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CHROMATOGRAPHIC PROFILE DEVELOPMENT BY HPTLC AND CHARACTERIZATION OF PHENOLICS IN ARECA NUT

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ABSTRACT
A.catechu kernel extract is evaluated for phenolic compounds (total phenolic and total flavonoid content). Extraction of phenolics in the kernel is carried out by water and the extract was screened for phytochemicals for the presence of alkaloids, flavonoids, tannins, saponins & terpenpoids determined by various chemical tests. Total phenolic content (TPC) was analyzed by Folin-Ciocalteu reagent assay method ($ë_{max}$765) and total flavonoid content (TFC) by aluminium chloride colorimetric method ($ë_{max}$415). Phytochemical screening revealed the presence of phytochemical groups with various concentrations. TPC and TFC in the aqueous extract was found to be 214.5 mg/g GAE and 184.12 mg/g RE, respectively. Further, chromatographic analysis was carried out by High Performance Thin Layer Chromatography (HPTLC) to evaluate the phenolic compounds and the densitometry was performed by scanning the developed plate at 270nm.

INTRODUCTION

Areca catechu L. (Palmae), commonly known as an important economical seed crop, is widely cultivated in tropical and subtropical areas, including India, Southeast Asia, East Africa and New Guinea. Areca nut is commonly known as betel nut, is the ripe fruit of the tree *A. catechu*. In Karnataka, as per the sources of the State Department of Horticulture, around 4.55 lakh acres (1.84 lakh hectares) is under arecanut cultivation which forms around 46% of the Country. It contributes to a total production of around 2.24 lakh tonnes that forms 47% of All India production (2009-10). It is important to note here that arecanut cultivation is undertaken in varied agro-climatic zones in almost 28 of 30 districts of the State. Top seven districts in Karnataka *viz*., Chikmagalur, Shimoga, Davanagere, Dakshina Kannada, Tumkur, Chitradurga and Uttara Kannada occupy 89% of the area under arecanut and contribute around 91% of areca produced in the State.

Phytochemical surveys are being seen as the first step towards the discovery and structural elucidation of useful natural organic constituents for textile or medicinal applications (Hostettmann *et al*., 2000). Crude plant extracts (e.g. infusion, tincture, decoction or others) are traditionally used by populations all over the world for medicinal purposes. Although their effectiveness and mechanisms of action have not been scientifically tested in the majority of the cases, they often mediate beneficial responses due to their bioactive chemical components (Barnes *et al*., 2007). The need to identify active chemical constituents in plant extracts requires phytochemical and analytical technique investigations.

A. catechu constituents are known through several experiments to have beneficial effects on skin, suggesting the possible use in cosmetics industries (Ashawat *et al*., 2007; Lee and Choi, 1999a, b; Lee *et al*., 2001; Padmaja *et al*., 1994). Areca nut is commonly used in folklore medicine for treatment of various diseases such as dyspepsia, constipation, beriberi and oedema. Areca nut before going into the market is processed such as boiling, to remove the outer coat of the kernel. The extract obtained during the boiling process is discarded as waste which is rich in several phenolic compounds and also has good colouring properties, which can be utilized as natural dye for textile applications. Therefore, a new commercial industry for the areca nut to reutilize the waste is emerging. For more economical and efficient utilization, the characteristics and composition of the commercial product needs to be scientifically studied. Various medicinal preparations use the Areca nuts (Staples and Bevacqua, 2006). These pharmacology activities are attributed to abundant phenolic compounds in the areca nuts. Although available studies have already demonstrated that areca fruit contains many phenolics and tannins (Zhang *et al*., 2008), whereas very scarce information is available about it to be used as natural dye source. The mode of action of plants producing dyeing effects on selected textile materials can be better investigated if the active ingredients are identified and characterized (Wanyama *et al*., 2011).

Boiling not only reduces the bitterness in the arecanut but also hastens the drying process. This liquor after the boiling process of the arecanut contains considerable quantities of tannins which is known as *chogaru*. It is a traditional practice to store a part of the liquor (1-5 ltrs) by arecanut farmers that is used to colour low quality arecanut and areca pieces and the rest of the liquor is thrown off. This slurry is stored as a stock solution for a year and a part of it is added while boiling the

newly harvested nuts. The sediments found in the liquor when dries are called arecanut dust. The dust and *chogaru* are traditionally used for tanning leather. The value for this *chogaru* can be enhanced by using this as colourant for textiles.

In the present study, the areca nut extract was screened for the presence of various phytochemical groups and further analyzed for the total phenolic and flavonoid contents. An HPTLC method was also developed in the present study, which is an efficient and effective method for the evaluation of phenolic compounds in the areca nut extract. This will give an insight of the phenolics present in the areca nut slurry, which is the extract to remove outer husk.

MATERIALS AND METHODS

Plant source

Areca nut - Sirsi local variety boiled slurry was collected from the Sirsi region (Uttar Kannada District) of Karnataka state, India for the present study.

Chemicals

Ammonia, aluminium chloride, chloroform, ethyl alcohol, ferric chloride, gelatin, hydrochloric acid, lead acetate, sulphuric acid, sodium hydroxide, sodium chloride, sodium carbonate and sodium nitrite were purchased from Rankem chemicals, Bangalore. The solvents acetone, ethyl acetate, methanol and formic acid were of analytical grade purchased from Thomas Baker Chemicals, Mumbai. Gallic acid and rutin reference standards were purchased from Sigma-Aldrich Co. (St.Louis, USA). Folin-Ciocalteu reagent, Dragendorff's reagent and HPTLC plates, Silica gel 60 F_{254} were procured from Merck KGaA (Darmstadt, Germany).

Sample preparation

The mature nuts were dehusked and boiled in water either as a whole or after cutting them into two or more pieces. The extract after the boiling process was collected and utilized for the further studies for evaluation of the phenolic compounds. The extract was filtered through whatmann paper no. 42 and were diluted with distilled water to get different concentration ratios as 25:75, 50:50 and 75:25 of areca nut extract and distilled water respectively. The extracts obtained were stored under refrigeration (8ºC) until further analysis within a week. Also, the levels of total constituents in plant derivates depend on the extraction process and its variables, such as the solvent used (Sideney *et al*., 2015).

Qualitative analysis

Phytochemical screening

The phytochemical tests for screening of various phytoconstituents like alkaloids, flavonoids, tannins, saponins and terpenoids in the areca nut boiled extract was analyzed by the method (Jyoti and Giridhar, 2015; Tejaswini *et al*., 2011).

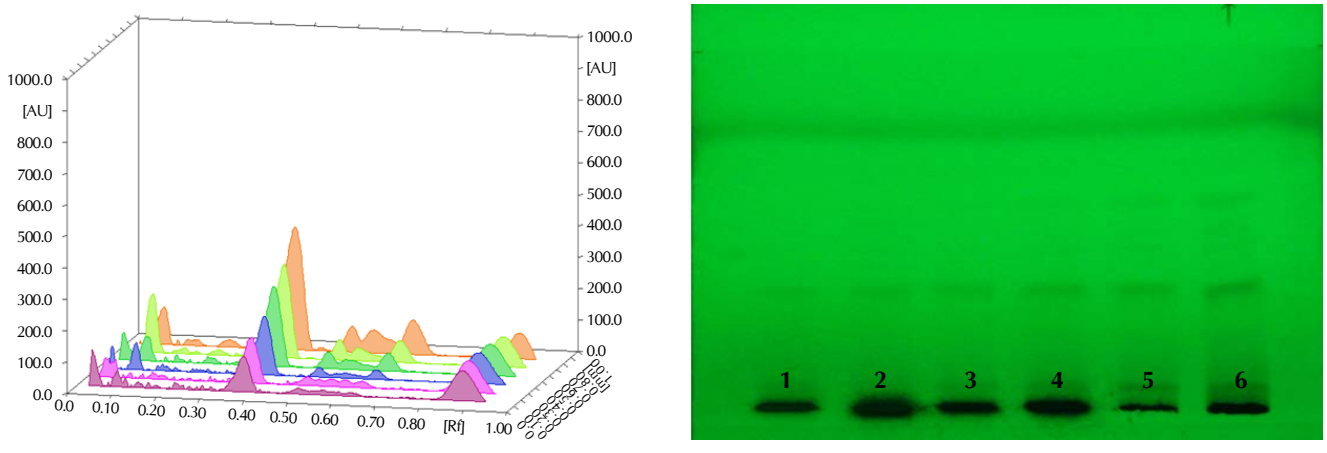
Quantitative analysis

Total Phenolic Content (TPC)

TPC in the extracts was determined by Folin-Ciocalteu assay method (Singleton and Rossi, 1986). Briefly, the solvent extract was diluted to appropriate volume and was mixed with 100µL of Folin-Ciocalteu reagent, incubated at room temperature for 3 minutes then 2mL of 10% Na_2CO_3 solution was added to the mixture. The resulting solution was incubated for 60 minutes at room temperature under dark condition, the absorbance was measured at ë765nm with the UV-Visible Spectro photometer (Biomate 3S, Thermo Scientific, USA). TPC was expressed as gallic acid equivalent (GAE) in milligrams per gm (mg/g) of sample.

Total Flavonoid Content (TFC)

TFC was determined by aluminium chloride colorimetric method (Yun *et al*., 2009). Aliquots (1ml) of appropriately diluted extracts or standard solutions were pipetted into 15ml polypropylene conical tubes containing 2ml double distilled H_2O and mixed with 0.15ml of 5% $NaNO_2$. After 5min, 0.15ml of 10% $AlCl_3.6H_2O$ solution was added and the mixture was allowed to stand for another 5min, and then 1ml of 1M NaOH was added. The reaction solution was well mixed, kept for 15min and the absorbance was determined at ë415nm using the UV-Visible Spectrophotometer (Biomate 3S, Thermo Scientific, USA). TFC was expressed as rutin equivalent (RE) in



Track 1 & 2: 1.5 & 3.0µl of *A.catechu* aqueous extract (1:3 ratio of extract & distilled water); Track 3 & 4: 1.5 & 3.0µl of *A.catechu* aqueous extract (1:1 ratio of extract & distilled water); Track 5 & 6: 1.5 & 3.0µl of *A.catechu* aqueous extract (3:1 ratio of extract & distilled water)

Figure 1: HPTLC Chromatogram & image profile of *A.catechu* extract at ë 270nm

Table 1: Phytochemical screening of *T.grandis* leaf extracts

Phytochemical group	Chemical test	*A.catechu* extract
Alkaloids	Dragendorff's	+ + +
	Wagner's	+ + +
Flavonoids	Ammonia	+
	Sodium hydroxide	+ + +
Phenolic compounds	Ferric chloride	+ + +
& tannins	Gelatin	+ + +
	Lead acetate	+ + +
Saponin	Foam	+ +
Terpenoids	Salkowski	+ + +

'+':positive, '-':negative, '+ + ':Dark colour, '+ + + ':Dark colour with precipitate.

Table 2: Chromatographic peaks of *A.catechu* extract at 270nm

Peak number	Extract R_f value	Area(AU)
1	0.05	95.1
2	0.08	2359.0
3	0.11	67.6
4	0.14	205.4
5	0.16	270.4
6	0.23	549.4
7	0.38	15478.3
8	0.51	2058.9
9	0.56	3096.1
10	0.65	3739.0

milligrams per gm (mg/g) of fresh sample.

HPTLC instrumentation and conditions

The chromatographic analysis was carried out by the analytical instrument High Performance Thin Layer Chromatography (HPTLC), (Camag, Muttenz, Switzerland). HPTLC system was comprised of Linomat V automatic sample applicator, vertical twin trough development chamber (10 cm x 10 cm, with metal lids), UV documentation cabinet and TLC Scanner III and winCATS TLC software (version 4.X).

RESULTS AND DISCUSSION

Table 1 records the phytochemical screening of the arecanut extract. It is observed from the Table that the extract revealed varying results for the different phytochemical group proved by different chemical tests. Alkaloids were present in high concentration proved by dragendorff and wagner test. The presence of flavonoids was positively proved by both the tests (ammonia and sodium hydroxide) in the extract, wherein ammonia test has less concentration of flavonoids compared to sodium hydroxide test. Also for phenolic compounds & tannins positive results were obtained with high concentration for all the tests, whereas saponins had positive results with medium concentration proved by foam test. Terpenoids were tested by Salkowski test which revealed positive results with high concentration. A similar study also quoted the results of phytochemicals in various medicinal plants with health beneficial effects (Sushmita *et al*., 2013).

Total phenolic content (TPC) of *A.catechu* extract was estimated using the Folin-Ciocalteu method, which relied on the transfer of electrons from phenolic compounds to Folin-Ciocalteu reagent in alkaline medium. TPC in aqueous extract of *A.catechu* kernel was found to be 214.5mg/g of gallic acid equivalent (GAE). Whereas total flavonoid content (TFC) in the extract was found to be 184.12 mg/g of rutin equivalent (RE).

The HPTLC densitometric method is widely accepted for separation and quantification of natural compounds such as flavonoids, phenolic acids, antraquinones, simple phenols, lignans and ginsenosides (Pereira *et al*., 2004; Srinivasa *et al*., 2004; Urszula *et al*., 2002). HPTLC was performed on silica gel 60 F_{254} HPTLC plates (Merck, Germany) 10x10cm plate with toluene, ethyl acetate, formic acid and methanol as mobile phase in the ratio 6:6:1.6:0.4 (v/v) respectively. The sample solutions of each extract were applied onto the plates as 8mm bands by means of a 10μL Hamilton microsyringe, distance between tracks (14mm), 10mm from the bottom edge, 15mm from the side edge. Plates were used without pretreatment. Chromatograms were developed in glass vertical twin-trough chambers (10 cm × 10 cm, with metal lids) previously saturated with mobile phase for 20 minutes and the development distance was 90 mm. After development, the plate was air-dried for 10min to remove the solvent vapours, heated in hot air oven at 120ºC for 5min and observed for the bands using UV documentation cabinet at 254nm.

Chromatographic profiles developed after scanning the HPTLC plate at ë254 nm is given in Fig. 1(a&b). The total number of peaks obtained, R_f values and area (AU) of the chromatographic peaks are given in Table 2. Number of peaks obtained for the *A.catechu* aqueous extract were 10. The peaks with R_f values 0.38 and 0.08 had the maximum area of 15478.3 and 2359.0 AU respectively, which were evaluated by comparing with the phenolic compounds reference standards.

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