of the extracts, when co-related. Except ethyl acetate, all the extracts exhibited significantly higher antioxidant activity in comparison to that of ascorbic acid (AA) used as standard.

Conclusion: The results obtained proves that the areca seed extracts especially ME, MWE and WE have higher antioxidant activity than the well established antioxidant ascorbic acid which suggests that the areca seed examined is a potential source of natural antioxidant. Among the various extracts the ME and MWE exhibited comparable antioxidant activity although they were obtained from two different extraction processes.

Keywords: - *Areca catechu* seed, Quantification of tannin, Total phenolic content, Reducing power and Hydrogen peroxide scavenging assay.

INTRODUCTION

The seed of *Areca catechu* L (Arecaceae) is commonly known as betel nut, extensively chewed in many tropical countries of the world (at least 10% of world population) [1, 2]. It has been a popular traditional medicine in China, Thailand, Sri Lanka, India[3], used in the treatment of different diseases like dyspepsia, constipation, beriberi, oedema, diarrhoea urinary, gynaecological disorders and to heal foot sore. Traditionally its powder has been used as dentifrice in tooth paste, as taeniacide (kills tapeworms) especially in animals[4].

The chemical constituents of Areca nut are mainly polyphenols including tannins, flavonoids and nine closely related alkaloids[5] including arecoline, arecaidine, arecaine, arecolidine, guvacine, isoguvacine, guvacoline, coniine, norarecoline belongs to pyridine group[6,7] besides carbohydrates, fats and minerals. Among the alkaloids, arecoline (0.12-0.24% in ripe nut) [8] has been reported to be the main alkaloid having cholinergic muscarinic agonistic activity and mostly present in the seed as salt of tannic acid. Lime facilitates its release from salt[9]. Total alkaloid content is about 0.45%[10]. About 8-18% polyphenolic compounds of the nut are tannins (catechol tannins or polymer of catechol) [11] and 7-15% non-tannins. The average tannin content in sun dried nut is 25%[12]. The polyphenolic content has been reported to be decreases with the maturity of the nut [13, 14, 15].

Polyphenols are the large numbers of natural phenolic compound found abundantly in plants, possessing antioxidant activity[16]. They are diverse in their chemical structure, nature and biological activity, capable of providing protection against oxidative stress and thus may plays a significant role in the prevention or improvement of several clinical conditions like: cancer, osteoporosis, neurodegenerative, cardiovascular diseases, diabetes mellitus etc[17, 18]. Plant materials rich in polyphenols are of interest for their significant health benefit and may be a potential drug or nutraceuticals.

The reactive oxygen species [(ROS) produced in the body through normal metabolic processes] and the free radicals [(FR) produced from environmental factors] are unstable, highly active chemical entities capable of oxidising essential cellular components resulting cell damage[19]. Normally bodies defence systems are able to inhibit this oxidative cell damages to some extent. But, when ROS or FR, produces beyond controllable limit, our body became unable to restore its normalcy leading to development of several chronic diseases.

Antioxidants can inhibit or delay the oxidation of an oxidizable substrate in a chain reaction, thereby appears to be important in the prevention of many diseases[20, 21, 22]. Several plant polyphenolic compounds are reported to be potential antioxidant and are less toxic and less carcinogenic then the synthetic antioxidants currently in use in many foods, beverages and medicines[23]. Thereby a great importance has been emphasized in finding out, effective natural antioxidants with low toxicity and carcinogenicity.

Plant polyphenolic compounds are stable as long as they are accumulated in living plant cells thus difficult to obtain in pure form[24]. Hence antioxidant potential of different extracts of areca seed have been evaluated and compared in this study.

MATERIALS AND METHODS

Chemicals

Tannic acid (Sigma Aldrich), Indigo carmine (Loba chemicals Pvt. Ltd. Mumbai, India), Methyl red (Merck), Folin-Ciocalteu reagent (Merck), Ascorbic acid (Fischer Chemic Ltd. Chennai, India), Sodium carbonate (Merck), Petroleum ether (Merck), Ethyl acetate (Merck), Methanol (Merck) and all other chemical and reagents used were of analytical grade.

Plant Material

Mature Areca catechu fruits were purchased from the owner of the Areca catechu plant, residing at Nagaon district of the state of Assam, India. Only healthy looking fruits, without infection or damage, were chosen for the examination. The husks was removed, nuts were sliced in to small pieces and dried under the sun for 30 days. The dried nuts were powdered in a mechanical grinder and utilised for extraction.

Preparation of Areca seed extracts

The powdered nut was extracted by two different methods: (I) Successive extraction of same amount of powder using solvents with increasing polarity. 400 gram of Areca nut powder was macerated in

petroleum ether for 7 days and the supernatant was separated by filtration using Whatman No.1 Filter paper, made in England. The residue was washed twice with the solvent, filtered and the filtrates were mixed. The remaining residue was then successively extracted with ethyl acetate, Methanol (100%) and water using the same procedure. (II) Soxhlet extraction: 200 gram of Areca powder was extracted separately with mixture solvent methanol-water (50:50) by continuous hot percolation process using Soxhlet apparatus. The solvent of each extracts were then evaporated in a rotary evaporator (HAHNVAPOR, Model No. HS-2005V, Made in Korea) to form concentrated thick mass and then dried using Freeze drier. All dried extracts were stored at 4^0c until use.

Quantification of tannins

Tannins in the extracts were quantified following the method described by Kumazawa et.al.2002.[25] with little modification. A weighed amount of different Areca seed extracts were dissolved in distilled water and filtered to get a clear solution. 20 ml of this solution was added to 25 ml of indigo carmine solution {1 gram indigo carmine in 1 litre acidic water (50 ml concentrated H_2SO_4/litre of water)} and then diluted to 500-700 ml water to get a clear solution. This solution was titrated with 0.04 N $KMn0_4$ and detected the end point when the colour of the solution changed to pale yellow. The volume of $KMnO_4$ solution consumed (A) was recorded.

Again, 20 ml of clear extract solution was added to 25 ml of Gelatine solution (0.2%), shacked for 15 minutes and filtered. The filtrate was added to 25ml of indigo carmine solution and then diluted to 500-700 ml of water. This solution was titrated with 0.04N $KMnO_4$ solution, till the colour of the solution changed to pale yellow (end point). The volume of $KMnO_4$ solution consumed (B) was recorded.

The amount of tannin oxidised with $KMnO_4$ was calculated using the following equation:

$$\% \text{ Tannin (as gallotannic acid)} = \frac{(A - B) \times (\text{gram tannins/ml } KMnO_4) \times 100}{\text{Gram extract in the sample solution taken}}$$

Where, A= total tannin like materials, B = Non tannin materials, (A-B) = True tannins.

Titre: 1ml of 0.04N $KMnO_4$ = 0.00168 gram tannin (as gallotannic acid).

Determination of total phenolic content (TPC)

Total phenolic content of the extracts were determined by the modified Folin-Ciocalteu method described by Wolfe et al. 2003[26, 27, 28]. Small amount (0.5ml) of extract solution (50µg/ml) was mixed with 5 ml of Folin-Ciocalteu reagent (previously diluted with water 1:10 v/v) and kept for 5 minutes. 4 ml of sodium carbonate solution (75 g/L) was added to it. The tubes were vortexed for 15 seconds and allowed to stand for 2 hours at room temperature. Absorbance was measured at 765 nm in UV- VIS spectrophotometer against blank (without sample). Standard solutions of Tannic acid of different concentrations (0.1- 0.5µg/ml) were prepared and absorbance was measured at 765 nm against blank to produce standard calibration curve. The total phenolic content was expressed as mg of tannic acid equivalent (TAE) /g of dry extract, using the following equation based on the calibration curve : $y = 0.012x$, $R^2 = 0.965$ where 'y' was the absorbance and 'x' was the tannic acid equivalent (TAE). All results were in average obtained from three parallel determinations.

Assay of Antioxidant Activity

Determination of reducing power

The reducing power of different Areca seed extracts were assessed by the method described by Oyaizu 1986[29, 30, 31]. 1ml of extracts of different concentrations was mixed with 2.5 ml of phosphate buffer (PH 6.6) and 2.5 ml of Potassium ferricyanide [K_3 Fe $(CN)_6$] solution (30mM). The mixture was incubated at 50^0C for 20 minutes and 2.5 ml of trichloroacetic acid (10%) was added which was then centrifuged at 3000 rpm for 10 minutes. The supernatant 2.5ml was mixed with 2.5 ml of distilled water and 0.5 ml of ferric chloride solution (6mM). Absorbance was measured at 700 nm after 10 minutes in UV-VIS spectrophotometer against blank without extract.The reducing power of the extracts were compared with that of Ascorbic acid as standard. Higher absorbance indicates higher reducing power.

Hydrogen peroxide scavenging assay

The ability of Areca seed extracts to scavenge hydrogen peroxide was determined, following method of Rouch et al. (1989)[32, 33]. A solution of hydrogen peroxide (40mM) was prepared in phosphate buffer (50mM PH 7.4) and its absorption was measured at 230 nm in spectrophotometer. Various concentrations (10-80µg/ml) of different Areca extracts were prepared. 0.6 ml of H_2O_2 solution was added and kept for 10 minutes. The absorbance was measured at 230 nm against blank containing phosphate buffer without H_2O_2 for background subtraction. Ascorbic acid was used as standard antioxidant. Different concentrations of ascorbic acid solutions were prepared and treated in the similar manner used for the extracts and then absorbance was measured at 230 nm against blank. Reduction of absorbance due to degradation of H_2O_2 with the increase concentrations were than recorded. The percentage of hydrogen peroxide scavenging activity for extracts and standard were calculated using the equation given below which were then plotted against concentrations for determination of IC_{50} values (the concentration at which 50% inhibition occurs) of different extracts and standard.

$$\% \ H_2O_2 \text{ scavenged} = \{(A_0 - A_1)/A_0\} \times 100$$

Where, A_0 = Absorbance of control, A_1 = Absorbance of standard or extract.

Statistical analysis:

Data obtained were analysed using one way analysis of variance (ANOVA) and expressed as mean ± standard deviation of three measurements using software GraphPad prism-5. A significant difference was considered at the confidence level of 5% ($p < 0.05$). Linear regression analysis was performed for calculating IC_{50} values.

RESULTS

About 8.875 g Powder was obtained from one areca seed (710 g / 80seeds) which was brown in colour. The percentage yield was found higher with 100% methanol then petroleum ether, ethyl acetate and water extracts respectively, when extracted using successive extraction process by cold maceration, from 400 g areca seed powder. But the yield was found highest with 50% methanol-water, when extracted by continuous percolation process separately using Soxhlet apparatus. The percentage yields found are shown in Table-1.

Preliminary phytochemical screening showed the presence of tannins in all the extracts. Percentage of tannin and total phenolic content was found higher in 100% methanol extract than in petroleum ether, ethyl acetate and in water extract when extracted successively by maceration. But found highest when extracted in Soxhlet apparatus using 50% methanol-water as extracting solvent. The percentage tannin and total phenolic content in different areca seed extracts obtained are shown in table-2. Total phenolic contents are depicted in Figure-1.

The antioxidant activity was analysed by evaluating reducing power and hydrogen peroxide scavenging ability of different extracts of areca seed. In the determination of reducing power the absorbance was found increased with the increase of concentrations which indicated the reducing ability of various extracts of areca seed. The highest reducing power was exhibited by methanol extract then petroleum ether, ethyl acetate, and water extracts obtained from successive extraction process. Ethyl acetate extract showed lowest reducing power in comparison to methanol and water extract respectively. Methanol-water extract obtained from continuous hot percolation process using Soxhlet apparatus exhibited highest reducing ability which is comparable to that of methanol extract obtained from successive extraction. The reducing powers of the extracts were compared to that of standard antioxidant ascorbic acid, which exhibited higher reducing power then all the extracts (Figure 2).

Table 1: % Yield of different areca seed extracts on the basis of extraction processes.

Sl. No.	Extracts	Process of extraction	% Yield
1	Petroleum ether	Successive extraction	1.40
2	Ethyl acetate	-do-	2.75
3	Methanol (100%)	-do-	10.35
4	Water	-do-	7.25
5	Methanol-water (50%)	Soxhlet extraction	27.30

Table 2: Percentage tannins and total phenolic content in different areca seed extracts.

Sl. No.	Extracts	% tannin in gram as gallotannic acid ± SD	Total phenolic contant in mg of tannic acid equivalent (TAE)/g of extract ±%SD
1	Petroleum ether	0.84 ± 0.02	1.00 ± 1.96
2	Ethyl acetate	10.00 ± 0.17	53.27 ± 2.04
3	Methanol	51.00 ± 0.82	146.70 ± 1.20
4	Water	27.00 ± 0.1	84.02 ± 1.40
5	Methanol-water (50%)	54.60 ± 0.43	155.80 ± 1.63

Each value represents mean ± standard deviation of three repeated determinations.

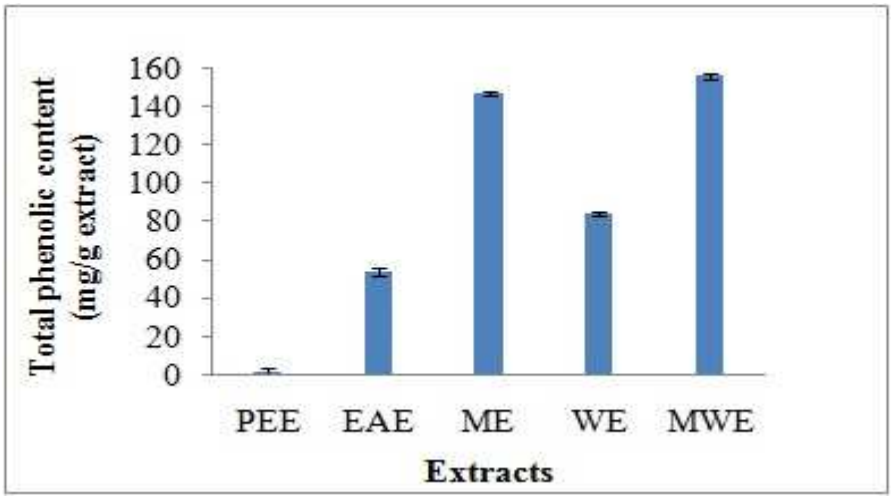

Fig. 1: Total Phenolic content of different areca seed extracts. Data expressed as mean ± standard deviation of three repeated determinations.

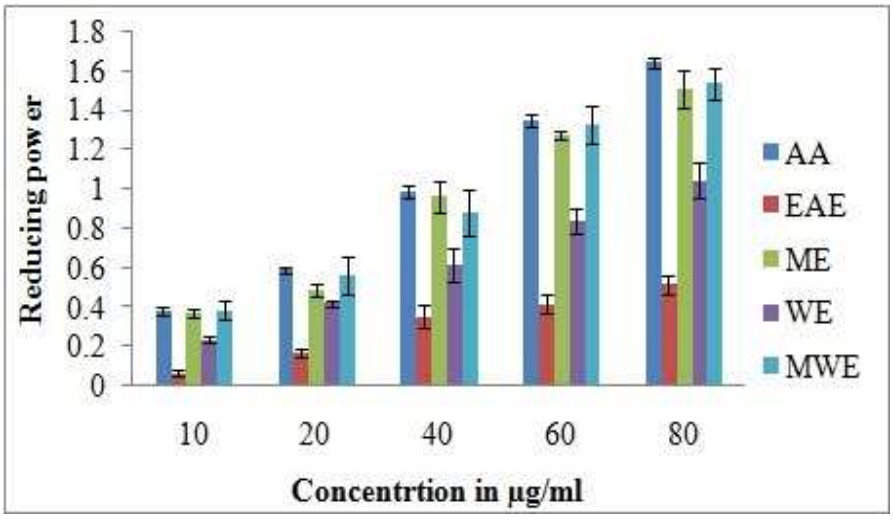

Fig. 2: Reducing power of areca seed extracts and Ascorbic acid (AA) as standard. Data expressed as mean ± standard deviation of three repeated determinations.

In the determination of Hydrogen peroxide scavenging activity the absorbance with methanol extract was found lowest than that of ethyl acetate and water extract obtained from successive extraction which indicated that methanol extract scavenged more hydrogen peroxide. Moreover methanol-water extract obtained from Soxhelt extraction exhibited comparable absorbance to that of methanol extract obtained from successive extraction. However all except ethyl acetate extract exhibited lower absorbance in comparison to that of standard antioxidant ascorbic acid (Figure 3).

The ME, MWE, WE except EAE exhibited higher scavenging ability (% inhibition) than that of standard antioxidant ascorbic acid (Figure 4, 5). The concentration at which 50% inhibition occurs or IC_{50} values of different areca seed extracts and ascorbic acid obtained in hydrogen peroxide scavenging assay are shown in Table-3.

The ME exhibited lowest IC_{50} value than all the extracts obtained from successive extraction. MWE and the ME showed nearly equal IC_{50} values although they were obtained from two different extraction processes. Moreover except EAE all the extracts showed lesser IC_{50} values than that of standard antioxidant ascorbic acid.

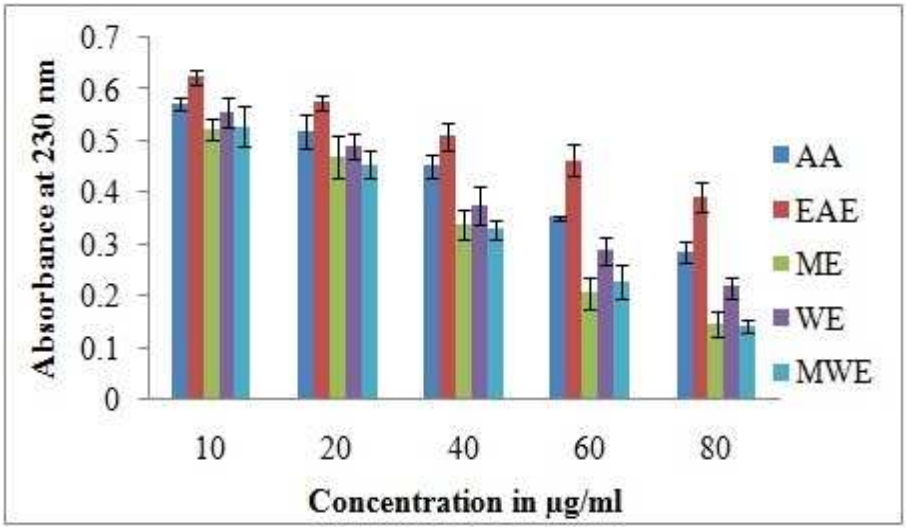

Fig. 3: Hydrogen peroxide scavenging activity of extracts and standard ascorbic acid.

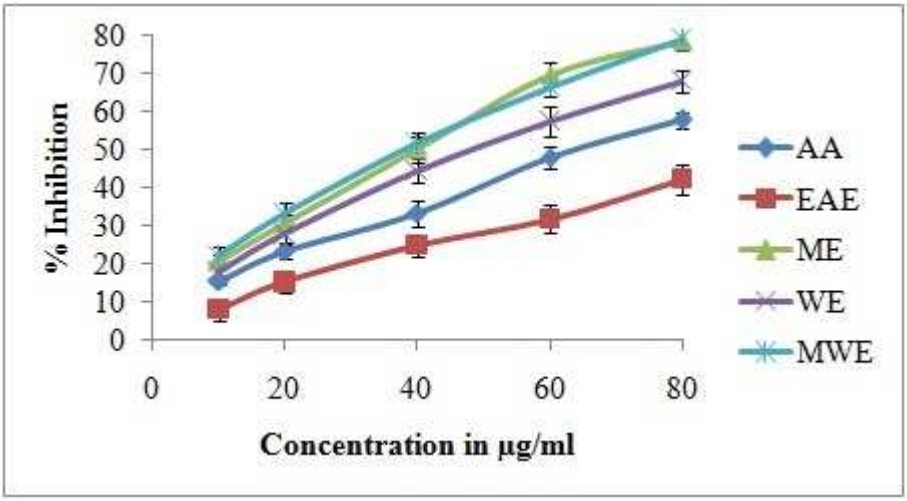

Fig. 4: Hydrogen peroxide scavenging ability of areca seed extracts and ascorbic acid (AA) as standard.

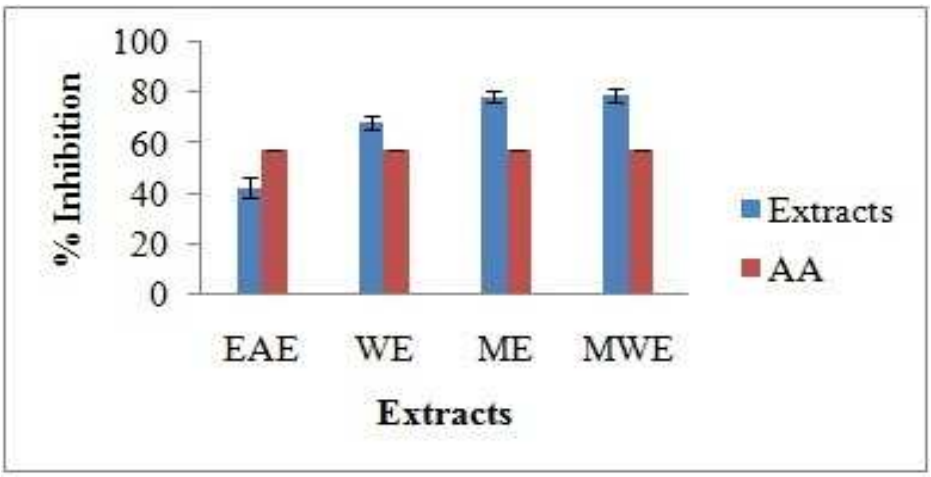

Fig. 5: percentage inhibition of hydrogen peroxide of areca seed extracts and ascorbic acid (AA).

Table 3: IC_{50} values of areca seed extracts and ascorbic acid. Data expressed as Mean ± Standard deviation of three repeated determinations.

Sl. No.	Standard / Extracts	IC_{50} (µg/ml)
1	Ascorbic acid (standard)	65.69±0.99%
2	Ethyl acetate extract (EAE)	96.39±1.67%
3	Methanol extract (ME)	41.87±1.98%
4	Water extract (WE)	51.44±1.41%
5	Methanol-water extract (MWE)	41.39±2.46%

Extracts with higher total phenolic content showed higher hydrogen peroxide scavenging ability (Figure 6) and lesser IC_{50} values (Figure 7) which indicated that there might be a co-relation between the polyphenolic content of the extracts and their scavenging activity. Moreover, the IC_{50} values of ME, MWE and WE except EAE were found less than that of ascorbic acid when compared (Figure-8), indicated their higher hydrogen peroxide scavenging ability than the well established antioxidant ascorbic acid.

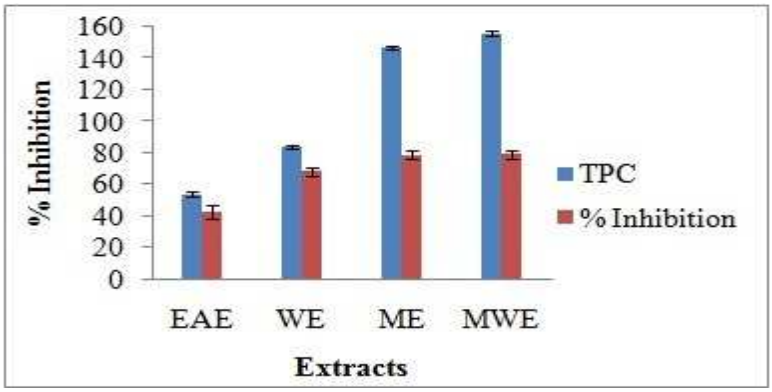

Fig. 6: Percentage inhibition of hydrogen peroxide of areca seed extracts and their total phenolic content (TPC).

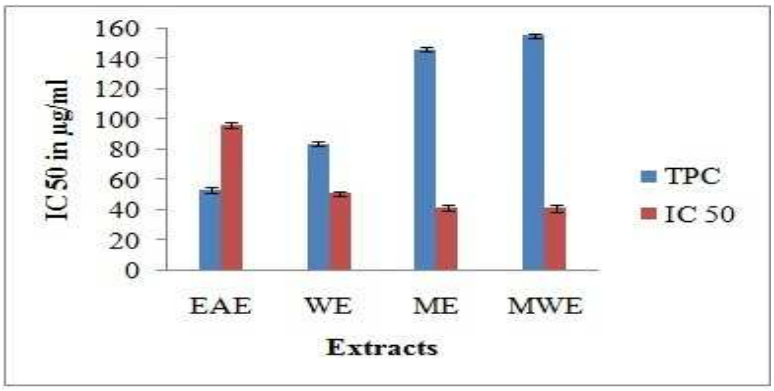

Fig. 7: IC_{50} values of crude extracts of areca seed and their total phenolic content (TPC)

Data expressed as mean ± standard deviation of three repeated determinations.

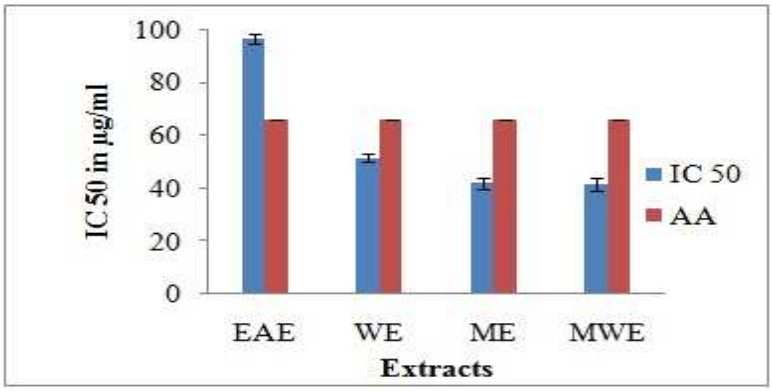

Fig. 8: IC_{50} values of crude extracts of areca seed and ascorbic acid (AA) as standard.

A significant correlation was observed in between the hydrogen peroxide scavenging ability and reducing power when plotted the data obtained in the same concentrations indicated the antioxidant abilities of the areca seed extracts. (Figure 9, 10, 11, 12)

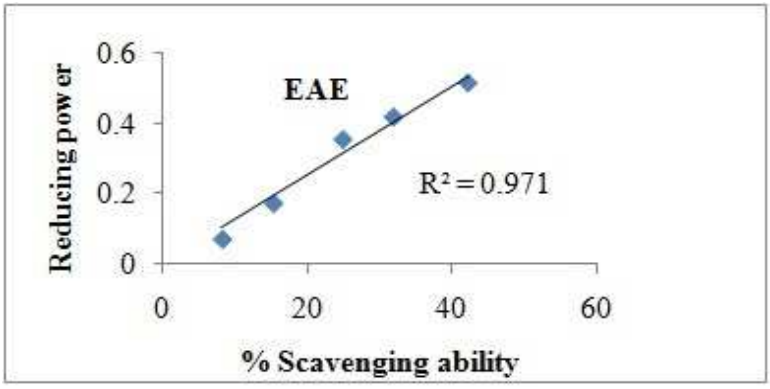

Fig. 9: Correlation between reducing power and percentage scavenging ability of ethyl acetate extract (EAE) of areca seed.

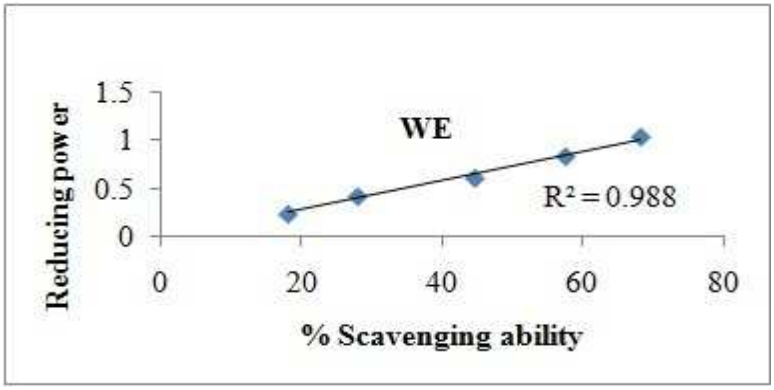

Fig. 10: Correlation between reducing power and percentage scavenging ability of water extract (WE) of areca seed.

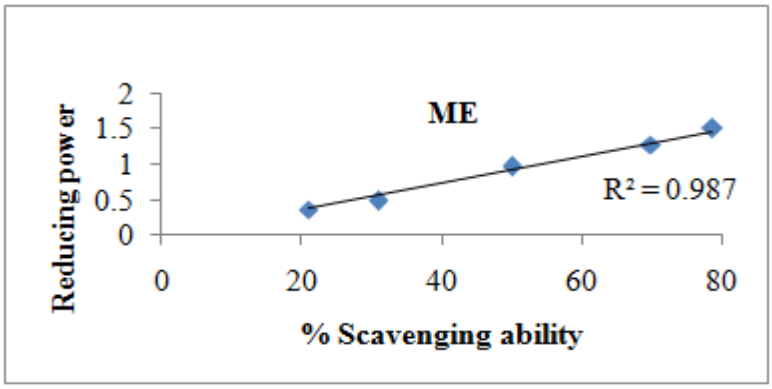

Fig. 11: Correlation between reducing power and percentage scavenging ability of methanol extract (ME) of areca seed.

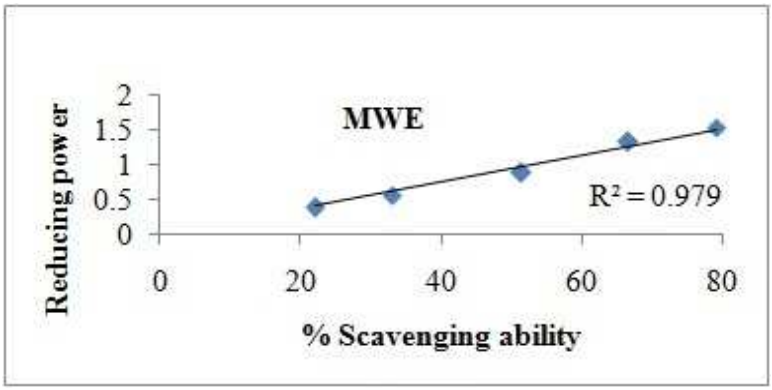

Fig. 12: Correlation between reducing power and percentage scavenging ability of methanol water extract (MWE) of areca seed.

DISCUSSION

When areca seed powder was extracted using petroleum ether, ethyl acetate, methanol and water as extracting solvents successively, the yield with methanol was found 10.35% which was highest among the solvents used. The yield with other solvents was found in the following order: water (7.25%) > ethyl acetate (2.75%) > petroleum ether (1.4%). Moreover the percentage tannin (51% g as gallotannic acid) and total phenolic content (146.7mgTAE/g extract) in methanol extract was also found more. Whereas, when extracted with 50% methanol-water in Soxhlet apparatus the yield was found highest (27.3%) but the % tannin (54.6%) and TPC (155.8 mg of TAE/g dry extract) were found only slightly more than that of methanol extract with only 10.35% yield, which indicates methanol is the best solvent for extraction of tannin from areca seed, in comparison to the other solvents utilised in this study. Tannins are high molecular weight phenylpropanoids (building units) formed from polymerisation of catechin (a family of polyphenolic Compound) practically insoluble in petroleum ether. But the petroleum ether extract showed the presence of tannin and other polyphenols, although only in a negligible amounts.

Various literature and research evidence suggests that the polyphenolic constituent of areca seed varies depending upon the geographical region where it is grown or cultivated, species and its maturity. The areca seed investigated in this study was collected from plant grown in the state of Assam, the north eastern part of India where it is a custom or ritual to chew it as betel quid for freshening mouth after tea or meal. Sufficient research data about its polyphenolic contents are not available to compare with our results. However, a little variation was observed when compared with the polyphenolic content of areca seed of other geographical regions, reported by various researchers. This variation may be due to differences in units, type of seed (fresh unripe, ripe, sun or shade dried etc.) and instruments used.

The antioxidant capability of different areca seed extracts are reportedly responsible for their polyphenolic content, which have potential reducing power due to presence of reductones (phenyl – OH group) that have the ability to donate a hydrogen atom to highly reactive free radicals resulting breaking of free radical chain reaction[34,35,36,37]. Different extracts of areca seed evaluated in this study, found to contain a considerable amount of polyphenolic compound like tannin and other non-tannin polyphenols. Their reducing potentiality was evaluated by estimating the ability to reduce Fe^{3+} (ferric) to Fe^{2+} (ferrous state) by donating an electron, which was observed by the change of yellow colour of the solution to prussian blue.The absorbance (at 700nm) of Ferrous complex formed was taken as a measure of reducing ability of the extracts which was found to increase with increasing concentrations. The extracts exhibited a considerable reducing power in concentration dependant manner which was compared with that of standard ascorbic acid. However the reducing power of ascorbic acid (1.57±0.028 at 80µg/ml) was found higher than all the extracts. Figure 2 shows that ME exhibited highest reducing ability among other solvents used in successive extraction. Moreover, the ME and MWE showed comparable reducing ability although they were obtained from two different extraction processes. The reducing power of different extracts obtained from successive extraction, found in the following order: ME (1.30±0.077) > WE (1.14±0.09) > EAE (0.61±0.05) at 80µg/ml. However at the same concentration reducing power of MWE obtained from Soxhlet extraction was found: 1.33±0.08 which is nearly equal to that of ME although the percentage yield was found higher (27.3%) than ME (10.35%). The reducing power of the extracts indicates the presence of compounds that are electron donors which can act as primary and secondary antioxidants[38, 39]. The extracts with high phenolic content exhibited higher reducing power which indicated that their antioxidant activity may be related to the polyphenolic compounds present in them.

The hydrogen peroxide scavenging abilities of the extracts were evaluated by determining their capability to convert hydrogen peroxide in to water. Polyphenolic and other compounds capable of donating electron might accelerate the conversion of H_2O_2 in to H_2O[40].The areca seed extracts found to contain polyphenolic compounds, thus they may be able to scavenged H_2O_2 when treated with them.

The absorbance of H_2O_2 in presence of extracts at different concentration was taken as a measure of scavenging activity. Figure 3 shows decrease of absorbance with increasing concentration of the extracts which indicates that H_2O_2 concentration decreases with increasing polyphenols present in the extracts. Figure 4 and 5 shows that the percentage scavenging ability of all the extracts except ethyl acetate are comparatively more than that of ascorbic acid. Moreover, the scavenging ability of areca seed extracts was found dependent on their total phenol content. Extracts with more phenol content scavenged most of the hydrogen peroxide (Figure 6). Methanol extract with TPC 146.7% ±1.2% scavenged 78.73% which is the highest among other solvents utilized in successive extraction. Water extract with TPC 84.02±1.40% scavenged 68.19%. EAE with TPC 53.27±2.04% scavenged 42.36% at 80µg/ml. However MWE obtained from Soxhlet extraction, with TPC 155.8 ±1.63% scavenged 79.14% at the same concentration. Furthermore, the extracts with higher phenolic content found to have lesser IC_{50} values (Figure 7) which indicates that the polyphenolic compounds present in the areca seed extracts might be related for their scavenging activity. Except ethyl acetate all the extracts exhibited lesser IC_{50} value in comparison to ascorbic acid (figure 8) indicating their scavenging potential. The IC_{50} values of the extracts were found in the following order MWE(41.39 ±2.46%) < ME(41.87±1.41%) < WE(51.44±1.98%) < EAE (96.33±1.67%). The ME and MWE exhibited nearly equal IC_{50} value although they were obtained from two different extraction processes. Standard antioxidant ascorbic acid was used as positive control to evaluate the antioxidant potentiality of the extracts. All the extracts particularly -ME, MWE and WE showed noticeable reducing power and higher hydrogen peroxide scavenging ability when compared with that of ascorbic acid.

The results obtained, provides considerable evidences that supports the antioxidant potential of the areca seed extracts. Moreover, Figure 9,10,11,12 shows a significant correlation between the reducing power and hydrogen peroxide scavenging ability of the areca seed extracts which further supports that the areca seed examined in this study possess potential antioxidant property.

CONCLUSION

The areca seed examined in this study, collected from the plant grown in the state of Assam, India, was found to contain high tannin and total polyphenolic content, exhibited considerable antioxidant activity. Total phenolic content of the extracts of the seed observed to be related to their antioxidant property. The methanol, methanol-water and water extracts showed high reducing power and significantly higher hydrogen peroxide scavenging ability than the well established antioxidant ascorbic acid that reflects the areca seeds antioxidant potentiality. The Methanol and Methanol-water extract exhibited comparable antioxidant activity although they were obtained from two different extraction processes. All these experimental evidences suggest that the seed is a potential source of natural antioxidant which may be helpful for the prevention or progression of diseases related to oxidative stress or may be a potent alternative to synthetic antioxidants. However, further investigations are required to identify the actual component responsible for the antioxidant activity and to ascertain its in-vivo activity prior to development it in to drug or nutraceuticals to extend its health benefit to the human being.

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