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Research Article

IN VITRO EVALUATION OF ANTIOXIDANT ACTIVITY OF *SPONDIAS MOMBIN* LEAF EXTRACT: DISCOVERING FUTURE AVENUES FOR AN AFFORDABLE AND EFFICIENT ANTIOXIDANT

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ABSTRACT

Spondias mombin is valued ethno medically in folkloric medicine. This medicinal plant according to traditional claim is said to cure various infectious and inflammatory ailments of gastrointestinal and genitourinary tract. Formation of reactive oxygen species (ROS) in inflammatory conditions is said to result in oxidative stress. The antibacterial and anti-inflammatory properties of *Spondias mombin* especially its leaves, have been linked to a range of compounds in it viz., anthraquinones, berberine, flavonoids, naphthoquinones, sesquiterpenes, quassiniods, indole and quinoline alkaloids. Hence, we aimed at exploring the antioxidant potential of the leaf extract of *Spondias mombin* by *in vitro* methods. The ethanolic extract of leaves was subjected to spectrophotometric analysis and DPPH methods to explore its reducing potential. The total phenolic and total flavonoid content was estimated by colorimetric methods. It was observed that ethanolic extract of *Spondias mombin* demonstrated a dose-dependent increase in the reducing property, with higher potency than the reference compound sodium metabisulphate. *Spondias mombin* shade dried leaf powder macerated and later extracted with hot water possessed a significantly high content of flavonoids than those extracted with other solvents. The leaf extract of *Spondias mombin* possesses significant antioxidant activity that may be attributed to its high flavonoid content which could afford protection in inflammatory conditions. This finding strengthens its widespread use in traditional medicine. Animal studies and clinical trials are necessary to validate these beneficial properties and translate them into clinical utility.

Keywords: Ethno medicine, poly phenols, flavonoids, antioxidant

INTRODUCTION

Phenolic compounds are the most widely distributed secondary metabolites, ubiquitous in the plant kingdom. The great majority of active phenolic compounds isolated from higher plants are flavonoids and phenolic acids. *Spondias mombin* or *Spondias purpurea* var. lutea is one such plant that carries high medicinal value in traditional medicine. It is a species of flowering plant in the family Anacardiaceae. It is native to the tropical Americas, including the West Indies which has been naturalized in parts of Africa, India, Sri Lanka and Indonesia and is rarely cultivated. In Goan Konkani, it is called Ambado. In Malayalam, it is called Ambazham, Junglee Aam in Hindi and Amra in bengali. The fruit pulp is either eaten fresh or made into juice, concentrate, jellies and sherbets. The young leaves, which taste slightly bitter and sour, are sometimes served raw together with certain types of Thai chilli pastes. *Spondias mombin*as a medicinal plant has a lot of potential, valuable, untapped resource of active drugs for treating diseases. All parts of the tree are medicinally important in traditional medicine. The tea made from the flowers and leaves is said to relieve stomach ache, biliary vomiting, genitor-urinary tract infections and in eye and throat inflammation. The fruit decoction is drunk as a diuretic, the decoction of the bark and the leaves is said to possess anti diarrheal property and thus used in the treatment of dysentery. Its medicinal uses also include conditions like hemorrhoids, gonorrhea and leucorrhea. The powdered bark is applied on wounds for healing. The antimicrobial, antibacterial, antifungal, and the antiviral properties of *Spondias mombin* have been reported[1-5]. Preliminary reports suggest that the phenolic acid, 6-alkenyl-salicylic acid in the leaf extract of *Spondias mombin* are responsible for its antibacterial property[6]. In another study, the anacardis acid derivative from the hexane extract of the plant was showed to possess beta lactamase inhibitory properties[7]. The anti-malarial activity of *Spondias mombin* discovered lately is said to have been linked to a range of compounds like anthraquinones, berberine, flavonoids, naphthoquinones, sesquiterpenes, quassiniods, indole and quinoline alkaloids present in the leaves[8]. The leaf extract has also shown anti-inflammatory activity in animal studies[9]. Formation of reactive oxygen species (ROS) in inflammatory conditions is said to result in oxidative stress and tissue damage. Hence, we aimed at confirming the previously suggested antioxidant potential of the leaf extract of *Spondias mombin* in our laboratory using spectrophotometry and also estimating its total flavonoid content by calorimetric methods, thus focusing at generating a stronger evidence for its beneficial medicinal properties.

MATERIALS AND METHODS

After receiving the approval from Institutional ethics committee, the study was commenced (January 2013).

Chemicals used

All chemicals and reagents used were of analytical grade and they (including standards quercetin, gallic acid and naringin) were obtained mostly from Sigma. Solvents used for extraction of plants were purchased from Fisher Scientific (India) Pvt. Ltd. Ready to use diagnostic kits (Aspen Labs Pvt. Ltd., Delhi-India).

Collection and identification of plant

The twigs from *Spondias mombin* tree were collected from a village in Dharwad district, Karnataka, India which was authenticated by the Department of Pharmacognosy, College of Pharmacy, Hubli, Karnataka, India. A voucher specimen with number PG 501-3 was deposited at the Pharmacognosy's herbarium. The twigs were thoroughly cleaned to remove adherent soil and other impurities, the leaves were shade dried and made into a coarse powder by rubbing in the palms.

Preparation of ethanolic extract of *Spondias mombin* leaves

250 g of shade dried leaf powder of *Spondias mombin* was extracted in Soxhlet's apparatus using petroleum ether for defatting and then it was extracted with 70 % ethanol. The solvent was evaporated on a water bath at a low temperature (50°C) and finally the residue was obtained.

Evaluation of *In-vitro* antioxidant activity

The following *in-vitro* models were carried out to evaluate antioxidant activity of *Spondias mombin*

- Reducing power assay
- DPPH (1, 1-Diphenyl-2-Picryl-hydrazyl) free radical scavenging activity.

Spectrophotometric assay of reducing power

The reducing power of 70 % ethanolic extract of *Spondias mombin* leaves were determined according to the method of Oyaizu[10]

Procedure

Different doses of 70 % ethanolic extract of leaves were mixed in 1 ml of distilled water so as to get 10 µg, 20 µg, 25 µg, 50 µg and 100 µg concentrations. This was mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide (2.5 ml, 1 %). The mixture was incubated at 50°C for 20 minutes. A portion (2.5 ml) of trichloroacetic acid (10 %) was added to the mixture, which was then centrifuged at 3000 rpm for 10 minutes. The upper layer of the solution (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1 %) and the absorbance (OD) was measured at 700 nm in double beam spectrophotometer. Increased absorbance of the reaction mixture indicates increase in reducing power. The % reducing power was calculated by using the formula.

$$\% \text{ increase in absorbance} = \frac{\text{Control OD} - \text{Test OD}}{\text{Control OD}} \times 100$$

The results are compiled in Table 1

DPPH (1, 1-Diphenyl-2-Picryl-hydrazyl) free radical scavenging activity

DPPH radical scavenging activity of *Spondias mombin* (70 % ethanolic extract) was measured by the method described by Sabir *et al*. Different concentrations of this extract (10, 20, 25, 50, 100 µg) was added to a 0.5 ml solution of DPPH (0.25 mM in 95 % ethanol). The mixture was shaken and allowed to stand at room temperature for 30 min and the absorbance was measured at 517 nm in a double beam spectrophotometer using DPPH solution as blank. Vitamin C (25 µg) was used as a standard compound in the DPPH assay[11]. Radical scavenging activity (RSA) was calculated as per the formula:

$$\% \text{ RSA} = (A_{DPPH} - A_S) / A_{DPPH} \times 100$$

(% RSA = percentage of DPPH discoloration that indicates the Radical Scavenging Activity, A_{DPPH} = absorbance of DPPH solution, A_S = absorbance of the solution when the sample was added at a particular level)

The results are compiled in Table 1.

***Spondias mombin* leaf extract preparation by Maceration technique**

Ten grams of the leaves were macerated [In this process, the shade dried coarse powder is placed in a stoppered container with the solvent and allowed to stand at room temperature for a period of at least 3 days with frequent agitation until the soluble matter has dissolved. The mixture then is strained, the marc (the damp solid material) is pressed and the combined liquids are clarified by filtration or decantation after standing]. Following this, the filtered liquid was transferred into three separate containers, extracted with 100 ml of cold water, hot water and 70 % methanol, respectively.[12] Leaf extracts prepared with three different solvents:

SM_{CW}: *Spondias mombin* (SM) shade dried leaf powder macerated for 48 h with cold water (distilled water stored at room temperature).
SM_{HW}: *Spondias mombin* shade dried leaf powder macerated for 48 h with hot water (50°C).
SM_{M15}: *Spondias mombin* shade dried leaf powder macerated with 70 % methanol for 15 days.

Estimation of total phenolics

The amount of total phenolic compounds in the extracts was determined colorimetrically with the Folin-Ciocalteu reagent, using a slightly modified method of Yu[13]. The reaction mixture contained, 0.1 ml sample extract diluted to 1 ml with distilled water, 0.5 ml of Folin-Ciocalteu reagent (1 N) and 1.5 ml of 20 % sodium carbonate solution and was incubated for 2 h at room temperature. The volume was raised to 5 ml with distilled water and the absorbance of blue colored mixture was measured at 765 nm (Spectronic 2202 UV-Vis Spectrophotometer). The concentration of total phenolic compounds was expressed as mg of gallic acid equivalents (GAE) per g of dried *Spondias mombin* extracts (three different extracts of cold water, hot water and methanol) using a standard curve of gallic acid described by the equation-

$$y = 0.0265x \ (R^2 = 0.9977).$$

Here, y = absorbance and x = concentration

Total flavonoid estimation
Estimation of total flavonoids by Aluminium chloride colorimetric method

The aluminum chloride colorimetric method was modified from the procedure reported by Woisky and Salatino[14]. Quercetin was used to make the calibration curve. 10 milligrams of quercetin was dissolved in 80 % ethanol making dilutions of 0.125, 0.25, 0.5, 0.75, 1.00, 1.25 and 1.5 mg/100 ml. The diluted standard solutions (0.5 ml) were separately mixed with 1.5 ml of 95 % ethanol, 0.1 ml of 10 % aluminium chloride, 0.1 ml of 1M potassium acetate and 2.8 ml of distilled water. After incubation at room temperature for 30 min, the color intensity recorded as absorbance of the reaction mixture was measured at 415 nm by using UV spectrophotometer. The amount of 10 % aluminium chloride was substituted by the same amount of distilled water in blank. Similarly, 0.5 ml of various extracts of *Spondias mombin* (1000 µg/ml) were made to react with aluminium chloride for determination of flavonoid content as described for quercetin.

2, 4-Dinitrophenylhydrazine (2, 4-DPH) Colorimetric Method

The current method was modified from the procedure described by Nagy and Grancai. Naringin was used as the reference standard. 20 milligrams of naringin was dissolved in methanol and then diluted to 125, 250, 500, 1000, 2000 ppm. One milliliter of each of the diluted standard solutions was separately reacted with 2 ml of 1 % 2, 4-dinitrophenylhydrazine reagent and 2 ml of methanol at 50°C for 50 min. After cooling to room temperature, the reaction mixture was

mixed with 5 ml of 1 % potassium hydroxide in 70 % methanol and incubated at room temperature for 2 min. Then, 1 ml of the mixture was taken, mixed with 5 ml of methanol and centrifuged at 1000 rpm for 10 min to remove the precipitate. The supernatant was collected and adjusted to 25 ml. The absorbance of the supernatant was measured at 495 nm. The various extracts of *Spondias mombin* (1000 µg/ml) were similarly made to react with 2, 4-dinitrophenylhydrazine for determination of flavonoid content (as described for naringin).

RESULTS

It is observed that 70 % of ethanolic extract of *Spondias mombin* have demonstrated dose dependent increase in the reducing property. While 25 µg of sodium metabisulphate (standard) has 140 % reducing property, this extract at 25 µg has more reducing property than compared to standard and 100 µg of *Spondias mombin* extract has shown maximum reducing power i.e., 580 %. The results are shown in Table 1. DPPH is an unstable nitrogen centered free radical that accepts an electron or hydrogen radical from suitable antioxidants and gets reduced to stable diamagnetic molecule along with stiochiometric loss of color. This phenomenon has been widely used by researchers as a quick and reliable parameter to assess the *in-vitro* antioxidant activity of crude extracts. From the DPPH radical scavenging activity of this extract is shown in Table 1, it is clear that this extract has shown a dose dependent scavenging activity of DPPH radical with a two-fold higher % RSA than the standard (Vitamin C). The amount of total phenolic compounds in the extracts determined colorimetrically with the Folin-Ciocalteu reagent is displayed in Table 2. The subclass of flavonoids is flavonols, flavones and flavonones. The flavonols of *Spondias mombin* complexes only with aluminium chloride and flavones and flavanones strongly react only with 2, 4-dinitrophenylhydrazine. Hence, the total flavonoid content was determined by adding up the flavonoid values obtained by each of these two methods. Results showed that, among the three different extracts of *Spondias mombin*, SM_{M15} contained the lowest level of total flavonoids and SM_{HW} contained the highest level of total flavonoids. The observations are recorded in Table 2.

Table 1: Reducing power and DPPH radical scavenging activity of 70 % Ethanolic Extract of *Spondias mombin* leaves (SMEE)

Reducing power activity			DPPH radical scavenging activity (RSA)	
Groups	**Absorbance Mean ± SEM**	**% Reducing activity**	**Absorbance Mean ± SEM**	**% RSA**
Control	0.056 ± 0.003	---	0.540 ± 0.010	---
Control + Std. 25 µg	**0.124 ± 0.003*****	**140**	**0.390 ± 0.005*****	**27.777**
Control + SMEE 10 µg	0.100 ± 0.005***	100	0.220 ± 0.011***	59.259
Control + SMEE 20 µg	0.113 ± 0.008**	120	0.196 ± 0.003***	63.703
Control + SMEE 25 µg	**0.150 ± 0.005***	**200**	**0.170 ± 0.005*****	**68.518**
Control + SMEE 50 µg	0.180 ± 0.015	260	0.116 ± 0.003***	78.518
Control+ SMEE 100 µg	0.343 ± 0.021**	580	0.103 ± 0.003***	80.925

Values are the mean ± S.E.M of three parallel measurements, Significance $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$, compared to standard, Std: Standard used is Sodium metabisulphate for reducing power and Ascorbic acid (Vitamin C) for DPPH radical scavenging activities

Table 2: Total flavonoid contents of *Spondias mombin* leaves determined by aluminium chloride and 2, 4-dinitrophenylhydrazine (2, 4-DPH) colorimetric methods and Total phenolic content

S. No.	Name of the sample	Total flavonoid contents[a]		Total (µg/ml)	Total phenolic content (mg GAE/g extract)
		$AlCl_3$[b] (µg)	2,4-DPH[c] (µg)		
1	SM_{CW}	40.16 ± 0.36	16.52 ± 0.55	56.68 ± 0.21	155.126 ± 12.546[d]
2.	**SM_{HW}**	**44.58 ± 0.35**	**29.85 ± 2.78**	**74.44 ± 2.72**	**461.698 ± 3.774[e]**
3.	SM_{M15}	32.20 ± 0.24	16.24 ± 0.28	48.45 ± 0.10	54.956 ± 3.027[f]

a: Results are presented as mean ± SEM of three parallel measurements, b: Levels calculated as quercetin equivalents, c: Levels calculated as naringin equivalents, Values within a column followed by different letters (d, e, f) are significantly different ($P < 0.05$), GAE – Gallic acid equivalents

DISCUSSION

Phytochemicals, especially phenolics are suggested to be the major bioactive compounds for health benefits. Phenolic compounds protect plants from oxidative damage and perform the same function for humans[15,16]. Several types of polyphenols (phenolic acids, hydrolysable tannins and flavonoids) show anti carcinogenic and anti mutagenic effects[17,18]. Flavonoids are considered to be very beneficial compounds due to their potent nature as antioxidants. Flavonoids are polyphenolic compounds found in small quantities in numerous plant foods, including fruit and vegetables, tea, wine, nuts, seeds, herbs and spices[19,20]. The flavonoids have aroused considerable interest recently because of their potential beneficial effects on human health - they have been reported to have antiviral, anti-allergic, anti platelet, anti-inflammatory, anti tumor and antioxidant activities[21]. Flavonoids are free radical scavengers, super antioxidants which prevent oxidative cell damage and have strong anticancer activity[22]. Flavonols are a class of flavonoids and their consumption has been associated with a variety of beneficial effects including increased activity of erythrocyte superoxide dismutase, a decrease in lymphocyte DNA damage, a decrease in urinary 8-hydroxy-2′-deoxyguanosine (a marker of oxidative damage) and an increase in plasma antioxidant capacity[23]. Ongoing studies on flavonoids-containing herbs suggest their role in the prevention of cancer and cardiovascular disease, treatment of various infectious and autoimmune disorders. Since our study strengthened the presence of antioxidants in the leaf extracts of *Spondias mombin*, it proves itspotential to repair free radical damages to the cells. Moreover, in synthetic form it can be marketed as antioxidant supplements during oxidative stressed conditions. By the virtue of presence of phyto-constituents like phenolic compounds and flavonoids, in our study too; the ethanolic extract of *Spondias mombin* has proven to possess significant antioxidant property and RSA *in-vitro,* more potent than the reference compound, thus confirming the earlier assumptions of *Spondias mombin* being an efficient antioxidant. Total polyphenols contents in examined the herb were earlier estimated by some authors. The results of those studies are difficult to compare with that obtained in this work, because of different manners of extraction and calculating methods; some authors gave only the amounts of polyphenols as mass (mg) in weight of dry extract and indicated information about extraction effectiveness (as mg of extract from 1 g of dry plant material). As antioxidants, flavonoids provide anti-inflammatory actions[24,25]; this may be the reason behind the folkloric use of *Spondias mombin* in

the treatment of various intestinal troubles[26]. The bioactivity of the polyphenols may be related to their ability to chelate metals, inhibit the lipooxygenase pathway and scavenge free radicals[27]. In food systems, flavonoids can act as free radical scavengers and terminate the radical chain reaction that occurs during the oxidation of triglycerides[28,29]. Antioxidant based drugs or formulations for the prevention and treatment of complex diseases like atherosclerosis, stroke, diabetes, Alzheimer's disease and cancer have appeared during the last 3 decades. Antioxidant based anti-ageing creams and skin care products are in the pipeline to enter the market[30].

CONCLUSION

The results of the study showed that the plant has high nutritive value which could attenuate physiological oxidative stress due to its high concentration of phenolic and flavonoids contents. Therefore the findings reveal the antioxidant potential of *Spondias mombin* giving an *in vitro* evidence for its possible antimicrobial and anti-inflammatory action, thus buttressing its invaluable position in traditional folklore medicine. The facts like *Spondias mombin* is an easily available and inexpensive herb in India and the availability of evidence on its clinical usefulness confer a promising and affordable therapeutic potential for free radical mediated diseases. Further in this direction, well-designed animal studies and controlled-clinical trials are warranted.

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