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Research Article

IN VITRO FREE RADICAL SCAVENGING POTENTIAL OF *ACORUS CALAMUS* L. RHIZOME FROM KUTTANAD WETLANDS, KERALA, INDIA

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ABSTRACT

Objective: The present study was aimed to carry out phytochemical screening and to assess the *in vitro* free radical scavenging ability of methanolic and aqueous extracts of *Acorus calamus* (Sweet flag) rhizome collected from Kuttanad wetlands, forming part of Vembanad-Kol wetland system, one of the Ramsar sites in Kerala, India.

Methods: The antioxidant potential of the rhizome was evaluated by using six *in vitro* assays, namely 2, 2-diphenyl-1-picrylhydrazyl (DPPH), hydroxyl radical, superoxide radical, reducing power, lipid peroxidation and metal ion chelating assay. The phenolic and flavanoid contents of methanolic and aqueous extracts were also analysed.

Results: The total phenolic contents of aqueous and methanolic extracts were 19.86 mg/g and 12.17 mg/g respectively. Similarly the flavonoid contents were 8.33 mg/g and 2.56 mg/g**.** The methanolic extract of the rhizome showed higher hydroxyl radical scavenging activity of 86.83 %, superoxide radical activity of 44.16 %, and also higher reducing power activity. The aqueous extract was a better scavenger of DPPH free radicals with 34.51 %, lipid peroxidation with 49.91 %, and metal ions chelating assays with 39.85 % activity.

Conclusion: The methanolic extract of *A. calamus* rhizome is a good free radical scavenger in case of reducing power, superoxide radical scavenging and hydroxyl radical scavenging assays. On the other hand, aqueous extract showed better radical scavenging ability in metal ion chelating, lipid peroxidation and DPPH radical scavenging assays.

Keywords: *Acorus calamus,* DPPH, Free radical scavenging, Lipid peroxidation, Phytochemical screening, Rhizome.

INTRODUCTION

Oxidative stress is the result of an imbalance of pro-oxidants (free radicals) and antioxidants in favour of pro-oxidants. Oxidative stress leads to extensive damage in cell structures in humans and this is considered as a major reason for the pathogenesis of several chronic diseases such as diabetes mellitus, cancer, inflammation and neurodegenerative diseases [1]. Antioxidant is a substance which prevents oxidative damage triggered by reactive oxygen species by interfering with the oxidation process by inhibiting the initiation or propagation of oxidizing chain reactions [2]. Antioxidant based drug formulations are widely used for the prevention and treatment of complex diseases like atherosclerosis, stroke, diabetes, Alzheimer's disease and cancer [3].

Natural antioxidants are secondary metabolites of plants and their antioxidant property is due to the presence of flavones, isoflavones, flavonoids, coumarin lignans, anthocyanin, catechins and isocatechins [4, 5]. Natural antioxidants are considered as safe and cause fewer adverse reactions than synthetic antioxidants [6]. Whereas, synthetic antioxidants (butylated hydroxytoluene, butylated hydroxyanisole, propylgallate and tertiary butyl-hydroquinone) are known to amend oxidative damages, they have restricted use because of their carcinogenic effect [7]. Therefore, in recent years, considerable attention has been directed towards identification of plant-based antioxidants, especially within biological, medical, nutritional and in agrochemical fields [8].

Acorus calamus L. (Family: Araceae) is a semi-aquatic, perennial, tuberous herb, with creeping rhizomes, sword shaped leaves and spadix inflorescence, having a long history of medicinal use in different healthcare systems worldwide, and particularly in Indian herbal traditions. While the scented leaves and rhizomes of the herb have been used traditionally in India as a medicine, the dried and powdered rhizome with a spicy flavour is used as a substitute for ginger, cinnamon and nutmeg for culinary purposes. The plant, locally known as 'Vayambu', is grown traditionally in the home gardens of Kuttanad wetland system in Kerala, and the plant is also found in isolated thickets along marshy habitats and fringe areas of ponds and canals as garden escape. In Ayurvedic system of medicine, the rhizomes of Sweet flag are used in the treatment of diseases like fever, asthma, bronchitis and also as a sedative. Further, the natives use the rhizome in treating cough [9].

The rhizomes of Sweet flag (*Acorus calamus* L.) is widely used in a number of ailments like epilepsy, mental ailments, bronchial catarrh, intermittent fevers and glandular, abdominal tumors, kidney and liver troubles, chronic diarrhea, dysentery, rheumatism, sinusitis and eczema**.** It is also used for appetite and as an aid to digestion. It is a highly valued herb because it acts as a rejuvenator for the brain and nervous systems [10] and is widely employed in modern herbal medicine due to its sedative, laxative, diuretic, and carminative properties [11]. It also possesses insecticidal, antifungal, antibacterial, tranquilizing, antidiarrhoeal, antidyslipidemic, neuroprotective, antioxidant, anticholinesterase, spasmolytic and vascular modulator activities [12]. Considering the presence of a wide range of bioactive compounds and broad spectrum of use, the present study was designed to analyse the antioxidant potential of methanolic and aqueous extracts of *A. calamus* rhizome gathered from the marshy habitats of Kuttanad wetlands, located along the coastal lowland zone of Kerala, India.

MATERIALS AND METHODS

Chemicals

Riboflavin, quercetin, butylated hydroxyl anisol (BHA), nitro blue tetrazolium (NBT), 2.2 Diphenyl -1-picryl hydrazyl (DPPH), deoxyribose, ferrozine, potassium ferricyanide and the solvents were procured from HiMedia Laboratories Pvt. Limited, Mumbai, India. Trichloroacetic acid (TCA), Folin Ciocalteau reagent, ethylene diamine tetra acetic acid (EDTA) and ascorbic acid were purchased from Sisco Research Laboratories, Mumbai, India. Thiobarbituric acid (TBA) was purchased from Rolex Chemical Industries, Mumbai, India. All the other chemicals and reagents used were of analytical grade and were prepared in deionized water.

Plant material and extraction

Rhizomes of *A. calamus* were collected from Kuttanad wetlands, Kerala, India. The plant was authenticated by Dr. T. Shaju, Plant Taxonomist, Division of Plant Systematics and Evolutionary Science, Jawaharlal Nehru Tropical Botanic Garden and Research Institute (JNTBGRI), Palode, Kerala, and the voucher specimens of the samples were deposited in the Herbarium of Environmental Resources Research Centre (ERRC), Thiruvananthapuram, Kerala. The rhizomes were washed, air dried in shade, powdered and subjected to cold extraction using methanol and water for 72 h and 24 h respectively. Extracts were filtered using a Whatman No. 1 filter paper and were evaporated to dryness under reduced pressure at 40ºC in rotary vacuum evaporator (IKA RV 10 digital). The extracts obtained were kept in sterile sample tubes and stored in refrigerator at 4ºC. The extract was dissolved in respective solvents prior to assay.

Fluorescence analysis

The fluorescence characteristics of the powered rhizome was studied under short UV (254 nm), long UV (366 nm) and visible light after treating with different solvents and chemicals following the method described by Kokoshi [13].

Phenol quantification

The total phenolic content of methanolic and aqueous extracts was determined using folin ciocalteu reagent [14]. Aliquots of the extracts were made up to 3 ml with distilled water. Then 0.5 ml folin ciocalteau reagent (1:1 with water) and 2 ml Na_2CO_3 (20 %) were added sequentially. The tubes containing the above reaction mixture were warmed for 1 minute, and cooled subsequently for measuring at 760 nm. The concentration of phenol in the test samples were calculated from the calibration plot and expressed as mg catechol equivalent of phenol/g of sample. Results were expressed as mg of catechin /1g of extract.

Flavonoid quantification

Total flavonoid content was determined by aluminium chloride method [15]. One ml sample and 4 ml of water were added to a volumetric flask. 0.3 ml of 5 % sodium nitrite, 0.3 ml of 10 % aluminium chloride was added after 5 min. Then after 6 min incubation at room temperature, 2 ml of 1 M sodium hydroxide was added to the reaction mixture and immediately made up to 10 ml with distilled water. The absorbance was read at 510 nm and the results were expressed as catechin equivalents (mg catechin /g dried extract).

Determination of *in vitro* antioxidant activities

DPPH radical scavenging assay

The hydrogen or electron donation ability of the plant extracts was measured from bleaching of the purple colour of DPPH [16]. 0.1 ml of extract with varying concentrations (25 - 2000 µg/ml) was added to 1.4 ml of DPPH solution. The mixture was properly shaken and incubated at ambient temperature in dark for 30 min and the absorbance at 517 nm was recorded.

Hydroxyl radical scavenging assay

Hydroxyl radical scavenging activity of the extracts was measured following the method of Halliwell [17]. One ml of the final reaction solution consisted of aliquots (500 µl) of various concentrations of the extract, 1 mM $FeCl_3$, 1 mM EDTA, 20 mM H_2O_2, 1 mM L-ascorbic acid, and 30 mM deoxyribose in potassium phosphate buffer (pH 7.4). The reaction mixture was incubated for 1 h at 37 °C, and further heated in a boiling water bath for 15 min after the addition of 1 ml of 2.8 % (w/v) trichloroacetic acid (TCA), and 1 ml of 1 % (w/w) 2- thiobarbituric acid. After cooling, the absorbance was measured at 532 nm against phosphate buffer blank.

Metal ion chelating assay

The ferrous ion chelating potential of the extracts at different concentrations was investigated based on the method described by Gulcin [18]. Different concentrations of the sample (25 - 2000 µg/ml) were added to solution of 2 mM iron (II) chloride (0.05 ml). The reaction was initiated by the addition of 5 mM ferrozine (0.2 ml) and the volume of the mixture was finally adjusted to 4 ml with ethanol, shaken vigorously and left standing at room temperature for 10 min. After the mixture had reached an equilibrium, the chelating activity of the solution was measured spectrophotometrically at 562 nm.

Reducing power assay

Ferric reducing power of extracts was determined following the method described by Oyaizu [19]. One ml of extracts at various concentrations 25 - 2000 µg/ml was respectively added to phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide (2.5 ml, 1%) followed by mixing and incubating at 50 °C for 20 minutes. Then, TCA (2.5 ml, 10 %) was added to the mixture and centrifuged at 3000 rpm for 10 min. 2.5 ml of the supernatant was taken out and mixed with 2.5 ml of distilled water followed by the addition of ferric chloride (0.5 ml, 0.1%), vortexed and the absorbance of the mixtures was read at 700 nm.

Lipid peroxidation assay

Antilipid peroxidation assay was performed with goat liver homogenate as per the protocol of Mandal [20]. 2.8 ml of 10 % goat liver homogenate, 0.1 ml of 50 mM ferrous sulphate and 0.1 ml of test sample 25 - 2000 µg/ml were added and the reaction mixture was incubated at 37 °C for 30 min. The reaction was terminated by adding 2 ml of 10 % TCA, 0.67 % TBA made in 50 % acetic acid and boiled for 1 hour at 100 °C, followed by centrifugation for 5 min at 10,000 rpm and supernatant was read at 535 nm against blank. Reaction mixture without test sample and $FeSO_4$ is used as control and mixture without extract act as induced.

Superoxide radicals scavenging assay

Scavenging activity of aqueous and methanolic extracts on superoxide anion radicals was spectrophotometrically determined based on the reduction of NBT which depends on light induced superoxide generation by riboflavin [21]. Different concentrations (25 – 2000 µg/ml) of *A. calamus* extracts were added to the reaction mixture containing EDTA (0.1 M), 0.0015 % NaCN, riboflavin (0.12 mM), NBT (1.5 mM) and various concentrations of extract and phosphate buffer (67 mM, pH 7.8) in a total volume of 3 ml. The tubes containing the reaction mixture were continuously illuminated with incandescent lamp for 15 minutes and the optical density was read at 530 nm before and after illumination against phosphate buffer as blank.

Assessment of percentage of inhibition

All analyses were carried out in triplicates and the data was presented as mean ± SD. Radical scavenging activity of extracts was expressed in terms of percentage of inhibition. DPPH, superoxide radical scavenging activity, hydroxyl radical scavenging activity and metal ion-chelating assay using the equation: % Inhibition = (Absorbance of control – Absorbance of sample)/Absorbance of control × 1 0 0, and the anti-lipid peroxidation percentage was calculated using the formula: % ALP = (Abs of Fe2+ induced peroxidation - abs of sample)/ Abs of Fe2+ induced peroxidation - abs of control x 100.

RESULTS

Fluorescence analysis

Under exposure to short UV the *A. calamus* rhizome powder showed various shades of green colour when treated with different chemicals. Different colours were also exhibited under long UV and visible light (Table 1).

Phenol quantification

The total phenol content was expressed as mg of catechin/g of extract and the corresponding values for *A. calamus* methanolic and aqueous extract was found to be 12.17 ± 1.47 mg/g and 19.86 ± 1.45 mg/g respectively.

Table 1: Fluorescent analysis of *A. calamus* rhizhome powder

Particulars of treatment	Under short UV light	Under long UV light	Under visible light
Powder as such	Light green	Cream	Cream
5% H_2SO_4	Light green	Green	Cream
5% HCl	Light green	Green	Cream
5% $Fecl_3$	Dark green	Black	Brown
5% NaOH	Dark green	Blackish green	Brown
5% KOH	Dark green	Blackish green	Brown
1N NaOH in water	Dark green	Pink	Brown
Ethanol	Dark green	Cream	Cream
Nitric acid	Yellowish green	Violet	Orange
Acetic acid	Green	Cream	Cream
Chloroform	Green	Violet	Brown
Acetone	Green	Cream	Cream
Petroleum ether	Green	Cream	Brown
Water	Green	Cream	Brown

Flavonoid quantification

The total flavanoid content of the *A. calamus* methanolic and aqueous extract was found to be 2.56 ± 0.76 mg/g and 8.33 ± 1.80 mg/g respectively and it was expressed in terms of catechol equivalents/g of dried extract.

DPPH radical scavenging activity

The scavenging effect of *A. calamus* methanolic and aqueous extract on DPPH radical was 18.91 % and 34 % respectively at a concentration of 2000 µg/ml (Figure 1). The standard BHA exhibited 98 % radical scavenging ability at 100 µg/ml concentration itself, which was very high when compared to both the rhizome extracts.

Hydroxyl radical scavenging activity

At 2000 µg/ml, the methanolic extract was found to exhibit better inhibition of 86.83 % (Figure 2) compared to aqueous extract (55.56 %) with an IC_{50} value of 1647.68 µg/ml. The scavenging activity obtained for BHA was 59 % at concentration 100 µg/ml.

Metal ion chelating activity

At concentration 2000 µg/ml, the percentages of inhibition of aqueous and methanolic extract were found to be 39.85 % and 26.66 % respectively (Figure 3) whereas, the standard BHA showed 82 % inhibition at concentration 100 µg/ml. The result of the present study suggested that the aqueous extract exhibited good chelating activity on ferrous ions than the methanolic extract.

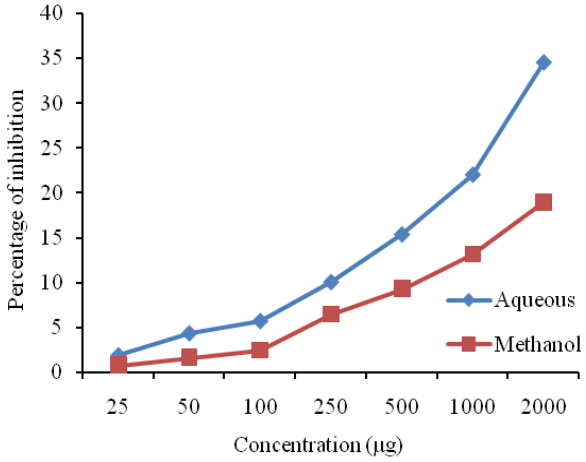

Fig. 1: DPPH radical scavenging activity of extracts

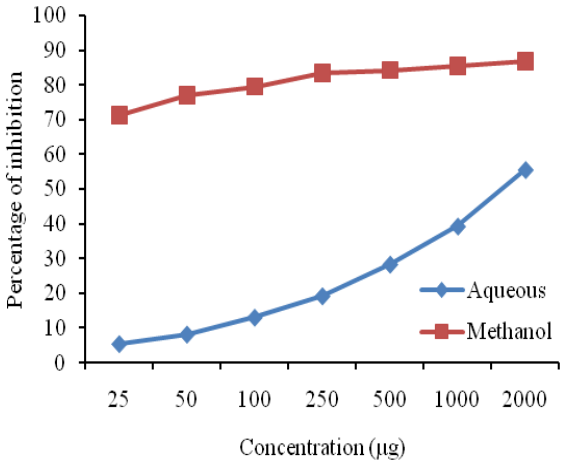

Fig. 2: Hydroxyl radical scavenging activity of extracts

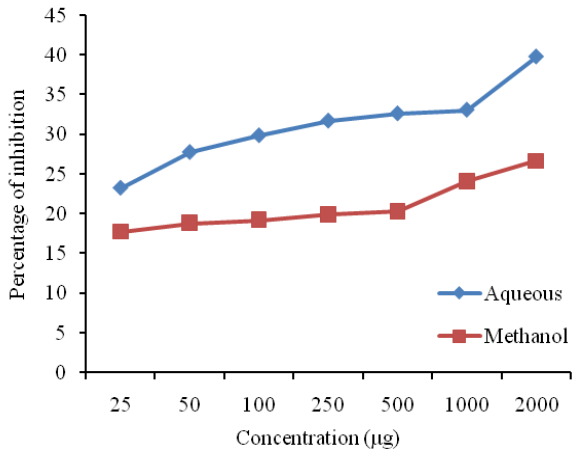

Fig. 3: Metal ion chelating activity of extracts

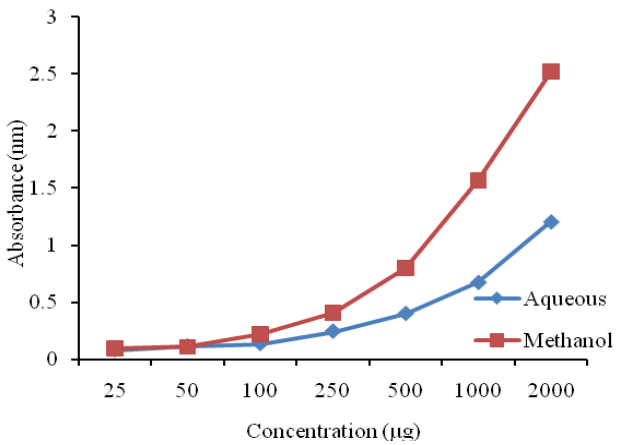

Fig. 4: Reducing power activity of extracts

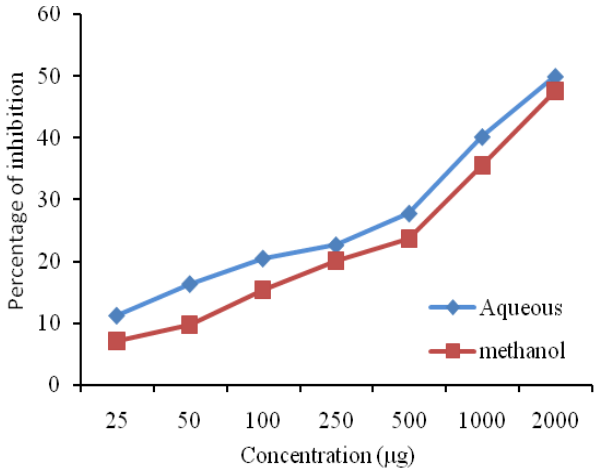

Fig. 5: Lipid peroxidation activity of extracts

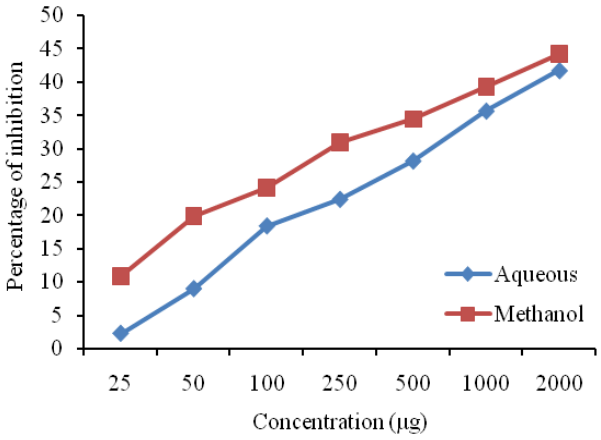

Fig. 6: Superoxide radical scavenging activity of extracts

Reducing power activity

Reducing power of both the extracts increased with an increase in concentration. The reducing power of the extracts was found to be dose dependent (Figure 4). The methanolic extract showed more reductive ability than the aqueous extract, which was capable for neutralizing the free radical. The reducing power ability of the standard BHA was more, than that of the extracts.

Lipid peroxidation assay

The aqueous extract exhibited good lipid peroxidation inhibition ability with 49.91 % (Figure 5) than the methanolic extract (47.6 %) and the standard quercetin showed 64 % inhibition at 100 µg/ml concentrations.

Superoxide radical scavenging assay

The superoxide radical scavenging ability of methanolic and aqueous extracts was 44.16 % and 41.76 % (Figure 6) respectively. The standard BHA exhibited 82 % inhibition of superoxide radicals at 100 µg/ml concentration which showed a wide variation when compared to the extracts.

DISCUSSION

The rhizome samples exhibited different colours when treated with different chemicals and exposed under short, long UV and under visible light. The characteristic fluorescent properties recorded in fluorescent analysis could be used as a standard in the identification and authentication of rhizome of *A. calamus* in its crude form and this will further reveal the presence of active agents by their various colour reactions to different chemicals and colour change under the ultraviolet rays [22]. The total phenolic and flavonaoid contents of methanolic extract recorded in the present study were lesser than those reported by Devi and Ganjewala [23] for *A. calamus* samples collected from Yercaud in Tamil Nadu, India. Similarly, IC_{50} values for DPPH radical scavenging, metal ion chelating and superoxide radical scavenging assays were 20.8 µg/ml, 33.3 µg/ml and 30.5 µg/ml respectively. So far there are no published reports regarding the antioxidant and phytochemical analysis of aqueous extracts of *A. calamus*. The phenolic and flavonoid compounds present in the samples collected from Kuttanad were low and this might be attributed to the low antioxidant property. The aqueous extract was found to be a better source of phenolics and flavonoids. Phenols are the important plant derived bioactive compounds because of their ability to scavenge free radicals due to their hydroxyl groups and it may directly contribute to their antioxidant potential [24].

The results of the present study showed that the aqueous extract was able to scavenge more DPPH radical when compared to methanolic extract. Unlike other reactive oxygen species generated *in vitro* such as the hydroxyl radical and superoxide anion, DPPH is a very stable free radical and it has an advantage of being unaffected by certain side reactions, such as metal ion chelation and enzyme inhibition [25]. The hydroxyl radical scavenging capacity of methanolic extract is higher enough to consider it as one possessing antioxidant potential and hydroxyl radical is implicated as one of the most damaging free radicals in the body and it can be the important mediator of damage to cell structures, nucleic acids, lipids and proteins [26]. The aqueous extract exhibited good metal ion chelating activity than the methanolic extract. Metal ion chelating ability plays a major role in antioxidant mechanism, since it reduces the concentration of the catalyzing transition metal in lipid peroxidation [27] and it also prevents oxy radical generation and consequent oxidative damage [28].

The methanolic extract of *A. calamus* showed more reductive ability than the aqueous extract, which was capable for neutralizing the free radical. Reducing power activity serves as an indicator of antioxidant ability and this is generally associated with the presence of reductones, which exhibit radical scavenging ability by preventing the chain reactions by donating a hydrogen atom and the reductones also react with certain precursors of peroxide, thus preventing peroxide formation [29]. The aqueous extract of *A. calamus* exhibited high anti-lipid peroxidation followed by the methanolic extract. The aqueous extract exhibit anti-lipid peroxidation by acting as scavengers of free radicals that form alkyl peroxyl and alkoxyl radicals or can donate hydrogen atom to alkyl peroxyl and alkoxyl radicals and thus terminate the chain reaction initiation [30]. The methanolic extract of *A. calamus* showed the highest superoxide radical scavenging activity followed by the aqueous extract. Meanwhile, Devi and Ganjewala [23] reported an IC_{50} value of 30.5 µg/ml for methanolic extract of *A. calamus* collected from Yercaud, Tamil Nadu. Although superoxide is the primary free radical in the biological system, this by itself is unreactive. However, the system converts it into more reactive oxygen species [31]. Comparatively, in the present study, methanolic extract was a better scavenger of superoxide radical when compared to that of aqueous extract.

Among the six *in vitro* assays conducted, aqueous extract showed good radical scavenging ability in metal ion chelating, lipid peroxidation and DPPH radical scavenging assays. The methanolic extract is a better scavenger in reducing power, superoxide radical and hydroxyl radical scavenging assays. This study indicated the presence of natural antioxidant compounds in *A. calamus* rhizome found in Kuttanad wetlands.

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