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Screening of phytochemical and free radical scavenging activity of *Acorus Calamus* L. rhizome

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Abstract
The accumulation of free radicals in the body is implicated in the development of various diseases due to oxidative stress. Current research endeavours are focused on preventing oxidative stress and mitigating the impact of free radicals through the use of antioxidants, primarily derived from plants rather than synthetic sources. In this study, *Acorus calamus*, a plant belonging to the Acoraceae family, was utilized to evaluate its phytochemical constituents and its ability to scavenge free radicals. To extract the *Acorus calamus* rhizome, four solvents—methanol, ethanol, chloroform, and water were employed. Preliminary phytochemical analysis was conducted using standard protocols. Additionally, the study assessed the free radical scavenging activity against DPPH, ABTS, FRAP, and nitric oxide. The results of the phytochemical screening indicated the presence of compounds such as alkaloids, flavonoids, sterols, proteins, and carbohydrates, with ethanol extract exhibiting higher concentrations compared to other solvents. The free radical scavenging activity demonstrated maximum efficacy in the ethanol extract, with a scavenging power of 89.53% for DPPH and 87.71% for ABTS, 88.24% for FRAP and 88.53% for Nitric oxide at a concentration of 100 µg/ml. These findings suggest that *Acorus calamus* rhizome has the potential to effectively scavenge free radicals, offering a promising avenue for preventing diseases associated with oxidative stress. This highlights the plant's potential as a valuable phytotherapeutic agent against a range of diseases and disorders.

Keywords: *Acorus calamus*, antioxidants, free radicals, phytochemicals, DPPH

Introduction
Traditionally utilized medicinal plants play a crucial role in promoting human health through therapeutic remedies. Natural products have proven to be a vast reservoir of diverse biomolecular structures, surpassing our current understanding [1]. Phytochemicals, which are bioactive compounds derived from plants, have the ability to alleviate various physiological disorders in humans and reduce the reliance on synthetic antibiotics.

The human body's normal cellular metabolic reactions, when exposed to a heightened environment and increased intake of xenobiotics, result in the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS). In specific pathophysiological conditions, ROS and RNS lead to oxidative stress, generating unstable molecules known as free radicals, which exacerbate numerous chronic and degenerative ailments [2]. The effective neutralization of cellular responses through antioxidants can mitigate oxidative stress and suppress free radical formation [3].

Phytoconstituents possess the capacity to scavenge free radicals by donating electrons or ions to unpaired electrons. Researchers are actively exploring potent antioxidants sourced from medicinal plants due to their financial sustainability and exceptional antioxidant properties without adverse effects. *Acorus calamus* L, a tall, perennial, grass-like monocot plant belonging to the Acoraceae family, has been a prominent fixture in Indian traditional medicines for centuries. Valued as a rejuvenator for the brain and nervous system, it is a key medhya drug known for enhancing memory power and intellect. The plant's rhizomes find widespread use in treating conditions such as epilepsy, mental ailments, chronic diarrhea, dysentery, fever, abdominal tumors, kidney and liver issues, and rheumatism. *A. calamus* leaves, rhizomes, and its essential oil known to have various biological activities, including antispasmodic, carminative, and regenerative properties [4]. In light of this background, the current study aimed to assess the potential of *A. calamus* as a natural source of phytochemicals and antioxidants.

Materials and Methodology
Plant collection and sample preparation
The rhizome of *A. calamus* were collected from the areas of Alappuzha, Kerala. The rhizome was washed entirely and let dry for 5-7 days at room temperature. The dried-out rhizome was round to powder and stored in screw-cap bottles until further analysis. Preparation of the extract, 20 g of sample was dissolved in 200 ml of various solvents (methanol, ethanol, chloroform, and water). It was then filtered and further concentrated by evaporation.

Phytochemical analysis
The extracts were subjected to preliminary phytochemical evaluation, which was done using standard color test methods [5].

Free radical scavenging activity
The free radical scavenging activities of the extracts were determined *in vitro* against a battery of radicals, namely DPPH, ABTS, FRAP and nitric oxide assay.

DPPH radical scavenging activity
1, 1-Diphenyl-2-picrylhydrazyl (DPPH) was obtained from Sigma Aldrich Co., St. Louis, USA. About 3 ml of graded concentration (25 - 100µg/ml) of extracts were taken in different test tubes, and 1 ml of 0.3mM DPPH methanol solution was added to these test tubes and shaken vigorously. Methanol served as the blank, and DPPH in methanol, without the rhizome extracts, served as the positive control. After 30 min incubation of samples at 25˚C in the dark, the absorption was measured at 517 nm. The inhibition percentage of DPPH was calculated as follows

Scavenging activity (%) = Abs (control) – Abs (sample) / Abs (control)x100
Abs (control)- absorbance of DPPH radical with methanol; Abs (sample)- absorbance of DPPH radical with sample extract [6].

ABTS radical scavenging activity
ABTS radical cations (ABTS+) were produced by reacting ABTS solution (7mM) with 2.45mM ammonium persulphate. The mixture was allowed to stand in the dark at room temperature for 12 to 16 hours before use. Aliquots (5µl) of the different extracts were added to 0.3ml of ABTS solution, and the final volume was made up to 1ml with ethanol. The absorbance was read at 745nm in a spectrophotometer, and the percent scavenging was calculated using the formula
Scavenging activity (%) = Abs (control) – Abs (sample) / Abs (control)x100 [7].

FRAP (Ferric reducing power assay)
Reaction mixtures were prepared by adding 2.5 ml of phosphate buffer (0.2 M, pH 6.6), 2.5 ml potassium ferricyanide (1%), and varying concentrations of extracts (25 - 100µg/ml). After the reaction, mixtures were incubated at 50°C in a water bath for 30 min, allowed to cool at room temperature (28°C), and 2.5 ml of 10% TCA (Trichloroacetic acid) was mixed into each reaction mixture, followed by the centrifugation at 2000 rpm for 10 min. The supernatant (2.5 ml) was separated in the test tube, added with 2.5 ml of distilled water and 0.5 ml FeCl3 (1.0%), and allowed to react for 10 min and absorbed at 700 nm [8].

Nitric oxide assay
Nitric oxide radical inhibition was estimated using Griess Illosvory reaction. In this investigation, Griess Illosvory reagent was generally modified by using napthyl ethylene diamine dihydrochloride (0.1% w/v) instead of 1-napthylamine (5%). The reaction mixture (3 ml) containing 2 ml of 10 mM sodium nitroprusside, 0.5 ml saline phosphate buffer and 0.5 ml of extracts (25,50, 100 µg/ml) were incubated at 25°C for 150 minutes. After incubation, 0.5 ml of the reaction mixture was mixed with 1 ml sulfanilic acid reagent (0.33% in 20% glacial acetic acid) and allowed to stand for 5 minutes for the completion of the reaction of diazotization. After this, a further 1 ml of the napthyl ethylene diamine dihydrochloride was added, mixed and was allowed to stand for 30 minutes at 25°C. The concentration of nitrite was assayed at 546 nm and was calculated with the control absorbance of the standard nitrite solution (without extracts or standards, but the same condition should be followed). Here buffer was used as blank solution and Ascorbic acid and quercetin were taken as standard solution. The percentage inhibition was calculated using the formula:
% scavenging activity = [(A control – A test or A std)/A control] * 100
where A control is the absorbance of control and A test or A std is the absorbance of test or standard, respectively [9].

Results and Discussion
The preliminary phytochemical screening was carried out on various solvents and revealed the presence of a wide range of phytoconstituents, including alkaloids, flavonoids, sterols, proteins, and carbohydrates which showed better result in ethanolic extract among the other three solvents (Table 1). This indicates that ethanol is highly capable of extracting secondary metabolites of *A. calamus* rhizome compared with all other solvents since the high polarity of the solvent accounts for the extraction of a wide range of compounds.

Table 1: Qualitative Phytochemical analysis of the extracts of *A.calamus* rhizome

Phytochemicals	Methanol	Ethanol	Chloroform	Aqueous
Alkaloids	-	+	+	-
Flavonoids	+	+	+	+
Sterols	+	+	-	+
Terpenoids	-	-	-	-
Anthraquinone	+	-	-	-
Anthocyanin	-	-	-	-
Proteins	+	+	-	-
Phenolic compounds	-	+	-	-
Quinones	-	-	-	-
Carbohydrates	+	+	+	+
Tannins	-	-	-	-
Saponins	-	-	-	-
Cardiac glycosides	-	-	-	-
Glycoside	-	-	-	-

+ Present - Absent

The ability of rhizome extracts to effectively neutralize free radicals, including DPPH (2,2- di (4-test-octyl phenyl) -1-picrylhydrazyl radical), ABTS (2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) radical cation, Nitric oxide scavenging activity along with their capacity to convert ferric (III) iron to ferrous (II) iron, was evaluated through antioxidant assays. A comparative assessment of *A. calamus* rhizome antioxidant activity was conducted using various solvents (refer to Table 2). The study's findings indicated that the ethanol extract of *A. calamus* rhizome demonstrated the highest antioxidant efficacy against both DPPH (89.53%) and ABTS radicals (87.71%), FRAP (88.24%) and Nitric oxide (88.53%). Subsequent solvents ranked in effectiveness as follows: Methanol (61.45%) in FRAP, Chloroform (84.73%) in Nitric oxide assay and Aqueous (79.41%) in FRAP. The polarity of the solvent plays a critical role in the presence of secondary metabolites and their antioxidant potential [10]. The range of radical scavenging ability observed varied from 36.22% to 89.53%.

Table 2: Free radical scavenging activity of *A. calamus* rhizome

Solvent	Concentration	Scavenging ability (%)			
		DPPH	ABTS	FRAP	Nitric oxide
Methanol	25	39.56	41.22	36.22	41.56
	50	45.09	44.21	49.32	46.09
	100	50.80	52.20	61.45	51.80
Ethanol	25	79.87	75.33	63.05	78.87
	50	82.22	82.07	75.37	83.22
	100	89.53	87.71	88.24	88.53
Chloroform	25	75.23	76.88	72.33	76.23
	50	80.66	79.63	77.88	81.66
	100	83.73	81.55	81.38	84.73
Aqueous	25	61.21	63.22	66.88	63.21
	50	63.35	66.33	68.29	66.35
	100	67.20	69.30	79.41	69.20
Standard	25	69.79	74.04	69.44	71.44
	50	77.43	80.09	73.21	75.21
	100	90.89	86.47	88.37	81.37

In the DPPH assay, the antioxidant demonstrated the ability to reduce and neutralize the violet-colored DPPH radical, transforming it into the stable yellow-colored compound 1,1-diphenyl-1,2-picryl hydrazine [1]. The antioxidant within *A. calamus* rhizome donates an H-atom to the DPPH radical, converting it to DPPH-H. Consequently, this reaction is characterized by a reduction in absorbance as DPPH loses its reactivity [11]. Similar interactions occur in the ABTS assay, where antioxidants interact with the generated ABTS radical, causing a decolorization of its blue hue. The FRAP assay is generally linked to the presence of reductones, known to exert antioxidant action by donating a hydrogen atom and breaking the free radical chain[12]. Coming to Nitric oxide, it is a freely diffusible gaseous free radical and its unregulated production results in nitrosative stress, leading to damages of proteins/DNA, cell injury and death [13, 14].

Bioactive compounds such as natural phenols and flavonoids play a crucial role in quenching reactive oxygen species, offering defense against oxidative stress, and inhibiting lipid peroxidation [15]. In this study, phyto compounds like flavonoids, phenolic acids, and phenolic diterpenes in the *A. calamus* rhizome extract naturally heightened radical scavenging activities. These phenolic components, rich in hydroxyl groups, including the o-dihydroxy group, exhibit potent radical scavenging effects and antioxidant power. Hydroxyl groups contribute significantly to hydrogen bond donation, aiding in the scavenging of free radicals, reduction of metal ions, and interaction with biomolecules [16]. A prior study on the ethanolic extract of *A. calamus* reported total phenolic contents of 2398.40 mg GAE/100g and 190.46 mg QE/100g dry rhizome extract [17]. The methanolic extract of the rhizome exhibited higher hydroxyl radical scavenging activity (86.83%), superoxide radical activity (44.16%), and reducing power activity. The aqueous extract demonstrated better scavenging of DPPH free radicals (34.51%), lipid peroxidation (49.91%), and metal ions chelating assays (39.85%) [18].

Elevated levels of nitric oxide are directly toxic to tissues, resulting in vascular damage and other ailments [19]. This toxicity is exacerbated upon reaction with the superoxide radical, forming the second reactive compound, peroxynitrite anion ($ONOO^-$)[20]. *A. calamus* inhibits nitrite formation during the radical (N) generation process by engaging in direct competition with oxygen. Moreover, the plant extract scavenges peroxynitrite, inhibiting the formation of the dangerous and highly reactive compound peroxynitrous acid (ONOOH) [21]. *A. calamus* exhibited activity comparable to the standard in these processes.

Conclusion

The present study reveals that the *A. calamus* rhizome harbours specific active phytochemical constituents that enhance antioxidant levels. The investigations, including the free radical scavenging assay and quantitative estimation of antioxidants, consistently showed a notable percentage of scavenging capacity attributed to potent antioxidants. This experimental evidence suggests the potential use of the plant in addressing human pathologies where free radicals play a crucial role. Nevertheless, further research is necessary to isolate and characterize the antioxidant constituents for a comprehensive understanding.

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