Research Paper

Extraction and Antioxidant Activity of Flavonoids from Seed Coat of *Borassus flabellifer* Linn using Orthogonal Array (L16(4⁴))

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This study aimed at optimizing the conditions required for maximal extraction of flavonoids from the seed coat of *Borassus flabellifer* and to evaluate antioxidant properties. Initially, the parameters like time, temperature, solvent concentration and material ratio were optimized using orthogonal design of experiments. Temperature was found to play a significant role in the maximum extraction of flavonoids and the optimum conditions were found to be 85°, 3 h, 70 % aqueous-ethanol and 1:20 material ratio. The crude samples were tested for antioxidant activity using the 1,1-diphenyl-2-picrylhydrazyl radical scavenging assay. Percent inhibition of the 1,1-diphenyl-2-picrylhydrazyl radical was found highest (95 %) at 50 µg/ml concentration of the extract of seed coat of *B. flabellifer*. Flavonoids were then purified using preparative thin layer chromatography. The purified sample did not reveal significant antioxidant activities. Hence, further purification of the sample is needed to obtain better results.

Key words: Antioxidant, seed coat, *Borassus flabellifer*, flavonoid, optimization

Since ancient times, plant products have been used to treat a variety of diseases as most of the bioactive compounds obtained from the plants appear to be less toxic to humans. Plant parts with medicinal importance are most commonly used for treatment of ailments such as cuts and wounds[1]. Those properties of many such plants remains unrevealed. One such plant is *B. flabellifer,* which belongs to family Arecaceae, commonly known as Palmyra palm or Asian toddy palm, a native of tropical Africa and widely distributed and cultivated in tropical Asian countries such as Thailand, Bangladesh, India, Myanmar, Sri Lanka and Malaysia[2]. This tree is tall and erect, grows up to the height of 30 m and it has large fan shaped leaves of diameter 0.9 to 1.5 m with a black stem and crown of leaves at the top, palmately fan shaped, petiole edges with hard horny spinescent serratures; flowers unisexual, male spadix branched female spadix simple; fruits large, subglobose drupes, on the greatly enlarged perianth[3]. There are three most economical and important species of genus *Borassus*, such as *B. aethiopum* Mart, *B. flabellifer* Linn and *B. sundaicus* Becc[4].

Previous studies reported optimum conditions for the extraction of flavonoids from red raspberry fruits. Factors like temperature, time, material ratio and solvent concentration were considered for optimization. Extraction of flavonoids was carried out by altering each parameter to find optimal condition in which the extraction is maximum. Orthogonal design was employed and the optimum extraction conditions were determined calorimetrically. The extraction of flavonoids was maximum at 80° for 3 h and the material ratio was 1:10 and 95 % aqueous-ethanol was the solvent[5]. Similarly, extraction, purification and characterization of flavonoids from *Opuntia milpa alta* skin was also reported. The parameters for extraction (volume of solvent, solvent to material ratio, temperature and time for extraction) were optimized by the orthogonal design[6].

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Optimization of these parameters was mainly done to maximize the yield of flavonoids. Previous studies reported that, optimization of extraction of phenolic compounds from cactus pear (*Opuntiaficus indica*) skin using response surface methodology. The extraction conditions, which have been optimized included time, temperature and solvent concentration. The extraction of phenolic compounds was found to be maximum at higher temperatures[7].

Flavonoids were extracted and quantified from waste tobacco leaves. The sample was extracted with 70 % aqueous-ethanol and then ethanol was evaporated at 50° using a rotary evaporator. The extract was then subjected to thin layer chromatography (TLC) and column chromatography using various mobile phases. TLC was done on silica gel plates and the mobile phase used here was ethyl acetate:aqueous-ethanol:water (65:10:15). After the solvent has run to about the edge of the plates, they were examined under UV-light. The flavonoids were visualized as + spots and three types of flavonoids namely, apigenin, rutin and quercetin were isolated[8]. Previously, phytochemical analysis and antibacterial activity from the methanol extract of the seed coat of palmyra palm was studied. The phytochemical analysis showed that the extract contained various phytochemicals including flavonoids, and saponins[9].

To evaluate the antioxidant potential of phytochemicals using 1,1-diphenyl-2-picrylhydrazyl (DPPH) method, the antioxidant activity of the given sample was determined based on the scavenging activity of stable radical chromogen DPPH[10]. Antioxidant assay of the aqueous-ethanol extract of the seed coat of *B. flabellifer* was determined using 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) and DPPH radical scavenging method and ascorbic acid was used as a standard[11]. Similarly, free radical scavenging activities of the fruits of *B. flabellifer* were studied. Soxhlet extraction of the fruit was carried out with water, methanol, petroleum ether and chloroform. The antioxidant activity was estimated using DPPH and ABTS assay. The aqueous extracts of the fruits were also subjected to the phytochemical screening and it revealed the presence of flavonoids, tannins and saponins[12].

MATERIALS AND METHODS

The tender seeds of *B. flabellifer* were purchased from the local market, Coimbatore district, Tamil Nadu. The seed coats peeled from the seeds were washed with distilled water and then dried under shade. The shade dried seed coats were powdered and stored at room temperature (28±2°) in air tight plastic bags for solvent extraction. Ethanol, methanol, ethyl acetate, chloroform, heptane, acetone, $AlCl_3$, 5 % sodium nitrite, 1 M sodium hydroxide, glacial acetic acid, 6 M sulphuric acid, 25 mM disodium hydrogen phosphate, 4 mM ammonium molybdate, ascorbic acid and quercetin were obtained from S. D. Fine-Chem Ltd., Mumbai, India. For TLC analysis, formic acid and silica gel G_{60} were obtained from Merck, Darmstadt, Germany. DPPH was obtained from HiMedia, India.

Solvent extraction:

The dried sample was extracted with aqueous-ethanol, acetone, chloroform and water. About 0.5 g of the sample was extracted with 25 ml of these solvents in a shaker at 37° and 100 rpm overnight. The maximum yield of flavonoids was obtained with aqueous-ethanol extract.

Estimation of flavonoids using $AlCl_3$ method:

The total flavonoid content present in the extract was quantified spectrophotometrically. Accurately measured 0.1 ml of the extract was made up to 5 ml using distilled water. To this, 0.3 ml of 5 % $NaNO_2$ was added and after 5 min 3 ml of 10 % $AlCl_3$ was added and mixed well. Finally, 2 ml of 1 M NaOH was added after 6 min and absorbance was measured at 510 nm[13]. Quercetin was used as a standard for constructing the calibration curve.

Optimization using orthogonal design:

Orthogonal design is a kind of experimental design, which is similar like that of response surface methodology and Benken-Box design to identify the key variables that are responsible for the expected function. Optimization of flavonoid extraction for *B. flabellifer* has yet not been reported. In this, an orthogonal array ($L16(4^4)$) was constructed to evaluate the effects of the following factors, temperature (A), time (B), material ratio (C), aqueous-ethanol concentration (D). Factors and experimental data are displayed in the Table 1 and 2. The data were analysed using Minitab 16.1 software.

Identification of flavonoids by TLC:

Flavonoids present in the crude extract were identified by TLC performed under the following conditions; adsorbent layer was silica gel 60, layer thickness 0.25 mm, layer size 20×10 cm, glass chamber

TABLE 1: ORTHOGONAL DESIGN - FACTORS AND LEVELS

Factors	Levels			
	1	2	3	4
Temperature (A)	55°	65°	75°	85°
Time (B)	1 h	2 h	3 h	4 h
Material ratio (C)	01:05	01:10	01:15	01:20
Aqueous ethanol concentration (D)	65 %	70 %	75 %	80 %

TABLE 2: ORTHOGONAL DESIGN AND EXPERIMENTAL RESULTS

No.	Temperature (A)	Time (B)	Material ratio (C)	Aqueous ethanol concentration (D)	Yield (mg/g)
1	1	1	1	1	1.62
2	1	2	2	2	1.1
3	1	3	3	3	0.74
4	1	4	4	4	0.96
5	2	1	3	2	1.31
6	2	2	4	1	0.46
7	2	3	1	4	0.48
8	2	4	2	3	1.52
9	3	1	4	3	1.95
10	3	2	3	4	2.19
11	3	3	2	1	1.73
12	3	4	1	2	1.86
13	4	1	2	4	4.24
14	4	2	1	3	1.56
15	4	3	4	2	6.73
16	4	4	3	1	5.33

25×25×14 cm, ascending separation technique, minimum 2 h chamber saturation state, length of run was 10 cm, solvent composition- ethyl acetate:glacial acetic acid:formicacid:water (100:11:11:26), margin between start point and plate edge: 2.0 cm above the lower edge of the plate and 1.5 cm was left from each side, samples were applied as spots using capillary tubes, quercetin as a standard. After completion of the solvent run, plates were examined under ultraviolet light (254 and 365 nm) for fluorescent spots to identify the flavonoids[13].

Isolation and purification of flavonoids by preparative TLC (PTLC):

PTLC was carried out in a similar manner as TLC using glass plates (20×20 cm) thickly coated with silica gel of 2 mm. Quercetin was used as the standard. After the solvent has reached a particular distance, the plate was dried and viewed under ultraviolet light for fluorescent spots, which indicate the flavonoids. The fluorescent spots coinciding with the standard quercetin were marked and scrapped off along with the silica gel. The powder was reconstituted in phosphate buffer (pH 7), followed by centrifugation at 10 000 rpm for 10 min. This step was carried out twice to ensure the complete removal of the adsorbent. The supernatant was then stored in sterile glass vials under refrigeration.

***In vitro* antioxidant capacity assays:**

Total antioxidant capacity assay is based on the principle of reduction of molybdenum (VI) to molybdenum (V) by the extract and the subsequent formation of green phosphate/molybdenum complex at acid pH 0.1 ml of the extracts of different concentrations (10-50 µg/ml) was prepared by dissolving appropriate volume of the extract in aqueous-ethanol. To all the tubes, 1 ml of the reagent solution was added. The tubes were capped with aluminium foil and incubated at 95° for 90 min. The tubes were then cooled to room temperature and the absorbance was measured at 695 nm against a blank. Ascorbic acid was used as standard[14]. The total antioxidant capacity was expressed as ascorbic acid equivalent using the formula: ascorbic acid equivalent (µM/g)=(T/S)×C×(V/P)×(RS/E)×(1×MW), where, T=OD of test solution, S=OD of standard, C=concentration of test (µg), V=volume of solvent used for extraction (ml), P=amount of powder (g), RS=volume of reagent solution (ml), E=volume of extract (ml), MW=molecular weight of ascorbic acid (176.13 g/g mol).

Determination of DPPH radical scavenging activity:

DPPH scavenging activity was measured by the slightly modified spectrophotometric method. The principle of this method is that DPPH radical is scavenged by antioxidants through the donation of protons forming reduced DPPH. The electrons become paired off and the solution loses colour depending on the number of electrons taken up. The colour changes from purple to yellow after reduction and the antioxidant activity is estimated by the decrease of absorbance at 517 nm[15]. The extracts of different concentrations (10-50 µg/ml) were prepared and the tubes were made up to 2 ml with aqueous ethanol. The solution of DPPH in aqueous ethanol (0.6 mM) was prepared fresh. To all the tubes, 0.5 ml of the DPPH solution was added. The solution in the test tubes were mixed well and incubated in dark for 30 min at room temperature and absorbance was measured at 517 nm. About 2 ml of aqueous-ethanol mixed with 0.5 ml of DPPH solution was taken as control. About 2 ml of aqueous ethanol was taken as blank. The percentage inhibition of the radicals due to the antioxidant property of the extract was calculated

using the formula: percent inhibition=[($A_{control}$–A_{sample})/$A_{control}$]×100.

RESULTS AND DISCUSSION

The flavonoid extraction in seed coat of *B. flabellifer* has the maximum yield of about 6.73 mg/g. The optimal extraction condition for this yield were found as temperature 85° (A_4), time 3 h (B_3), material ratio 1:20 (C_4), aqueous ethanol concentration 70 % (D_2) i.e. A_4>C_4>B_3>D_2. The results were analysed in two and one way ANOVA and found that temperature is the significant variable for flavonoid extraction. Table 1 shows orthogonal design with factors and variable levels. The results of variable factors and levels were showed in Table 2.

The effect of temperature in the flavonoid yield was evaluated in the present study using four temperature settings at 55°, 65°, 75° and 85°. Flavonoid yield gradually increased with the rise in temperature from 55° to 85°. Temperature was proved to be a significant variable by analysis of one way and two-way ANOVA shown in fig. 1. The increase in flavonoid yield suggested that the diffusion rate of solvent and solubility of flavonoid increases with increasing temperature. Previous studies have also reported that increasing the temperature of solvent increased spreadabilty of the solute in the solvent as the viscosity and surface tension of the solvent reduced at higher temperatures[13,16].

Time is also one of the main factors affecting extraction. To achieve maximum yield, different extraction times were investigated. The optimal time duration for flavonoid extraction is found to be 3 h (fig. 2). This might be due to thermal degradation of flavonoid with

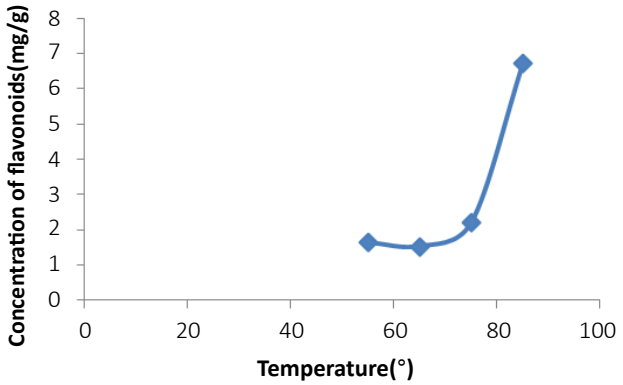

Fig. 1: Effect of temperature on extraction of flavonoids
The flavonoid yield gradually increased with increasing temperature from 55° to 85°. Temperature was found to be the significant variable by analysis of one way and two-way ANOVA

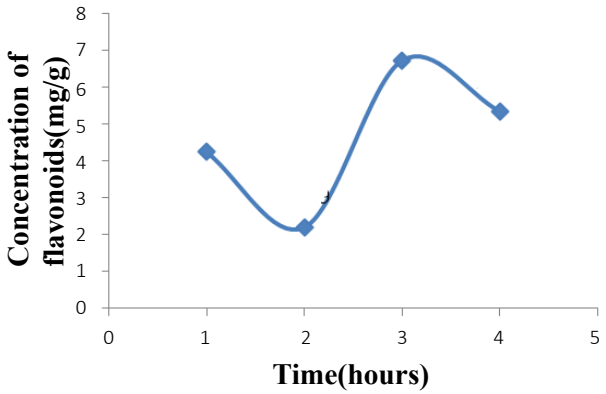

Fig. 2: Effect of time on extraction of flavonoids
The optimal time duration for flavonoid extraction is found to be 3 h

increase in time or because of the reduction of adhesion of flavonoids to the walls of glass tubes, which hinder the extraction process. Similar results revealed that the optimized time duration is well associated with the previous investigation[13].

The flavonoid extraction from the *B. flabellifer* was maximum at 1:20 material ratio. This may be because when the solvent volume increased, excessive diffusion of solvent causes swelling of plant cells creating pores so that more flavonoids get extracted out from the cell. Previous investigation reported that when more solvent was used, faster extraction rate could be obtained[17]. This is because there is an increase in the concentration difference inside the plant cell and external solvent, which allowed more solvent to diffuse into the cell causing maximum flavonoid extraction. Fig. 3 shows material ratio on extraction of flavonoid from seed coat of *B. flabellifer*.

Aqueous ethanol of different strengths due to varying polarity is likely to extract different flavonoid contents. Flavonoid extraction was found to be maximum with 70 % aqueous ethanol, however, with higher or lower alcohol strengths than 70 % lower yields of flavonoids were obtained. Previous studies reported that extraction is maximum at 50 % aqueous ethanol concentration[17]. The reason for this is that aqueous ethanol being a polar solvent, water present can absorb heat energy facilitating heat transfer through the system and increasing extracting efficiency. Furthermore, the reason for maximum extraction is that flavonoid has low polarity with aqueous ethanol having high affinity to solubilize it. Fig. 4 shows effect of aqueous ethanol concentration in flavonoid yield.

On observing the TLC and PTLC plates under ultraviolet light, the fluorescent spots of the sample,

which coincided with that of the standard quercetin were observed. The retention factor R_f value of the standard (0.95) matched with the R_f value of the sample (0.93). This revealed the presence of flavonoids in the crude sample. R_f was calculated as the distance travelled by the compound to that by the solvent[18]. The regions in the plate where the flavonoids were present were scraped off along with the silica gel and then centrifuged with phosphate buffer (pH 7) at 10 000 rpm for 10 min. The supernatant was then lyophilized.

The free radical scavenging property of crude extract of *B. flabellifer* were assessed using DPPH method and the results were shown in fig. 5. The amount of DPPH radical decreased in the presence of crude extract. On increasing the concentration of extract (10 µg to 50 µg), the percent inhibition increased from 80.42 to 93.23. The data obtained reveal that the extracts are free radical inhibitors and thus contain a potent antioxidant.

In previous studies, methanol extracts of the bark exhibited maximum inhibition of 77 % at the concentration of 900 µg/ml[19]. In our present study the same percent inhibition was obtained at the low concentration of 10 µg/ml. Also, the investigation made on the antioxidant property of *B. flabellifer*, aqueous extract of pulp exhibited inhibition of 40 % at 100 µg/ml[12]. This may due to the presence of higher amounts of flavonoids in our crude extract or due to more flavonoid solubilizing capacity of aqueous-ethanol. Previous studies reported that the methanol extract of the seed coat of *B. flabellifer* exhibited 60 % inhibition at 10 µg/ml, which is 1.5 fold lower than the result obtained in the present investigation (80 % inhibition at 10 µg/ml). This might be due to the better optimization of flavonoid extraction by using orthogonal design than the conventional shake flask method[11].

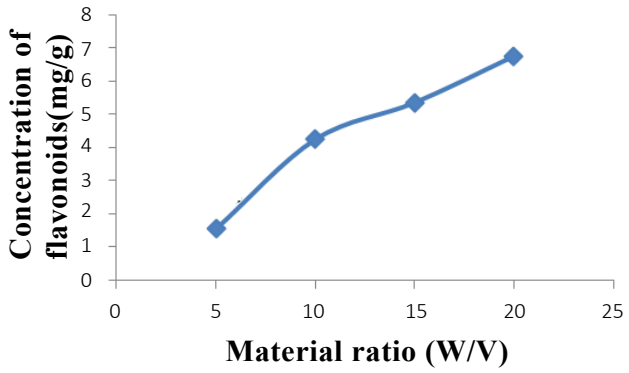

Fig. 3: Effect of material ratio on extraction of flavonoids
The flavonoid extraction from the *B. flabellifer* was maximum at 1:20 material ratio

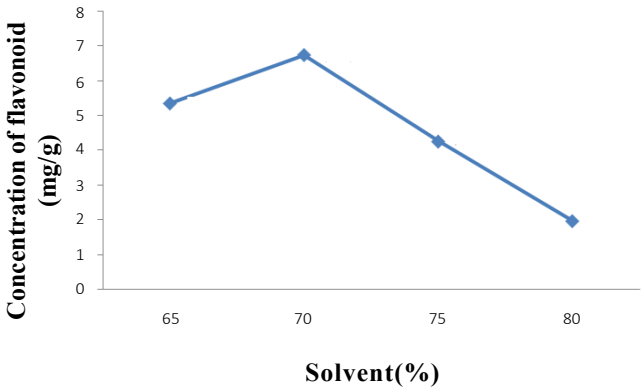

Fig.4: Effect of solvent (%) on extraction of flavonoids
The flavonoid extraction was maximum at 70 % aqueous ethanol; at higher or lower concentration the yield was suppressed

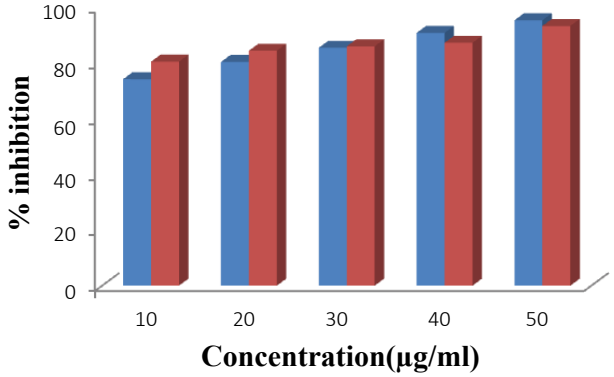

Fig. 5: Antioxidant activity of aqueous-ethanol extract by DPPH radical scavenging method
■ Ascorbic acid; ■ crude extract

This study concluded that the optimum conditions required for maximum flavonoid extraction from the seed coat of *B. flabellifer* are 85°, 70 % ethanol and material ratio of 1:20 for 3 h. The presence of flavonoid was confirmed by TLC and it is further purified by PTLC. The crude extract was found to possess antioxidant property even at low concentration. This suggested the potent antioxidant potential in the extract. Further purification of the flavonoids present in the seed coat of *B. flabellifer* is needed, so that the seed coat could serve as a better source of this bioactive compound.

Conflict of interest

We declare that we have no conflict of interest.

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Nil.

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