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Areca catechu Slurry: A Rich Source of Phenolics and Flavonoids

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In present study, the qualitative and quantitative analysis of phenolic compounds and flavonoids in arecanut slurry based on UV spectrometry and LC-MS were carried out. Results revealed that the arecanut slurry powder extract exhibited the presence of different phenolic groups such as alkaloids, flavonoids, phenolic acids, tannins, saponins and terpenoids. Further, total phenolic content (TPC) and total flavonoid content (TFC) of aqueous extract of areca slurry powder was found to be 214.50 mg/g (GAE) and 184.12 mg/g (RE), respectively. LC-MS analysis depicted the presence of vanillic acid in considerable amounts, which is a benzoic acid derivative used as a flavouring agent. Meanwhile, catechin was profoundly present in the aqueous extracts of arecanut slurry powder among all the other flavonoids. The arecanut slurry powder extracts exhibited substantial amount of vanillic acid and catechin, which are known to be beneficial in various pharmacological studies.

Keywords: *Areca catechu*, LC-MS, Phytochemicals, Total phenolic content, Total flavonoid content.

INTRODUCTION

Phytochemicals are used as templates for lead optimization programs that are intended to make safe and effective drugs. In developed countries, 25% of the medicines are based on plant extracts and their derivatives [1,2]. Plant phenolic compounds are typical botanical gifts from nature derived through a long history of scientific investigation. They represent the most abundant and widely available class of plant natural products. The plant phenolics are known to occur in high concentration in large number of plants and exhibit a protective role against several oxidative diseases [3].

Areca catechu L. (Arecaceae) is a widely distributed plant species in South Asia and commonly referred to as betel nut in India [4,5]. Arecanut palm occupies a prominent place among the cultivated crops in the Indian states of Assam, Kerala, Karnataka, Meghalaya, Tamil Nadu and West Bengal. Arecanut being the fourth most consumed addictive stimulant after caffeine, nicotine and alcohol, is chewed, alone or in a variety of ways *viz*., adding tobacco, slacked lime or other flavouring ingredients by at least 600 million people in India and Southeast Asia [6,7]. Paan, a preparation combining betel leaf with arecanut (*Areca catechu L.*), slaked lime and a combination of delicious condiments, sweetening agents and tobacco is consumed across South Asia [8,9]. In southeastern part of China, unprocessed fresh arecanut is treated with maltose and lime. It is cut into pieces and chewed with a few drops of cassia oil [10].

Arecanut (the seed of *A. catechu* L.) has been used as a traditional medicine for centuries to treat different diseases, including tapeworm infestation, abdominal distension, diarrhea and edema, while the husk of *A. catechu* L. serves as a valuable traditional medicine for treatment of dysuria, constipation and beriberi [5,11]. The plant is reported to have multiple therapeutic properties like, masticatory, anthelmintic, aphrodisiac [12], antihypertensive [13], wound healing [14], hypoglycemic [15], antidepressant [16] and anti-HIV [17]. The nut has been reported to produce sense of wellbeing, a hot sensation in the body, increased sweating, salivation [18] and heightened awareness, prevention of hunger and an increased capacity to work [18,19]. It was cited for its various medicinal properties, especially antibacterial and antiviral activity [20-22]. The extract of *Areca catechu* has stimulant effect on central nervous system, which is mostly due to presence of these alkaloids [14,23,24].

Previous investigations have focused on alkaloids obtained from *Areca catechu* L., with some studies reporting the determination of alkaloids in arecanut products available in India and China using HPLC [25], However, no information on analysis of phenolic compounds after thermal processing of arecanut slurry is available. Further, the role of other major constituents in arecanut slurry *viz*., phenol, which has significant positive effect on human body has not been explored. Previously, an attempt was made to evaluate different phenolic compounds in the arecanut slurry by HPTLC method [26]. However, for further confirmation of these phenolic compounds, the mass spectrometry characterization was essential. Therefore, a systematic method for the analysis of both phenolic compounds and flavonoids in the arecanut slurry was examined in present study.

Qualitative and quantitative analyses based on UV spectrometry and LC-MS for characterization of phenolic compounds was assessed in the present investigation. A total of 14 phenolic acids; gallic acid, ferulic acid, caffeic acid, syringic acid, vanillic acid, *p*-coumaric acid, *p*-hydroxybenzoic acid, salicylic acid, gentisic acid, protocatechuic acid, *o*-coumaric acid, 2,4-dihydroxybenzoic caid, chlorogenic acid, *trans*-cinnamic acid and 8 flavonoid compounds *viz*. rutin, myricetin, catechin, naringenin, quercetin, hesperetin, apigenin and luteolin were analyzed by LC-MS method.

EXPERIMENTAL

Arecanut slurry was procured from arecanut farmers from Uttar Kannada district, India. Sirsi local is the tall variety popularly used by the farmers in the district. Slurry of the variety was hence taken for the present study.

Chemicals, reagents, solvents and gelatin were procured from Rankem Chemicals and Thomas Baker Chemicals, India. The reference standards like gallic acid, ferulic acid, caffeic acid, syringic acid, *p*-coumaric acid, *p*-hydroxybenzoic acid, vanillic acid, salicylic acid, gentisic acid, protocatechuic acid, *o*-coumaric acid, 2,4-dihydroxybenzoic acid, chlorogenic acid, *trans*-cinnamic acid, rutin, myricetin, catechin, naringenin, quercetin, hesperetin, apigenin, luteolin were procured from Sigma-Aldrich Co. (St. Louis, USA). Folin-Ciocalteu reagent and Dragendorff's reagent were procured from Merck KGaA, (Darmstadt, Germany).

Extraction of phenolics/bioactive components: Areca slurry was boiled in water, dried in hot air oven at 45 ± 2 ºC and the powder obtained was stored in a refrigerator for further analysis. The slurry powder (2 g) was reconstituted in 25 mL of the distilled water. After 24 h, the contents were centrifuged at 10,000 rpm for 20 min. The process was repeated for the same source with another fresh 25 mL solvent. The supernatants were filtered and the fractions were pooled and made upto 50 mL with water. The extract was further utilized for the analysis of phenolic compounds.

Qualitative analysis-phytochemical screening: Phytochemical screening for the identification of phenolic groups like alkaloids, flavonoids, tannins, saponins and terpenoids was carried out in the extracts obtained using different chemical tests.

Alkaloids

Wagner's test: Few drops of Wagner's reagent were added by the side of test tube to 1 mL of extract of arecanut slurry. A reddish-brown precipitate confirms the test as positive.

Dragendoff's test: Few drops of Dragendorff's reagent were added to 1 mL of extract. A prominent yellow precipitate indicates the presence of alkaloids.

Flavonoids

Ammonia test: 1 mL of extract of arecanut slurry was treated with 0.5 mL of ammonia solution. An appearance of yellow colouration indicates the positive result.

Sodium hydroxide test: Few drops of 20% NaOH solution were added to 1 mL of arecanut slurry extract. On addition of HCl, the changed yellow colour of the extract turns to a colourless solution that depicted the presence of flavonoids.

Phenolic compounds

Ferric chloride test: Few drops of neutral 5% $FeCl_3$ solution were added to 1 mL of extract of arecanut slurry. A dark green colour indicates the presence of tannins.

Lead acetate test: Lead acetate solution (10%, 3 mL) was added to 1 mL of extract of arecanut slurry. Appearance of bulky white precipitate confirms the presence of phenolic compounds.

Saponins: 1 mL of extract of arecanut slurry was shaked vigorously with 15 mL of distilled water for 15 min. The formation of persistent foam indicates the presence of saponins.

Terpenoids: Terpenoids were identified by employing Salkowski test. In brief, 1 mL of extract of arecanut slurry was treated with 0.5 mL of chloroform and conc. sulphuric acid. A reddish brown precipitate indicates the presence of terpenoids.

Total flavonoid content (TFC): TFC is determined by a colorimetric method with minor modification [26]. Aliquots (1 mL) of diluted extracts or standard solutions was pipetted into 15 mL polypropylene conical tubes containing 2 mL double distilled water and mixed with 0.15 mL of 5% $NaNO_2$. After 5 min, added 0.15 mL of 10% $AlCl_3 \cdot 6H_2O$ solution to the mixture and allowed to stand for another 5 min and then 1 mL of 1 M NaOH was added. The reaction solution was mixed well, kept for 15 min and the absorbance was determined at 415 nm using UV-vis spectrophotometer (Biomate 3S, Thermo Scientific, USA). TFC was finally calculated using the standard rutin curve and expressed as mg rutin equivalent (mg RE) per gram (g) of sample.

Total phenolic contents (TPC): TPC in the extract of arecanut slurry was determined using Folin-Ciocalteu reagent assay method [27] with little modification. The extract was diluted 1:10 with distilled water and used for the estimation. 1 mL of extract was mixed with 100 µL of Folin-Ciocalteu, incubated at room temperature for 3 min and then mixed with 2 mL of 10% Na_2CO_3 solution. The resulting solution was further incubated for 60 min at room temperature under dark. The absorbance was measured at 765 nm using the UV-vis spectrophotometer (Biomate 3S, Thermo Scientific, USA). TPC is expressed as gallic acid equivalent (GAE) in milligrams per gram (mg/g) of sample.

Sample preparation: The sample after extraction and drying process was redissolved in methanol (HPLC grade)

and made upto 1 mL. The sample was diluted in the ratio 1:10 using the mobile phase (methanol:water/formic acid). The diluted extracts were injected into the system for LC-MS analysis.

LC-MS analysis: LC-MS analysis was performed using an Acquity UPLC system coupled to a Synapt G2-HDMS mass spectrometer system (Waters Corp., MA, USA). For the chromatographic separation a C18 reversed-phase column (5 µm, 4.6 × 150 mm; Waters Corp., MA, USA) was used with a gradient of methanol/acetonitrile (from 95:5:0.1 to 0:100:0.1 in 6 min). The mass spectral data were acquired in electrospray in negative mode. The capillary voltage and fragment or voltage were maintained at -3000 V and 125 V, respectively. Nitrogen was applied as the desolvation gas at a flow rate of 5 L/min. Mass spectra were acquired by multiple residue monitoring (MRM) of scanning mode.

RESULTS AND DISCUSSION

The extract of arecanut slurry powder exhibited the presence of alkaloids in significant (dense) amounts which was confirmed by both Dragendorff's and Wagner's tests. Alkaloids are the large group of small organic compounds, mainly derived from amino acids containing nitrogen found vastly in plants. Sodium hydroxide test confirmed the presence of flavonoids in excess amounts confirmed by the dark colour precipitation. Similarly, phenolic acids, tannins and terpenoids were also significantly present that was confirmed by various tests (Table-1).

It is clear from Fig. 1 that TPC of the aqueous extract of areca slurry powder was 214.50 ± 5.58 mg/g (GAE), while the TFC was found to be 184.12 ± 3.09 mg/g (RE). Solvents play a vital role in the extraction process. The extraction efficiency and quality of phenolics is dependent on the type of solvent, its concentration that varies with the sources and its physical state. Arecanut wastes contain 50-60% of tannin and they act as precipitants of gelatins, alkaloids, glycosides, heavy metals and proteins [28].

TABLE-1
PHYTOCHEMICAL SCREENING

Phytochemical group	Chemical test	Presence of components (A*)
Alkaloids	Dragendorff's	+++
	Wagner's	+++
Flavonoids	Ammonia	+
	Sodium hydroxide	+++
Phenolic compounds & tannins	Ferric chloride	+++
	Gelatin	+++
	Lead acetate	+++
Saponins	Foam	++
Terpenoids	Salkowski	+++

A* = Aqueous extract, '+' = presence of compound (scarcely), '–' = absence of compound, '++' = Dark colour (moderately), '+++' = Dark colour with precipitate (densely)

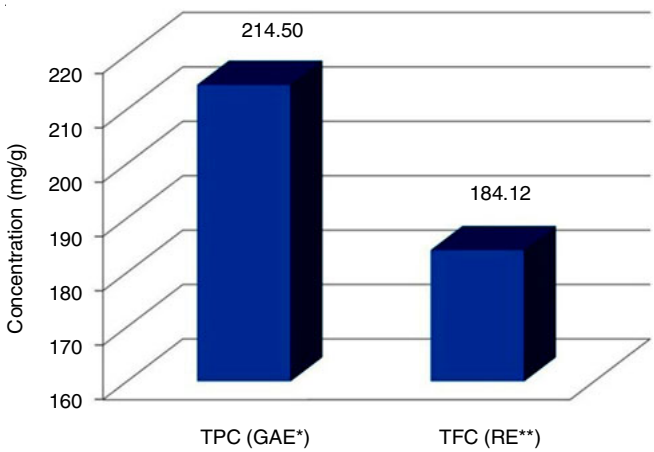

Fig. 1. TPC and TFC of arecanut slurry extract

LC-MS characterization of phenolic acids (Fig. 2) depicted that among the 14 phenolic acids, arecanut slurry extract contained considerable amounts of vanillic acid (Table-2). Catechin was profoundly present (14739.53 µg/g of sample) in the aqueous extracts of arecanut slurry powder (Fig. 3), followed by

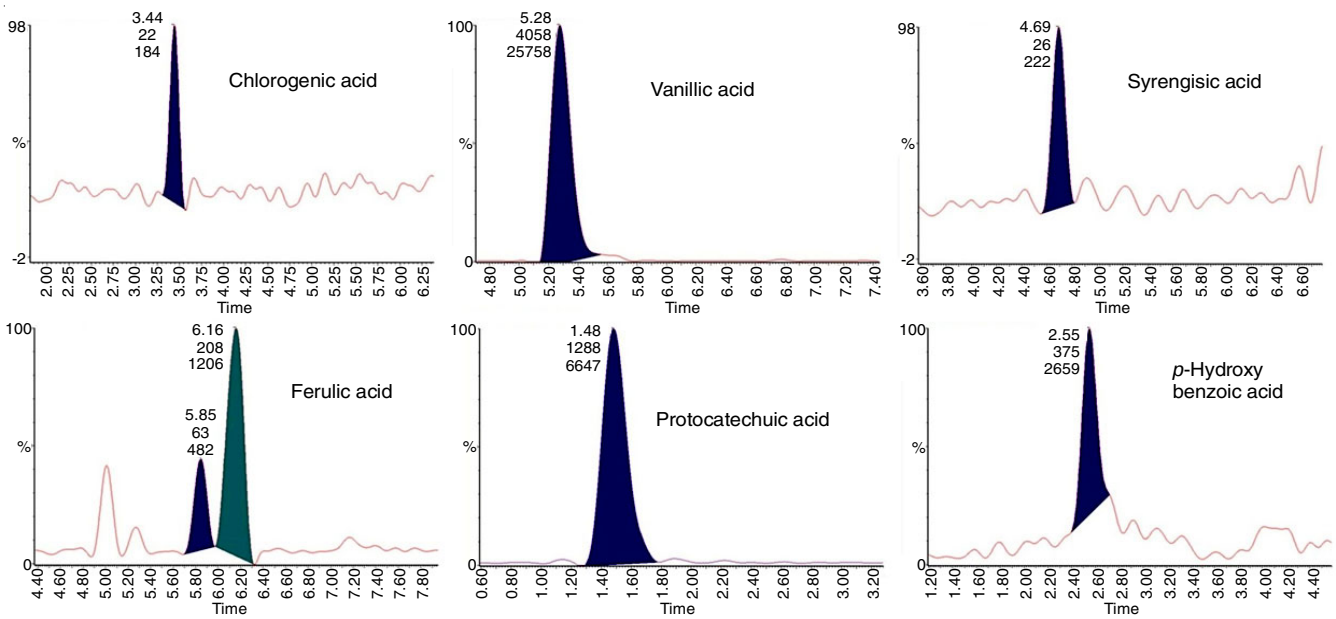

Fig. 2. LC-MS chromatographic profiles of phenolic acids in extract of arecanut slurry powder

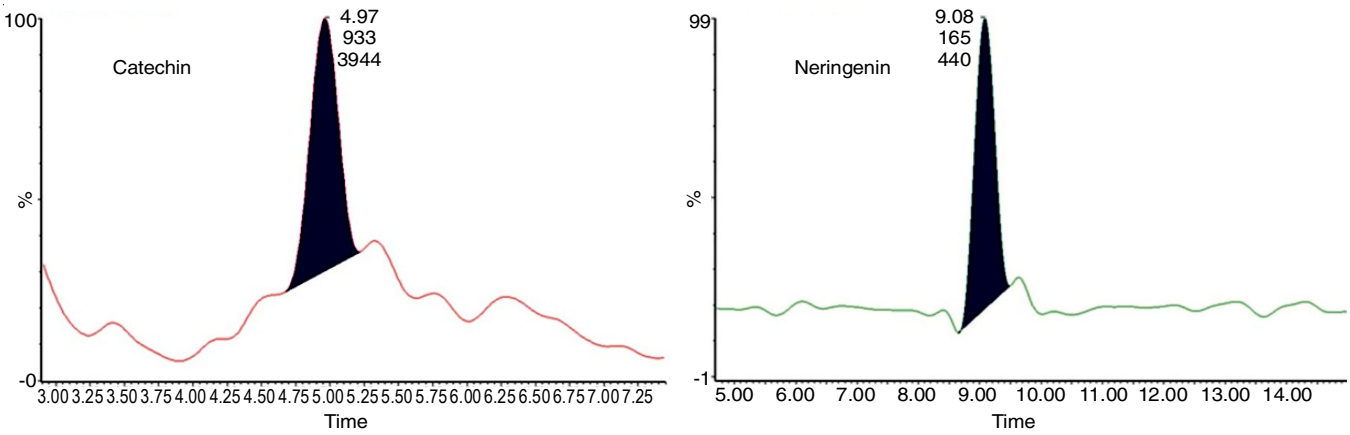

Fig. 3. LC-MS chromatographic profiles of flavonoids in the extract of arecanut slurry powder

TABLE-2
LC-MS ANALYSIS OF PHENOLIC ACIDS
IN THE ARECANUT SLURRY EXTRACTS

S. No.	Phenolic acids	Arecanut extract (µg/g of sample)
1	Syringic acid	0.283
2	Ferullic acid	0.169
3	Caffeic acid	0.006
4	Gallic acid	0.096
5	Vanillic acid	33.519
6	*p*-Coumaric acid	0.034
7	*p*-Hydroxybenzoic acid	0.161
8	Salicylic acid	0.016
9	Gentisic acid	0.004
10	Protocatechuic acid	1.203
11	*o*-Coumaric acid	0.002
12	2,4-Dihydroxybenzoic	0.010
13	Chlorogenic acid	0.009
14	*trans*-Cinnamic acid	0.133

*All values are expressed as µg/g of sample

quercetin (248.01 µg/g of sample), naringenin (155.43 µg/g of sample), hesperetin (80.73 µg/g of sample) and other flavonoids (Table-3).

TABLE-3
LC-MS ANALYSIS OF FLAVONOIDS
IN THE ANALYZED SAMPLES

S. No.	Flavonoids	Arecanut (µg/g of sample)
1	Rutin	15.77
2	Myricetin	30.02
3	Catechin	14739.53
4	Naringenin	155.43
5	Quercetin	248.01
6	Hesperetin	80.73
7	Apigenin	11.62
8	Luteolin	32.68

*All values are expressed as µg/g of sample

Conclusion

In present work, extract of *Areca catechxu* exhibits a high source of flavonoids and phenolics. The results are quite encouraging as the arecanut slurry powder extracts exhibited substantial amount of vanillic acid and catechin, which are known to be beneficial in various pharmacological studies.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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