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Antioxidant potential of young pods of *Acacia catechu* wild collected from Jabalpur region

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ABSTRACT

Acacia catechu is a multipurpose tree that produces nitrogen rich fodder and green manure, high quality fuel wood and charcoal, strong durable poles and timber. Apart from the most useful products i.e. Kutch or "Kattha", which is strong antioxidant, astringent, anti-inflammatory, antibacterial and antifungal in nature, other plant parts have not been investigated for other biological activities. The present study showcases a comprehensive investigation on young pods of *Acacia catechu* collected from Jabalpur. The pods were extracted sequentially with solvents of decreasing polarity in order to extract all the possible phytochemicals. The results show good antioxidant activity in ethyl acetate extract and was able to scavenge peroxide and DPPH free radicals. The activity was comparable to ascorbic acid.

Keywords: *Acacia catechu*, antioxidants, phytochemicals, DPPH activity, peroxide radicals

1. Introduction

The oxidation of important biological molecules gives rise to free radicals (superoxide, hydroxyl radicals, nitric acid) and other reactive oxygen species (ROS) i.e. hydrogen peroxide, hypochloric acid and proxynitrite. These are produced during aerobic metabolism in the body. These free radicals can cause oxidative damage to amino acids, lipids, proteins and DNA[1,2]. Such oxidation can damage the cell's surface, alter its DNA or completely kill the cells. Radical scavengers have attracted special interest because they can protect the human body from free radicals that may cause many diseases, including cancer, and contribute to the aging process[3]. Antioxidant activities are the main area of focus when a plant or plant part is used as a food as well as a drug simultaneously[4]. An antioxidant is a molecule capable of inhibiting the oxidation of other molecules. They are radical scavengers which protect the human body against free radicals that may cause pathological conditions such as ischemia, anaemia, asthma, arthritis, inflammation, neurodegeneration, Parkinson's disease, mongolism, premature ageing and dementia[5].

Medicinal plants constitute the main source of new pharmaceuticals and health-care products. A whole range of plant derived dietary supplements, phytochemicals and provitamins that assist in maintaining good health and combating diseases are now being described as functional ingredients and nutraceuticals. Since plant based ingredients are commonly used as a food source, and mostly the plant based secondary metabolites are taken with the food, which perform therapeutic as well as medicinal role in the body. Especially the Indian food; which is prepared according to the Ayurveda; advocate the inclusion of such plant based ingredients that provide therapeutic inputs to the body along with the nutrition. The antioxidant abilities of phytochemicals have been shown by allyl sulphides (onion leaf, garlic), carotenoids (fruits i.e. carrots), flavonoids which give colour and flavour to various fruits and vegetables and polyphenols (tea, grapes) etc[6]. This is why it is long established fact for beneficial roles of fruit and vegetables in the human diet providing protection against cellular damage caused by exposure to high levels of free radicals.

Recent studies have shown that a number of plant products including polyphenols, terpenes and various plant extracts exert an antioxidant action. In the context of present scientific knowledge and day-by-day increasing interest in identifying newer substances and products, India stands tall for its rich diversity, good traditional & folk knowledge and developing infrastructure. This has made a way for the Indian scientists and the world to make good use of its natural resources. Not only newer natural resources are being explored, but the traditional knowledge is being counterchecked by the present day technologies. Because of biological variations, light & temperature conditions and other abiotic factors, studying the regional fauna and resources are of high importance.

The present study is focused upon a rather less known food source; young pods of *Acacia catechu* Willd, commonly known as Khair (family: Leguminosae, sub family: Mimosaceae) in India. It is a common tree growing mostly on barren land where the light intensity is higher. This multipurpose tree has attracted researchers from the ancient times because of various products that have proved to be therapeutic, medicinal and pharmacological importance. Though the pods of *Acacia catechu* are not a regular source of food, studies have proved the nutrition potential of these pods with high protein and carbohydrate content [7]. In the light of urgency of newer natural antioxidant compounds to be discovered day by day with their improved biological activity, *Acacia* provides a valuable insight for such discoveries.

2. Materials and Methods

2.1 Collection of plant material

The trees of *Acacia catechu* were found in good quantity in the forest area of Dumna Nature Reserve, Jabalpur, India. The plants were identified using their morphological identification keys and confirmed by the local forest officers. The young pods of *A. catechu* from fully grown trees were June-July 2012.

The young pods were air dried under shade for one week or longer. Once dried up to constant weight, the pods were ground using a mixer grinder. The powder was passed through a test sieve having 100 µM pore size (Sonar, India) and the remaining course powder was again grinded and sieved till the material could not be ground further. The fine powder of less than 100 µM was immediately stored in an air tight container for further use.

2.2 Extraction of young pods

For the extraction of antioxidant compound(s), a systematic extraction procedure was adopted using solvents of varying polarity. Extraction was done sequentially with water, methanol, ethyl acetate and petroleum ether.

For aqueous extraction, 10 g of dried plant powder was suspended in 250 ml of cold distilled water. After two hours, the suspension was stirred for two hours on a magnetic stirrer (Remi, India) and again was kept in refrigerator for two hours. The cycle was repeated for 48 hours. After 48 h the suspension was filtered through an eight layered muslin cloth. The remaining powder was extracted again for 48 h with 250 ml of fresh distilled water to ensure complete extraction and filtered. The remaining powder (residue) was dried and used for further extraction.

The residue obtained after the aqueous extraction was extracted sequentially with methanol, ethyl acetate and petroleum ether using Soxhlet extractor. With each solvent, 10 cycles of extraction were performed. The residue left after each extraction served as a source material for next extraction. In this way, the phytochemicals from young pods of *Acacia catechu* were extracted in four solvents. All the extracts were dried under vacuum and kept refrigerated until use.

2.3 Total phenolic content

The total phenolic content was determined by the Folin-Ciocalteu method described by Wu *et al*. [8] with some minor modification. For this, 0.3 ml of plant extract was added to 1.5 ml of Folin-Ciocalteau reagent and 1.2 ml of 75% (w/v) sodium carbonate solution in test tubes. The tubes were vortexed for 15 sec and allowed to stand for 30 min at room temperature. Absorbance was measured at 765 nm with the spectrophotometer. Results were expressed as milligram of tannic acid equivalent per gram of extract weight using the standard curve of tannic acid.

2.4 Evaluation of in-vitro antioxidant activity

2.4.1 Peroxide scavenging activity

The ferric thiocyanate method was used to determine the amount of peroxide at the initial stage of lipid peroxidation [9]. The peroxide reacts with ferrous chloride ($FeCl_2$) to form a reddish ferric chloride ($FeCl_3$) which in turn reacts with ammonium thiocyanate and produce ferric thiocyanate. The initial solution is denser and produces higher absorbance at 500 nm. In this method the concentration of peroxide decreases as the antioxidant activity increases.

For FTC method 4 mg of each extracted powder was dissolved in 4 ml of methanol by stirring and filtered into dark bottles. 4.1 ml of 2.52% of linoleic acid (Sigma, USA) in methanol, 8 ml of 0.05 M phosphate buffer (pH 7.0) and 3.9 ml of water was placed in the same dark vial with a screw cap and then placed in an oven at 40°C in dark. To 0.1ml of this solution, 9.7ml of 75% of ethanol, 0.1ml 30% of ammonium thiocyanate and 0.1 ml of 0.02 M ferrous chloride in 3.5% of hydrochloric acid was added. Exactly 3 min after the addition of ferrous chloride to the reaction mixture, the absorbance was measured at 500 nm. A negative control with methanol only and a positive control with ascorbic acid (4 mg ml^{-1}) were placed simultaneously. The process was repeated every 24 h until the absorbance of the positive control reached maximum. The percent reduction of peroxide ions were calculated using the following formula-

$$\% \text{ activity} = 100 - (A_1/A_0) \times 100$$

Where:,

A_0=Absorbance of methanol reaction.

A_1=Absorbance in the presence of sample extract.

2.4.2 DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity

An easy rapid and sensitive method for the antioxidant screening of plant extracts is free radical scavenging assay using 2,2,diphenyl1-1-picrylhydrazyl (DPPH) stable free radical spectrophotometrically[10,11]. DPPH is dark violet in color and in the presence of an antioxidant, DPPH radical obtains one more electron and the absorbance decreases[12]. The color turns from purple to yellow as the molar absorptivity of DPPH radical at 517 nm reduces when the odd electron of DPPH radical becomes paired with hydrogen from a free radical scavenging compound (antioxidant) to form the reduced DPPH-H. The resulting de-colorization is stoichiometric with respect to number of electrons captured.

The scavenging effect of plant samples as well as Vit C corresponding to quenching intensity of 2,2-diphenyl-1-picrylhydrazyl (DPPH) was carried out. The 500µl of each extract (1 mg ml^{-1} in ethanol) was mixed with the same volume of DPPH solution (60 µM in ethanol) and allowed to stand for 2 h at room

temperature in dark (until stable absorption values were obtained). The absorbance was then measured at 517 nm using a spectrophotometer. The ascorbic acid in the same concentration was used as positive control.

The percentage scavenging effect was determined by comparing the absorbance of the solution containing the test sample to that of negative control solution (ethanol) without test sample taking corresponding blanks. The experiments were performed at least three times and the mean values are shown.

$$\% \text{ antioxidant activity for DPPH} = (A\text{-}A_x)/A \times 100$$

Where,

A- Absorbance of DPPH solution with ethanol.

Ax- Absorbance of DPPH solution with test solution

2.5 Thin layer chromatography

A chromatographic run sample was prepared by dissolving 10 mg of dried extract or ascorbic acid in 1 ml of methanol. The suspension was filtered through a 0.22 µM membrane filter (Axiva, India). The filtrate was loaded on to the activated Silica gel G (SRL, India) plate. The antioxidant compounds were separated using solvent mixture of methanol and chloroform (85:15 v/v). Once the run is completed, plates were air dried for 15 min and the plates were sprayed by 0.04% DPPH solution in methanol using a spray gun for 5 sec. The image was observed under visible light at exactly 2 min after spraying using a white light illuminator. The area of bright yellow bands against the purple background determined DPPH radical scavenging activity.

3. Results

The young pods of *Acacia catechu* collected from Jabalpur, India were analyzed for their potential of scavenging free radicals. The total phenolic content could be detected only in methanol and ethyl ether extracts and were 0.152 and 0.041 mg tannic acid equivalent.

Table 1: Peroxide scavenging activity of different extracts of young pods of *A. catechu* using Ferric thiocyanate method. The absorbance was taken for six days continuously.

Day	Negative control	Positive control (Ascorbic acid)	Pod extracts			
			Aqueous	**Methanol**	**Ethyl acetate**	**Petroleum ether**
1	0.10	0.00	0.09	0.12	0.09	0.08
2	0.35	0.14	0.18	0.22	0.14	0.13
3	0.49	0.22	0.28	0.39	0.22	0.19
4	0.58	0.31	0.35	0.45	0.29	0.26
5	0.69	0.37	0.46	0.56	0.36	0.31
6	0.78	0.41	0.57	0.67	0.41	0.40
% activity		**47.43**	**26.92**	**14.10**	**47.43**	**48.71**

Table 1 shows that when different extracts of young pods were subjected to peroxide scavenging assays *in vitro*, the aqueous extract was able to scavenge 26.92 % of peroxide radicals while the positive control; ascorbic acid scavenged 47.43% peroxide ions. The methanolic extract was least effective with ability to scavenge only 14% peroxide ions. Ethyl acetate and petroleum ether extracts showed good peroxide scavenging activity and showed 47.43 and 48.71% activity respectively.

When DPPH free radical scavenging activities were assayed *in vitro*, ascorbic acid scavenged 64% of DPPH free radicals. In comparison, aqueous and methanolic extracts could scavenge only 10 and 6% ions respectively. Ethyl acetate and petroleum ether extracts again showed promising activity by scavenging 42.5 and 52.5% DPPH free radicals respectively (**table 2**).

Table 2: DPPH free radical scavenging activity of different extracts of *Acacia catechu* pod. The difference in absorption (ΔA_{520}) was recorded after 2 h.

	Neg. Ctrl	Ascorbic acid	Pod extracts			
			Aqueous	**Methanol**	**Ethyl acetate**	**Petroleum ether**
ΔA_{520}	**0.02**	**1.28**	**0.2**	**0.12**	**0.85**	**1.05**
DPPH scavenging activity %	**-**	**64.0**	**10**	**6.0**	**42.5**	**52.5**

Fig 1 shows that when used in same concentration, ascorbic acid was able to scavenge DPPH free radicals more than the peroxide radicals. However, the aqueous, methanol and ethyl acetate extracts of young pods of *Acacia catechu* were able to scavenge peroxide radicals more than the DPPH free radicals. Petroleum ether extract scavenged both the ions with almost same caliber.

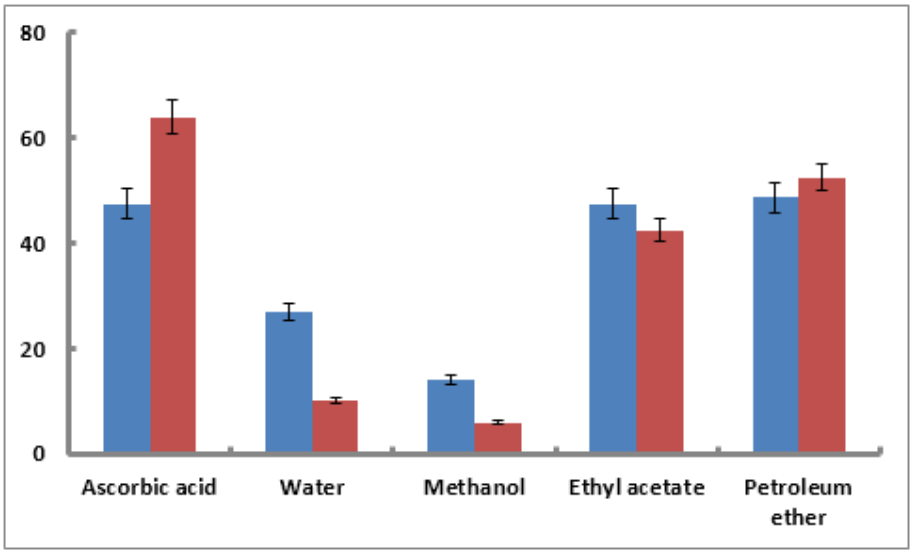

Fig 1: Comparative scavenging activity of ascorbic acid and different extracts of young pods of *Acacia catechu* against peroxide free radicals (blue bars) and DPPH free radicals (red bars). Data are presented as mean percent inhibition of three replicates ± SD.

Since the petroleum extract showed highest activity among all four extracts, this extract was subjected to thin layer chromatography. Ascorbic acid in the same concentration was used as a control. The ascorbic acid produced an antioxidant band with Rf value of 0.91, while petroleum ether extract showed single continuous band with maximum intensity at 0.78 (**Fig 2**).

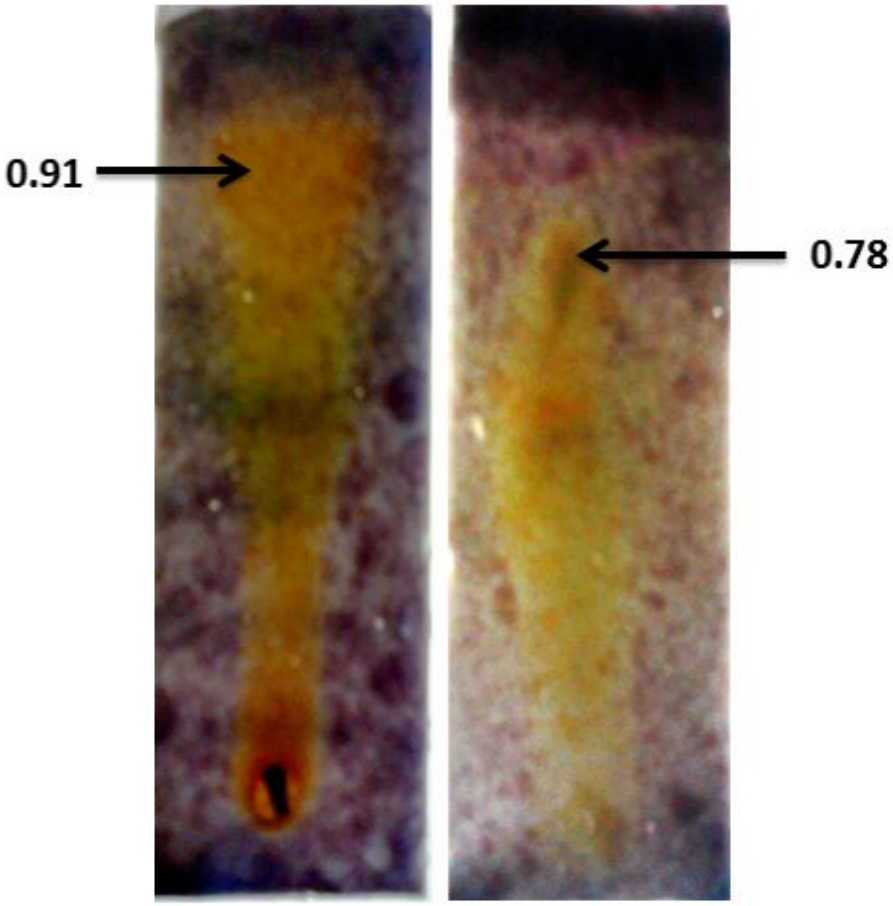

Fig 2: Thin layer chromatogram of ascorbic acid and petroleum ether extract of pods of *Acacia catechu*. The chromatogram was developed with DPPH free radical spray. The ascorbic acid shows R_f of 0.91 while petroleum ether extract of pods showed R_f of 0.78.

4. Discussion

The present study was focused on identifying the antioxidant potential of a lesser known food source in India; young pods of *Acacia catechu*. Though *Acacia catechu* is an important economic plant, its pods are not used as a regular food source in India and most parts of the world. However, in Mexico, the flat, green pods of acacia tree are eaten regularly. The pods are sometimes light green or deep red in color—both taste the same. These pods are eaten raw with guacamole, sometimes cooked and made into a sauce.

Since, this plant is endemic to most parts of the India, including Jabalpur, the availability of its pod is round the year. The young pods have been showed with promising amount of fiber as well as protein, fat, carbohydrates and nutritionally important minerals. Thus, the young pods of *Acacia catechu* can be used as a rich food source. These young pods were also rich in various phytochemicals and hence can be a good source of medicines too.

The results suggest that the ethyl acetate and petroleum ether fractions were able to scavenge both peroxide and DPPH free radicals. The *in-vitro* activities were comparable to a well known antioxidant compound; ascorbic acid. Both the extracts have also shown presence of sterols, triterpenes and cardiac glucosides as major secondary metabolites (data not shown).

Antioxidant activity of phenolic compounds is well known [13]. However, our data suggest that the total phenolic compounds were very less in ethyl acetate extract and in undetected amount in petroleum ether extract. Hence, the possibility of presence of some complex or non-phenolic antioxidant compound(s) cannot be ruled out. The thin layer chromatography data also suggests that there is one prominent antioxidant compound in petroleum ether fraction. Although, some of the major phenolic antioxidant compounds have been isolated from this tree, their presence or absence in young pods is not well established.

A. Catechu contains 2-12% catechins, 25-33% phlobatannin, 20-30% gummy matter, quercitrin, and quercetin [14]. The acacia tree has epicatechin that forms white acellular crystals which are soluble in hot water and alcohol and give a green color with ferric salts. Catechu tannic acid is an amorphous phlobatannin, which is formed from epicatechin by loss of the elements of water. It readily yields the phlobaphane catechu-red. The seed fatty acids contained 0.1% of c 18:1 epoxy acids. The bark and root contain flavanoids: quercetin, 3-methylquercetin, dihydrokaempferol, taxifolin; steroids: poriferasterol; triterpenoids: lupenone and lupeol[15]. Flavonoids were isolated from the leaf: quercitrin, hyperin, quercetin-3-O-arabinofuranoside[16].

Since, no study has compared the antioxidant potential of young pods of *Acacia catechu* against peroxide radicals; our study elaborates the use of *Acacia catechu* for peroxide scavenging activity. This activity is more useful to delay the rancidity of fats. Edible oils are rapidly oxidized in presence of oxygen and generate peroxide free radicals which are responsible for rancidity of the fats. The *Acacia catechu* show promising in vitro activities against peroxide radicals and hence can be used as an admixture to edible oils to prevent rancidity. DPPH free radical is used to investigate antioxidant potential of any given compound in vitro. The compounds able to scavenge DPPH free radicals are considered as good antioxidants.

The present study establishes the new source of antioxidants from a common tree. However, much work is needed in this direction to ascertain the antioxidant activity against other reactive oxygen species.

5. Conclusion

The present study concludes that the young pods of *Acacia catechu* exhibit good amount of peroxide and DPPH free radical scavenging activity and has good potential to be exploited as source of antioxidants as well as food.

6. Conflicts of Interests

Authors have no conflicts of interests.

7. Acknowledgement

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