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PHYTOCHEMICALS OF *CALAMUS TRAVANCORICUS* AGAINST THE FREE RADICAL INDUCED DEGENERATIVE DISORDERS

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ABSTRACT

The biological activity of the extract of *Calamus travancoricus* Bedd.ex. Becc. & Hk.f. has so far remained unknown. The present work on the in vitro antioxidant activity of the ethanolic fruit extract of this plant is the first investigation. The study deals with phytochemical screening and antioxidant activity of the ethanolic fruit extract of *C. travancoricus*. Phytochemical analysis of the extract revealed the presence of terpenoids, steroids, tannins and Flavanoids. The total phenolic content of the extract was found to be 85.3mg per gram of the extract. The free radical scavenging activity of the extract was confirmed in a DPPH assay, which showed the stronger radical scavenging effect with IC_{50} value of 25 µg /ml. The extract exhibited dose dependent increase in the absorbance indicated that the fruits of *C.travancoricus* possess concentration dependent reducing power. The study clearly indicated that the extract was found to possess scavenging effects on superoxide anions at concentration dependent manner with 66% of inhibition. Likewise, the extract showed significant inhibition of hydroxyl radicals in a concentration dependent manner with an IC_{50} value of 75.3 µg /mL.

KEYWORDS: *Calamus travancoricus*, Antioxidant activity, Phytochemicals, Free radicals.

INTRODUCTION

In human body system, free radicals are produced due to oxidative stress developed during normal metabolic processes or upon exposure to various environmental or chemical factors which subsequently damage the macromolecules viz., DNA, proteins and lipids. Oxidative

stress is one of the key factors for various diseases like cancer, diabetes, arthritis, inflammation etc.[1] Natural products have been reported to store large amount of antioxidants other than ascorbate, tocopherol and carotenoids. Antioxidants delay or prevent free radical catalyzed reactions. Phenolic compounds of plants are responsible for the antioxidant activity.[2] The traditional medicinal methods, especially the use of medicinal plants, still play a vital role to cover the basic health needs in the developing countries. The medicinal value of these plants lies in phytoconstituents that produce a definite physiological action on the human body. The most important of these bioactive constituents of plants are alkaloids, tannins, flavonoids terpenoids, saponins etc.[3] A variety of free radical scavenging antioxidants exists within the body of which many of them are derived from dietary sources like fruits and vegetables.[4] Synthetic antioxidants like butylated hydroxy anisole (BHA), butylated hydroxyl toluene (BHT), tertiary butylated hydroxy quinone and gallic acid esters have been suspected to be carcinogenic. Hence, strong limitations have been placed on their use and there is a trend to replace them with naturally occurring antioxidants. Therefore, it is a dire need to explore the antioxidants from natural sources having fewer side effects.

The genus *Calamus* (commonly called cane or rattan) is considered economically very important. Rattans are extensively used for making furniture, fancy articles etc. Rattans are used in Ayurvedic system of medicine for curing various diseases like Cough, Edema, Herpes, Diabetes, Rabies etc.[5] *Calamus* oil extracted from the roots contains palmitic acid, iso-eugenol, calamine, calamol, etc. and is used in perfumery and for flavouring liquors. The ripe fruit pulp of *C. floribundus* is edible and is a dyspepsiac. The tender shoots of *C. erectus*, *C. floribundus* and *C. latifolius* are eaten as vegetable and also as a cure for stomach ulcer and muscular sprain.[6] *Calamus* is a paleotropical genus with about 370 species distributed throughout the world. They are mainly found in the tropical rain forests and constitute an integral part of the tropical forest ecosystem. Indian rattans are distributed in tropical wet evergreen forests and semi-evergreen to moist deciduous forests from almost sea level to 1500m altitude with rainfall ranging from 1500-3000mm. Rattans are perennials, generally clustered, high climbing spiny palms. They are dioecious, flowering is annual and pleonanthic. In most of the species of Indian rattans, the flowering generally starts between October-January and fruits mature between April-June. With these backgrounds, to explore the medicinal value of the *C. travancoricus* the present study was undertaken using various *in vitro* models.

MATERIALS AND METHODS

Chemicals

Ascorbic acid, gallic acid, potassium ferricyanide, trichloroacetic acid, ferric chloride, folin-ciocalteu reagent, phenazine methosulfate (PMS), Nicotinamide adenine dinucleotide (NADH), Nitro blue tetrazolium chloride (NBT), thiobarbituric acid etc. were purchased from Merck India Ltd., Mumbai and 1,1- diphenyl-2- picryl hydrazyl (DPPH) was obtained from Sigma (St. Louis, USA). All the reagents and solvents used were of analytical grade.

Plant material and preparation of extract

The fruits of *C. travancoricus* were collected from Kodagu district, Karnataka, India. The collected fruits were thoroughly washed and dried in an oven at 40^{o}C for a week. The dried fruits were powdered using a grinder and extracted with ethanol using Soxhlet apparatus. The extract was filtered through a Whatman No.1 filter paper and the filtrate was concentrated in Rotary vaccum evaporator. Dried extract was stored at 4°C for further studies.

Phytochemical screening and determination of total phenolic content

The ethanolic fruit extract was studied for the presence of phytoconstituents such as flavonoids, tannins, saponins, steroids, alkaloids, terpenoids and glycosides using different phytochemical tests as the method described by Ayoola *et al.*[7] The total phenolic contents were determined by Folin Ciocalteu method.[8] Briefly, 2 mL of the sample was mixed with 2 ml of Folin–Ciocalteu reagent, followed by addition of 2 mL of 7.5%, w/v solution of sodium carbonate. The absorbance was measured at 765 nm after keeping in the dark for 30 min. The result was expressed as mg of gallic acid equivalents per gram of extract utilizing a calibration curve of gallic acid.

DPPH radical scavenging assay

The free radical scavenging capacity of the extract was determined as per the method described by Blois.[9] Ascorbic acid was used as a reference standard. Briefly, One ml of freshly prepared DPPH solution (0.004% w/v) was mixed with 1 ml of sample solution and standard solution at different concentrations (20-100µg/ml) separately. These solution mixtures were kept in dark for 30 minutes and optical density was measured at 517nm using a spectrophotometer. The % inhibition was calculated using the formula given below.

% inhibition = [(A – B)/A] × 100

Where A = optical density of the blank and B = optical density of the sample.

Reducing power assay

Antioxidant activity by reducing power assay was elucidated using the previously reported method.[10] Briefly, 1ml of extract at different concentration was mixed with 2.5ml of phosphate buffer and 2.5ml of potassium ferric cyanide in test tubes. The mixture was incubated for 20 min. at 50 °C. After incubation 2.5ml of trichloroacetic acid was added to the mixture followed by centrifugation at 5000 rpm for 10 min. The upper layer (2.5ml) was mixed with 2.5ml of distilled water and 0.5ml of ferric chloride. The absorbance was measured at 700nm. Increase in absorbance of the reaction mixture indicated the reducing power of sample. Ascorbic acid was used as positive control.

Superoxide radical scavenging assay Superoxide anions were generated using PMS-NADH system. The superoxide anions are subsequently made to reduce nitro blue tetrazolium chloride and the absorbance was measured at 560nm.[11] Briefly the reaction mixture contains 1mL of nitro blue tetrazolium,1mL NADH in 100mM phosphate buffer (pH 7.8) and 0.1mL of sample solution at different concentrations (20-100µg/ml). The reaction was started by adding 100µl of PMS and incubated at 25 C for 5 minutes. The absorbance of the mixture was measured at 560nm against blank. Ascorbic acid was used as reference standard. The percentage inhibition was determined by comparing the results of control and test samples using the following formula.

% inhibition = [(A – B)/A] × 100

Where A = optical density of the blank and B = optical density of the sample.

Hydroxyl Radical Scavenging Activity

The method described by Aruoma and Halliwell was used to determine the hydroxyl radical scavenging capacity of the extract.[12] Different concentrations of the extract were added to the reaction mixture in a final volume of 1 mL in potassium phosphate buffer (pH 7.4). This mixture was incubated at 37◦ C for 1 hour and then mixed with 1 mL of 2.8% trichloroacetic acid and 1 mL of 1% thiobarbituric acid. The mixture was then heated in a boiling water bath for 15 minutes and absorbance was taken at 532 nm. Ascorbic acid was used as a standard. The percentage of inhibition was calculated as described above.

STATISTICAL ANALYSIS

All the experimental results were mean ± SD of three parallel measurements. Linear regression analysis was used to calculate the IC_{50} values.

RESULTS AND DISCUSSION

Phytochemical screening and determination of total phenolic content

The preliminary phytochemical analysis of the extract revealed the presence of terpenoids, steroids, tannins and Flavanoids (Table 1). Determination of total phenolic contents revealed the presence of 85.3mg/g of phenolic compounds. The phenolic concentration of the extract was expressed as milligram of gallic acid equivalents per gram of extract. Polyphenolic compounds are very important plant constituents because of their free radical scavenging ability.[13] It is suggested that polyphenolic compounds have inhibitory effects on mutagenesis and carcinogenesis in human beings.[14] The antioxidative characteristics might be attributed to the presence of polyphenolic compounds.

DPPH radical scavenging assay

DPPH is relatively stable nitrogen centered free radical that easily accepts an electron or hydrogen radical to become a stable diamagnetic molecule.[15] In the present study, strong free radical scavenging effect of the extract was confirmed in a DPPH assay with maximum percentage inhibition of 92% (Table 2). The extract showed the stronger scavenging effect which is comparable to standard antioxidant ascorbic acid. The IC_{50} value of ethanolic extract and Ascorbic acid were found to be 25and 12µg/ml respectively. DPPH radicals react with suitable reducing agents as a result of which the electrons become paired off forming the corresponding hydrazine.[16] Hence, the free radical scavenging capacity of an extract may serve as a significant indicator of its potential antioxidant activity.

Reducing power assay

The results of the present study indicated that reducing power of the extract increased with increasing concentration which is comparable to standard ascorbic acid (Table 3). The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity.[17] Reducing power is to measure the reductive ability of antioxidant and it is evaluated by the transformation of Fe3+ to Fe2+ in the presence of the sample extracts. The ability to reduce Fe3+ may be attributed by hydrogen donation from phenolic compounds.

Superoxide radical scavenging assay

Superoxide anion is a harmful reactive oxygen species as it damages cellular components in biological systems.[18] The extract showed potent superoxide radical scavenging activity with increasing concentration which is comparable to standard ascorbic acid. Maximum

percentage inhibition of the extract and the standard ascorbic acid was found to be 66% and 92% respectively (Table 4). Superoxide anion plays an important role in the formation of reactive oxygen species such as hydrogen peroxide, hydroxyl radical and singlet oxygen, which induces oxidative damage in lipids, protein, and DNA. The results of the present investigation clearly suggested that the extract is a potent superoxide radical scavenger.

Hydroxyl Radical Scavenging Activity

Results of this assay showed the ability of the extract to inhibit hydroxyl radical-mediated deoxyribose degradation. The fruit extract of *C. travancoricus* showed significant inhibition of hydroxyl radicals generated by Fenton's reagent in a concentration dependent manner with an IC_{50} value of 75.3 µg /mL which is comparable to ascorbic acid (Table 5). In living organisms, hydroxyl radicals and superoxide radicals are being continuously formed in a process of reduction of oxygen to water. Hydroxyl radicals are the major active oxygen species causing lipid peroxidation and enormous biological damage like reduction of disulfide bonds in proteins, specifically fibrinogen, resulting in their unfolding and scrambled refolding into abnormal spatial configurations.[19]

Table 1: Showing the phytochemical constituents of ethanolic fruit extract of *Calamus travancoricus*.

Terpenoids	Saponins	Steroids	Alkaloids	Glycosides	Tanins	Flavanoids
++	--	++	--	--	++	++

++ Presence of constituent

-- Absence of constituent

Table 2: DPPH free radical scavenging activity of ethanolic fruit extract of *Calamus travancoricus* and Ascorbic acid.

Concentration of the extract and ascorbic acid (µg)	% inhibition of the extract	% inhibition of ascorbic acid
20	45 ± 1.1	87.1 ± 1.1
40	68 ± 1.3	93.0 ± 1.4
60	75 ± 0.6	96.0 ± 0.9
80	84 ± 1.2	98.1 ± 1.2
100	92 ± 0.9	99.1 ± 0.8

Values are shown in mean ± SE

Table 3: Reducing power activity of ethanolic fruit extract of *Calamus travancoricus* and Ascorbic acid.

Concentration of the extract and ascorbic acid (µg)	Reduction potential (absorbance) of the extract	Reductional potential (absorbance) of ascorbic acid
20	0.44 ± 0.02	0.56 ± 0.04
40	0.72 ± 0.03	0.98 ± 0.03
60	0.98 ± 0.02	1.28 ± 0.05
80	1.39 ± 0.09	1.96 ± 0.2
100	1.88 ± 0.05	1.98 ± 0.06

Values are shown in mean ± SE.

Table 4: Superoxide anion scavenging activity of ethanolic fruit extract of *Calamus travancoricus.*

Concentration of the extract (µg)	% inhibition of the extract	% inhibition of ascorbic acid
20	19 ± 0.5	46 ± 0.63
40	28 ± 0.04	58 ± 1.2
60	43 ± 1.1	76 ± 0.9
80	52 ± 0.9	88 ±1.4
100	66 ± 1.2	92 ±0.8

Values are shown in mean ± SE.

Table 5: Hydroxyl radical scavenging activity of ethanolic fruit extract of *Calamus travancoricus.*

Concentration of the extract (µg)	% inhibition of the extract	% inhibition of ascorbic acid
20	20 ± 0.04	48 ± 0.4
40	32 ± 0.08	57 ± 1.02
60	41 ± 1.2	74 ± 0.09
80	54 ± 0.09	82 ±1.24
100	63 ± 1.4	93 ±0.08

Values are shown in mean ± SE.

CONCLUSIONS

The results obtained in the *in vitro* models such as reducing power assay, DPPH radical scavenging assay, superoxide radical scavenging assay and hydroxyl radical scavenging activity clearly indicated that, the ethanolic fruit extract of *Calamus travancoricus* exhibited strong free radical scavenging activity when compared with standard ascorbic acid. The free radical scavenging activity of the extract may be due to the presence of phenolic compounds such as flavonoids, steroids, terpenoids and tannins. The results of this study showed that the

extract can be used as easily accessible source of natural antioxidants and as a possible food supplement. The fruits of *Calamus travancoricus* could be served as a new source of nutraceuticals with potential applications to reduce the level of oxidative stress and related health benefits. This study established a significant scope to develop a broad spectrum use of *Calamus travancoricus* in herbal medicine and as a base for the development of novel potent drugs against the oxidative stress related health disorders in human beings.

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