Phytochemical screening for various secondary metabolites, antioxidant and anthelmintic activity of *Coscinium fenestratum* fruit pulp- A new bio source for the novel drug discovery

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Abstract

Coscinium fenestratum (Gaertn.) Colebr. (CF, Family: Menispermaceae) is an important endangered woody plant in India. The plant contains various major secondary metabolites for treatment of various disease conditions. In this present study the dried fruit pulp was subjected to aqueous, methanol and mixed aqueous and methanol (1:1) solvents extraction followed by phytochemical investigations, estimation of alkaloids, phenolics, flavonoids, antioxidant potentiality and anthelmintic activity was carried out. Preliminary phytochemical screening of CF fruit extracts revealed the presence of alkaloids phenols, flavonoids, tannins, steroids and resins which are responsible for biological properties. Combined aqueous and methanol extract resulted significant anthelmintic and antioxidant properties in a dose-dependent manner. DPPH free radical scavenging assay and H_2O_2 assay exhibited IC_{50} values of 42.38 ± 0.012 µg/ml and 46.80 ± 0.011 µg/ml respectively. Thereafter the anthelmintic activity was carried out against *Pheretima posthuma* and *Taenia solium* with the extract at varied concentrations of 25, 50, 100 and 150 mg/ml and compared with standard albendazole (25 and 50 mg/ml) and saline water (0.9%) as control. All the extracts exhibited concentration dependent paralytic effect, followed by death on the test organism but

significant activity showed by combined methanol and aqueous extract. The findings of this study indicate first time that the CF fruit pulp has therapeutic values with prominent antioxidant and anthelmintic property in order to find possible isolated compounds as a bio sources for future novel antioxidants in food and pharmaceutical formulations.
Key words: Antioxidant study, Anthelmintic activity, *Coscinium fenestratum*, Extracts, phytochemical study

Introduction

Secondary metabolites are important plant constituents for effective therapeutic activities. It was reported that presence of specific group of compounds show specific medicinal actions and sometimes traditionally reported but not have much or any scientific validations. In these activities antioxidant and anthelmintic activities are very important. Rapid production of free radicals lead to oxidative damage to biomolecules and results in serious disorders viz. degenerative disorders, cancer, diabetes, neural disorders and ageing and hence antioxidant plays a vital role to block the free radicals production [1, 2]. In other hand infections with parasitic worms are serious problem to the humans and produce various diseases in worldwide and helminthes is one of them. There are various types of worms viz. round worms or nematodes include intestinal worms, filarial worms that cause lymphatic filariasis, onchocerciasis, platyhelminths or flatworms include the flukes, tapeworms etc [3]. These worms causes lymphatic filariasis, onchocerciasis, cysticercosis, malnutrition, anaemia, eosinophilia, pneumonia etc. which are life threatening. As per the report of WHO more than two billion people are suffering from parasitic worm infections globally [4]. Treatment with synthetic drugs causes many side effects and helminthes becomes resistant. Hence there are demands on natural plant secondary metabolites for treatment and prevention of this chronic problem.

Coscinium fenestratum (Gaertn.) Colebr. (CF) is a woody plant belongs to the family Menispermaceae. The plant is commonly known as tree turmeric or false Calumba due to yellow stem. CF is found in Asian countries like India, Malaysia, Vietnam, Myanmar, Singapore, Thailand and Sri Lanka [5, 6]. In India, the plant is endangered and located in the Western Ghats areas, especially in high rainfall evergreen forests of Karnataka, Kerala and Tamil Nadu at altitude of 500-750 m [7, 8]. The tree requires long seed germination time and takes 14-15 years to mature and

flowering. Hence availability of fruits and seeds are very rare and these lead to endangered red labeled species due to overexploited from natural habitats, zero cultivation thought and uprooted before their reproduction stage for its medicinal importances [4]. Leaves and roots are traditionally used for treatment of ulcers, skin diseases, eye disorders, inflammation, hypertension, jaundice, diabetes and snake bites [9-11]. Thereafter various solvent extracts of leaves and roots of CF have reported multiple beneficial pharmacological activities viz. hepatoprotective, immunoprotective, hypoglycemic, anti tumor activities, dressing wounds, against ulcers, in cutaneous leishmaniasis and non toxic to mammals [12, 13]. Stem and root extracts are also showed the antioxidant and antimicrobial potential [14]. Traditionally CF is used as one of the ingredients in several ayurvedic preparations like soap, bath gels, face wash and bath oil etc, and in cosmetic industry as facial masks, fairness creams, body lotions etc [4]. Furthermore stem extract of CF reported significant effect on stimulating insulin secretion [15]. These activities due to presence of the important alkaloid containing phytoconstituents like berlambine, dihydroberlambine, noroxyhydrastine, berberine (an isoquinoline alkaloid) and other constituents like ceryl-alcohol, saponin, hentriacontane, sitosterol glucoside, palmitic acid, oleic acid etc. that are isolated from stem and roots of the plants [16]. Recently ecdysterone was identified and isolated from stem and leaves of CF and estimated through HPLC and LC-MS [17]. The availability of various phytoconstituents in root and stem, forced to select the various solvent extraction for the fruits. It was reported that the constituents varied with the solvent extraction and the zone of collection of the raw materials [18-20]. Till now no literature has revealed the medicinal importance of fruit and the constituents of pulp may be due to improper collection or less availability of the fruits and seeds. Furthermore it is essential to know about the cause of delayed germination too. Based on the secondary metabolites availability in the plant, the present study was carried out to know about the phytoconstituents present and their estimation followed by novel antioxidant and anthelmintic activities were performed first time from the various extract of dried fruit pulp.

Materials and methods:

Collection and Identification of fruits: 100 numbers of CF fruits were collected from Dr. Gokul S, CIMAP Research Centre, Allalasandra, GKVK Post, Bangalore -65

(Latitude: 12° 58' N and Longitude: 77° 38' E), and was authenticated by Dr. P.E. Rajasekharan, Principal Scientist, IIHR, Bangalore. The fruits are stored as herbarium in Pharmacognosy Department of Krupanidhi College of Pharmacy, Bangalore (Herbarium No: CF-317/KCP/2016-17).

Morphological study of fruits and seeds:

50 fruits are randomly selected and measured for diameter using vernier caliper with the measurement readings in centimetres (cm) and it is precise up to 2 decimal places. Thereafter seed diameters are also measured after removal of pulp and resinous matters to know about the probability for late germination. Furthermore whole morphological study was carried out by observed color, odor, size, shape, extra features etc.

Preparation of Extracts:

Fresh fruits of CF plant were shade dried for several days and in between observed for fungal infections. The dried pulps were ground to a course powder and 250 g of the same was soxhlet extracted with light petroleum ether for 4 hrs and defatted the materials. Further the same successively extracted with four solvents viz. chloroform, methanol (80%), aqueous and equal ratio mixture of aqueous and methanol (80%) (1:1). Reflux method was used for all extracts separately for 7-8 hrs (after drying each time of extraction) preparation and finally yield was calculated after removal of the solvents by rotary evaporation (at 45^0 C) and dried extract was stored in refrigerator (at 4-5^0 C) for further investigations.

Phytochemical screening:

The preliminary phytochemical analysis of the plant extracts were performed using standard protocol given by Khandelwal, 2003 [21] and Kokate, 2005 [22] to identify the presence of alkaloids, flavonoids, steroids, glycosides, cardiac glycosides, anthraquinones, tannins and saponins.

Based on the presence of phytoconstituents, following estimations of secondary metabolites were carried out.

Determination of Alkaloid:

1 mg of the each plant extracts separately were dissolved in dimethyl sulphoxide (DMSO) and further 1ml of 2 N HCl was added and filtered. The solutions were separately transferred to a separating funnel with the addition of 5 ml of bromocresol

green solution and 5 ml of phosphate buffer. Then the mixture was shaken properly with 1, 2, 3 and 4 ml chloroform and collected in a 10 ml volumetric flask and diluted to the volume with chloroform. A set of reference standard solutions of atropine (10, 20, 30, 40, 50 and 60 µg/ml) were prepared in the same manner. The absorbance for test and standard solutions were determined using UV spectrophotometer at 470 nm. Blank sample was prepared for error correction. Finally the total alkaloid content was calculated as mg of atropine equivalent (AE)/g of each extract [23].

Total Phenolic Content

The total phenolic compounds in all three fruit pulp extracts of CF were determined by Folin-Ciocalteu's method. During reaction blue color is formed and the blue color is measured spectrophotometrically [24]. 1 ml of sample (1 mg/ml) was mixed with 1 ml of Folin-Ciocalteu's phenol reagent. After 5 min, 10 ml of a 7% Na_2CO_3 solution was added to the mixture then 13 ml of deionized distilled water was added and mixed thoroughly. The mixture was kept in the dark for 90 min at 23°C for the reaction (blue color form). Gallic acid was used as a standard and the standard solution was prepared as per the same method followed for the sample (10, 20, 30, 40, 50 and 60 µg/ml). Then absorbance was taken at 765 nm. The total phenolic content was determined from extrapolation of calibration curve which was made by preparing gallic acid solution and was expressed as mg of gallic acid equivalent (GAEs) per g of extract (GA mg/g). The estimation of the phenolic compounds was carried out in triplicate. The following formula was used for the calculation:

$$T = (C \times V)/M$$

Where, T = total content of phenolic compounds, mg/g plant extract, in GAE; C = concentration of gallic acid established from the calibration curve (µg/ml); V = volume of extract (ml); M = weight of water extract of the plant (g).

Total Flavonoid Content

Total flavonoid content was measured by Aluminium chloride colorimetric assay as described by Park et al., 2008 [25]. In a 10 ml test tube, 0.3 ml of extracts, 3.4 ml of 30% methanol, 0.15 ml of $NaNO_2$ (0.5 M) and 0.15 ml of $AlCl_3.6H_2O$ (0.3 M) were mixed properly. Further after few minutes, 1 ml of NaOH (1 M) was added. Same way the standard solution was also prepared using rutin as standard. The standard curve for

total flavonoids was made using rutin standard solution (10, 20, 30, 40, 50 and 60 µg/ml). The solution was mixed well and the absorbance was measured against the reagent blank at 506 nm. The total flavonoids were expressed as mg of rutin equivalent (RuE)/ g of dried extract.

Antioxidant assays

Each sample was dissolved in 80% methanol to make a concentration of 1 mg/ml and then diluted to prepare the series concentrations for antioxidant assays.

DPPH Assay

All the fruit pulp extracts were tested for their free radical scavenging activity against the stable free radical 1,1-diphenyl-2-picryl-hydrazyl (DPPH). The ability to bleach DPPH by the extracts was quantified using spectrophotometer. The method was described as per Brand-Williams et al., (1995) method [26]. 1ml of 0.1mM DPPH solution in methanol was mixed with 1ml of plant extract solution of varying concentrations (25, 50, 100, 150 and 200µg/ml). Corresponding blank sample were prepared using mixed 1ml methanol and 1ml DPPH solution methanol and L-ascorbic acid was used as reference standard (1-100 µg/ml) and the experiment was done in triplicate. The decrease in absorbance was measured at 517nm after 30 minutes in dark (for reaction) using UV-Vis spectrophotometer. The inhibition % was calculated using the following formula. The IC_{50} value of the sample i.e. the concentration of sample required to inhibit 50% of the DPPH free radical, was calculated using the calibration curve by linear regression. The percentage scavenging was calculated using the following formula,

DPPH Scavenging effect (%) = [($A_{control}$ – A_{sample} / $A_{control}$) x 100]

Hydrogen peroxide-scavenging activity:

The hydrogen peroxide assay was described as per the method described by Nabavi et al., 2009 method [27]. Hydrogen peroxide solution (2 mmol/l) was prepared in phosphate buffer (pH 7.4). 0.6 ml of extract at various concentrations (25, 50, 100, 150 and 200 µg/ml) was added to hydrogen peroxide solution. For each concentration, a separate blank sample was prepared. Absorbance of hydrogen peroxide by UV visible spectrometer at 230 nm was determined followed by after 10 minutes reading calculated for blank solution containing phosphate buffer without hydrogen peroxide.

The percentage inhibition of H_2O_2 scavenging activity was calculated using the below formula:

% scavenging activity = [1- (Absorbance of test / Absorbance of control)] X 100

Anthelmintic property:

Chemicals and drugs

All chemicals and drugs were obtained commercially and were of analytical grade. Albendazole was purchased from local market of Bangalore. Dimethyl formamide (DMF) were purchased from Merck, Germany.

Selection of experimental organisms

The assay was performed preliminary on adult earthworm, *Pheretima posthuma*, belong to class Oligochaeta. Due to easy availability and its anatomical and physiological resemblance with the intestinal round worm parasites of human beings, they have been widely used for preliminary evaluation of anthelmintic activity. Thereafter tapeworm (*Taenia solium*, Family: Taeniidae) was selected for assurance of anthelmintic activity. Earthworms were collected from moist soil of medicinal garden of Krupanidhi College of Pharmacy, Bangalore and tape worms were collected from local slaughter house (Infested intestines of porks), Yeshwanthpur, Bangalore. Both of them are separately washed with normal saline to remove all foreign matters from body and further used for anthelmintic study. The earthworms of 3-5 cm in lengths and 0.1-0.2 cm in width and tapeworms of 6-8 cm length were used for all the experimental protocol. Albendazole (25 mg/ml and 50 mg /ml) was used as standard solution (prepared by dissolved in DMF) and each of the test solutions of CF fruit extracts (25, 50, 100 and 150 mg/ml) were evaluated for anthelmintic activity.

Methods:

For the evaluation of each plant extract, four worms were placed in a Petri dish containing 20 ml solution of crude extracts separately in the said concentrations and then introduced the worms in the solution. The same method was used for each case.

Observations

An observation made for the time taken for paralysis and death of individual worms during the completion of the investigation. When there was no movement of any part of the body, then time noted for the paralysis condition followed by the death time was noted when no movement of any part of the body even after shaken vigorously and also

followed by fading away of their body colors of worms. Death was also ascertained when dipped in warm water at 50^0 C for those worms [28]. Experiment was carried out as per the guideline of the Institutional Bio safety and Ethical Committee [29].

Statistical analysis

Data are expressed as mean ± SD from three replications. For antioxidant assays and anthelmintic activity one way ANOVA test followed by Tukey's test ($P < 0.05$) was used to analyze the differences among IC_{50} of various extracts for different antioxidant assays. The IC_{50} values were determined using the Graph Pad Prism 5 software. Correlation coefficient (r) was carried out among the extract and the activities. P values less than 0.05 were considered to be statistically significant.

Result and discussion

Morphological study of the fruits and seeds:

Vernier caliper was used and measured the diameter for 50 numbers of CF fruits and seeds separately (Figure-1 and 2) and the diameter was recorded in Table-1 and 2 respectively. The color of the fruits (Figure-3) is greyish ash without any odor. Size are varies from 10-14 cm in diameter and shape is Globulus, tapering towards embryonic site. Each fruit contain a single seed. Fruit pulp is made up of resinous matter (Figure-1). In contrast, color of seeds is off white to gray without odor. Size is 4-6 cm in diameter and shape is subglobulus, divaricate. The seeds are very hard to break (Figure-4). It was observed that due to high content of alkaloids i.e. berberine in the seed and the embryonic opening part is closed with the resinous matters that causes delayed germination. Average diameter of fruit: 12.35 cm; Minimum diameter of fruit: 10.6 cm; Maximum diameter of fruit: 13.7 cm. Furthermore average diameter of seed: 5.03 cm; Minimum diameter of seed: 4.1 cm; Maximum diameter of seed: 6.3 cm.

Yield of the extract:

The yield and color of the crude extract obtained from the extracted fruit pulp of CF are depicted in Table 3 and Figure-5 and 6.

The yield of the extracts was found higher percentage (5.76%) in combined extract of methanol and aqueous solvents followed by methanol extract (5.12%) and aqueous (4.12%). Earlier literatures are also reported that combined extracts increased yield of the crude extracts than the individual extract in particular plant species [19, 30].The present investigation was also resulted the similar. This indicated that the CF fruit pulp extracts was also dependent of type of solvent used. Literature survey

revealed that the extraction yield increases with increasing polarity of the solvent used in extraction. Hence the combined use of water and organic solvent may facilitate the extraction of soluble chemicals in water and/or organic solvent and due to that the yield of secondary metabolites is higher than individual solvent extraction [31]. The results of this study are in agreement with the extraction yields of some medicinal plants [32, 33].

Phytochemical Screening

Various chemical tests were performed to detect the presence of secondary metabolites. The result was tabulated in Table-4. Based on the availability of the secondary metabolites, further estimation of vital plant constituents' viz. total alkaloids, phenols and total flavonoid were determined for methanol, aqueous and combined aqueous and methanol extracts.

Total Alkaloids:

The alkaloid content was determined in CF fruit pulp extracts and expressed in terms of atropine equivalent as mg of AE/g of extract (the standard curve equation: $y = 0.014x + 0.106$, $R^2 = 0.994$). The highest concentration of alkaloid was measured 77.02 mg/g in combined extract. This is because of high solubility of the alkaloids in the combined extract rather than individual ones (Table-5) [31, 33].

Total phenolic content:

The total phenolic contents in all the extracted CF fruit pulp was determined using the Folin-Ciocalteu's reagent is expressed in terms of gallic acid equivalent (mg of GA/g of extract, the standard curve equation: $y = 0.012x + 0.166$, $R^2 = 0.991$). The highest concentration of phenols was measured in combined aqueous and methanol extract followed by methanol and aqueous extract. It was reported that high solubility of phenols in polar solvents provides high concentration in the extracts [34, 35] and the same trend followed in the present in investigation where combined aqueous and methanol solvents increased solubility of phenolic compounds than individual solvents (Table-6).

Total Flavonoid content:

The concentration of Flavonoids was determined using spectrophotometric method for all the three extracts and the content of flavonoids was expressed in terms of rutin (Ru) equivalent (mg of Ru/g, the standard curve equation: $y = 0.011x + 0.041$, $R^2 = 0.993$). In this case also the same trend followed as above. The highest flavonoid

concentration (98.03 mg Ru/g) was recorded for combined extract followed by methanol extract. This result was due to the solubility. It was reported that the concentration of flavonoid in plant extracts depends on the polarity of solvents used in the extract preparation [36]. The similar result obtained in the same study (Table-7).

Based on the estimation of the total alkaloids, phenols and flavonoids, the further investigation was carried out to reveal an antioxidant study. Phenolic and flavonoid compounds are known to have correlation with antioxidant activities [37, 38]. Furthermore the present study revealed about the higher content of these compounds and due to presence of them the CF fruit pulp may have the scavenging activity i.e. mainly due to their redox properties, which play an important role in adsorbing and neutralizing free radicals and decompose peroxides [39]. In focus of that two different methods were used to revealed antioxidant activity.

Antioxidant assays

An antioxidant is defined as any substance that inhibits oxidative damage to a target molecule [40]. Antioxidant compounds like phenolic acids, polyphenols and flavonoids reduce free radicals like peroxide, hydrogen peroxide or lipid peroxyl and thus inhibit the oxidative mechanisms which lead to degenerative diseases [41]. Based on that following few methods were studied for the CF fruit pulp extract.

DPPH method: Free radical scavenging activity of all the crude extracts of CF fruit pulp was quantitatively determined using a DPPH assay along with IC_{50} values. IC_{50} value represents the particular concentration of test extract that inhibit the activity of 50%. The result was tabulated in figure-7. The effect of antioxidants on DPPH is thought to be due to their hydrogen donating ability [42]. DPPH is a purple color dye having absorption maxima of 517 nm and upon reaction with a hydrogen donor the purple color fades or disappears due to conversion of it to 2, 2-diphenyl-1-picryl hydrazine and hence absorbance is decrease. Combined aqueous and methanol extract showed maximum % inhibition (68.43%) followed by methanol extract (58.24%) at 200 µg/ml concentration whereas L-ascorbic acid showed 92.17% inhibition. The potency of scavenging activity for all the extracts was determined by IC_{50} values. Combined extract (Aqueous + methanol) showed lower IC_{50} value of 42.38 µg/ml followed by methanol extract (52.43 µg/ml, Table-8) when compared to standard L-ascorbic acid (4.87 µg/ml).

Hydrogen peroxide radical scavenging assay: Hydrogen peroxide is a weak oxidizing agent and by oxidation of essential thiol (-SH) groups it can inactivate a few enzymes. The generation of H_2O_2 in minimum quantity in biological systems is significant to determine. Naturally-occurring iron complexes inside the cell are react with H_2O_2 in vivo and highly reactive hydroxyl radicals are generates that causes the origin toxic effects [43]. The scavenging activity of CF fruit extracts was evaluated and compared with Ascorbic acid and the results are tabulated in figure-8. It was reported that H_2O_2 Scavenging activity of extracts depends on the phenolics content, which can donate electrons to H_2O_2 [44] and the present study revealed the content of phenolics which showed scavenging activity of H_2O_2. Among the three extracts combined extract (Aqueous + Methanol) followed by methanol extract showed good activity in depleting H_2O_2 with an IC_{50} value of 46.80 and 54.22 µg/ml respectively (Table 8). The percentage of H_2O_2 scavenging activity of combined extracts (Aqueous + Methanol) was found to be 61.07 % followed by methanol extract (59.23%) at 200 µg/ml concentration as compared with standard L-ascorbic acid (90.13 %).

Correlation matrix: There is a direct correlation between the % yield, content of secondary metabolites and the antioxidant study was observed in this present investigation. The result was depicted in table-9.

The results from Table-9, indicates that the % yield of extract in particular solvents (CF fruit pulp) has direct correlation with the content of secondary metabolites and even antioxidant activities. Furthermore the Table-8 indicated that antioxidant activity dependent on IC_{50} values which are inversely correlated which is similar result of earlier research findings [45]. Based on the presence of various phytochemicals viz. alkaloids, phenols, tannins, flavonoids and due to strong antioxidant activity of CF fruit extracts, the further anthelmintic activity was carried out first time. Many scientific literatures already revealed that presence of phenols, tannins, flavonoids leads to anthelmintic activity [46, 47].

Anthelmintic activity:

Preliminary anthelmintic activity was carried out using various extracts of CF plant on adult earthworm (*Pheretima posthuma*) followed by on tape worm (*Taenia solium*) at the dose of 25, 50, 100 and 150 mg/ml and compared with control (0.9% Normal Saline) and albendazole (25 mg/ml and 50 mg /ml) as standard. Results

revealed combined aqueous and methanol extract of CF fruit pulp showed significant anthelmintic activity (26.01minutes for death of worms) than others with respect to paralysis followed by death for earth worms at 150 mg/ml concentration and with the same combined extract produced death of tape worms at 37.32 minutes which in near to standard drug (28.11 minutes) (Table-10). Table -10 revealed that albendazole at 50 mg/ml concentration, showed 18.30 and 19.20 minutes for paralysis and 25.20 and 28.11 minutes for death of earth worms and tape worms respectively.

Literature review reported that tannins and phenolics are known to interfere with the energy generation in parasites with the mechanism of uncoupled oxidative phosphorylation [46] and causes death by binds with free proteins in the gastrointestinal tract of host animal or glycoprotein on the cuticle of the parasite. Furthermore, estimation of alkaloids, phenolics and flavonoids in CF fruit extracts was resulted high content in combined methanol and aqueous extract that supported strong anthelmintic activity [31]. The established result was reported similar as per the earlier scientific research [48, 49]. In the present study, earthworm was selected for preliminary study because they are more sensitive followed by tape and round worms [50]. Earlier literatures established anthelmintic activity of *Coscinium fenestratum* stem aqueous extract against round worms and earthworms [51] and death time resulted more than 63 minutes whereas in our present study fruit pulp extract showed naround 37.32 minutes, lesser than stem extract. The result must be due to the effect of solvent used in extraction.

Conclusion:

The present investigation has concluded that CF fruit pulp has therapeutic activities due to the presence of secondary metabolites and has significantly established anthelmintic activity with estimated total alkaloids, phenols and flavonoids. The solvent system played a vital role for the activities in which combined aqueous and methanol extract showed most significant antioxidant and anthelmintic activity as compare to individual extract of methanol and aqueous. This is the first time report on CF fruit pulp extract which may be further explored for its phytochemical profile to recognize the active constituent responsible for anthelmintic activity.

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