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A comparative study of the *in vitro* antioxidant property of different extracts of *Acorus calamus* Linn

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ABSTRACT

Acorus calamus Linn. (Acoraceae) is a medicinal plant widely distributed in India and other eastern countries. The root, rhizome extracts and aromatic oil from the plant has been previously screened for its antioxidant potential. But whole plant extract is yet to be screened for its possible bioactivity and antioxidant property to the best of our knowledge. Therefore, screening of antioxidant property of whole plant extract of A. calamus was chosen for the study. The antioxidant property of ethanol, hydro-ethanol and aqueous extracts was determined by phytochemical screening, estimation of total phenolics and flavonoids and in vitro antioxidant screening models viz. DPPH scavenging activity, nitric oxide scavenging activity, hydroxyl radical scavenging activity, superoxide radical scavenging activity, reductive ability and ferric reducing antioxidant power (FRAP) along with HPTLC analyses. The antioxidant activity of the extracts was compared to standard ascorbic acid to assess its free radical scavenging potential. The ethanol extract showed marked quantity of phenolics and flavonoids and promising antioxidant activity followed by hydro-ethanol and aqueous extracts. IC_{50} values for the scavenging activities of DPPH, nitric oxide, hydroxyl radical, superoxide radical exhibited by the ethanol extract were 54.82µg/mL, 118.802 µg/mL, 109.85 µg/mL, 38.3 µg/mL respectively. The reductive ability and FRAP values of the extracts were found to increase in a concentration dependent manner. The ethanol extract of A. calamus showed promising in vitro antioxidant activity indicating its potency for therapeutic applications.

Key words: *Acorus calamus* Linn., antioxidant activity, HPTLC, DPPH, FRAP.

INTRODUCTION

Free radicals are toxic by-products formed naturally as a result of aerobic metabolism in our body. They are any species capable of independent existence and contain one or more unpaired electrons which react with other molecules by oxidation or reduction reaction [1]. Free radicals include reactive oxygen species (ROS) and reactive nitrogen species (RNS) [2]. Generation of oxidants due to endogenous factors (metabolism, infections, exercise, ageing) or exogenous factors (exposure to radiations, metal catalysed reactions and oxygen free radicals as pollutants in the atmosphere) that are beyond the antioxidant capacity of a biological system gives rise to oxidative stress [3]. The generation of oxidative stress is harmful to the body and may cause peroxidation of membrane lipids

leading to loss of membrane integrity and cell death, denaturation of proteins including enzymes, ion channels and strand breakage in DNA [4]. Therefore, they can be related to certain pathophysiological conditions such as arthritis, haemorrhagic shock, coronary artery diseases, cataract, cancer, AIDS as well as age related degenerative brain disorders [5]. Antioxidants are compounds that can decrease oxidative stress and minimize the incidence of pathological conditions caused by the oxidants. The mechanism of action of these antioxidant compounds includes inhibition of the enzymes or chelation of trace elements involved in free radical production, scavenging of reactive species and up-regulation or protection of antioxidant defense [6]. Antioxidants are produced naturally in the human body and there is symmetry between the oxidants generated and the antioxidants present. However, due to oxidant overproduction, inadequate supply of nutritional supplements and/or derisory antioxidant argument this equilibrium is hindered resulting in oxidative hassle [7, 8]. Therefore, a dietary source rich in antioxidant is recommended. Antioxidants taken as supplements are particularly important in reducing the cumulative oxidative damages. Among currently available drugs, synthetic antioxidants do have potential side effects and carcinogenicity which can be minimized to a great extent through the administration of natural compounds. Therefore, interest in medicinal plants and their therapeutic potential as antioxidants is fast increasing. Still there remain a large number of natural drugs which are yet to be explored scientifically [9, 10].

Acorus calamus Linn. (fam: Acoraceae) commonly known as Sweet flag, Sweet Sedge, Myrtle Flag is a semi-aquatic, perennial, aromatic herb with creeping rhizomes originating in Asia but now widely distributed in Europe, North America and Africa. It is also found indigenously in the marshy tracts of Kashmir, Shirmaur (Himachal Pradesh), Manipur and in Naga Hills of India. The rhizome, root and leaf yield a light brown to brownish yellow volatile aromatic oil known as calamus oil. The alcoholic extract and the essential oil have been reported to possess *in vitro* antimicrobial properties and antiulcer activity [11]. The alcoholic extract of the plant is also reported to exhibit antiviral properties [12], insecticidal properties [13]. The rhizome is reported to be used as an aromatic stimulant, an expectorant, a carminative, an anti-spasmodic, an emetic, a laxative and a diuretic. Reportedly, an infusion of the rhizome is successfully used in the treatment of dysentery, dyspepsia, intestinal worms, cough and fever [14]. Furthermore, the rhizome infusion is also used as a central nervous system relaxant, a stomachic [15], an appetite stimulant, an anthelmintic, a vermifuge, an antibacterial agent, a sedative, an analgesic [16] and a contraceptive [17]. Asarone has been reported to be the major bioactive constituent of the volatile calamus oil. Isomeric forms of asarone commonly found are α,β,ɣ of which α and β asarone are mostly responsible for the bioactivity of *Acorus calamus*. The concentration of asarone in calamus oil depends on the part of the plant used for the extraction of the oil and the ploidy [18]. A-asarone has been reported to show anticarcinogenic properties [19]. Traditional uses of this plant in NE India include use of fresh rhizome of the plant against cold particularly in infants and also as a strong insect repellant. Rhizome paste of the plant is applied on the body of the harvester of honey-sacs to get rid of honey-bees. Protective effects of *Acorus calamus* on free radical scavengers and lipid peroxidation in discrete regions of brain against noise stress exposed rat [20] is reported. Pharmacological profile of *Acorus calamus* [21] was also studied. Its blood pressure-lowering and vascular modulator effects of is mediated through multiple pathways [22]. The antispasmodic effect of *Acorus calamus* is mediated through calcium channel blockade [23]. There are reports on the ethanol extract of *Acorus calamus* rhizomes on central nervous system [24]. *In vitro* free radical scavenging activity of root and rhizome extracts of *Acorus calamus* was reported In few instances, cpSSR was successfully explored to know the diversity of *Acorus calamus* collected from Northern India [25]. The present study was conducted with an aim to investigate the *in vitro* antioxidant potential of the whole plant extract of *Acorus calamus* along with phytochemical screening. The study was conducted with three different solvents, viz. ethanol, hydro-ethanol and aqueous. Although, the antioxidant potential of root and rhizome extracts of *Acorus calamus* has been reported earlier, *in vitro* antioxidant analysis for the whole plant extract of *Acorus calamus* has not yet been reported in literature. Use of the whole plant as antioxidant can be instrumental in saving the plant from extinction by preservation of the root and rhizome of the plant for further regeneration.

MATERIALS AND METHODS

Preparation of extracts

The plants were authenticated at the herbarium of Department of Agronomy, Assam Agricultural University by Taxonomist Dr. I. C. Barua. For preparation of the extracts, about 500gm of the powdered sample was dipped in sufficient amount of ethanol (100% for ethanolic, 80% for hydro-ethanolic and distilled water for aqueous extracts) for several days in an air tight flask. Subsequently, it was filtered and the filtrate was centrifuged. The supernatant was then transferred to a rotary evaporator (Rotavapor R-210, Buchi) for removal of the solvent. Finally, the liquid

was transferred to a lyophilizer (Heto PowerDry LL3000, Thermo Electron Corporation) at -80^{O}C. The extract was then dried in a hot air oven for several days at moderate temperature until completely dry. Yield % was found to be 5.02, 2.55, 2.54 for ethanol, hydro-ethanol and aqueous extracts respectively.

Chemicals and Reagents

Sulphuric acid, chloroform, methanol, hydrogen peroxide(H_2O_2), O-phosphoric acid, sodium nitrate ($NaNO_2$) was purchased from Merck India. Deoxyribose, thiobarbituric acid (TBA), trichloroacetic acid (TCA), ethylenediaminetetraacetic acid (EDTA), 2-diphenyl-1-picryl-hydrazyl (DPPH), sodium nitroprusside, gallic acid, quercetin, nicotinamide adenine dinucleotide (NADH) were obtained from Sigma-Aldrich USA. ferric chloride, sodium hydroxide, copper acetate, ascorbic acid, tripyridyl-s-triazine (TPTZ), nitroblue tetrazolium (NBT), phenazine methosulphate (PMS), potassium ferricyanide (K_4FeCN_6), potassium persulphate, sodium nitroprusside (SNP) were purchased from HIMEDIA. All other reagents were of analytical grade. Solvents for HPTLC analysis were obtained from Merck (HPLC grade).

Phytochemical Analysis

The extracts were subjected to preliminary phytochemical screening to detect the presence of different chemical groups of compounds such as steroids, phenolics, tannins, flavonoids, glycosides, diterpenes, triterpenes and alkaloids [26, 27, 28, 29].

Determination of total *in vitro* antioxidant activity

Determination of total phenolics

Total phenolic content of the extracts were determined using Folin-Ciocalteau method with some modifications [30]. Aliquots of the extracts were mixed with 9 mL of distilled water taken in 25 mL volumetric flasks. To that 1 mL of Folin-Ciocalteau reagent was added and mixed. 10 mL of 7% Na_2CO_3 was added after an incubation period of 5 min. The solution was then diluted to 25 mL and allowed to stand for 90 min at room temperature. Absorbance was measured at 750 nm using UV-Vis Spectrophotometer (Multiskan Thermo Fischer Scientific Model 1119300). Total phenolic content was expressed as mg/g gallic acid equivalent.

Determination of total flavonoids

Total flavonoid content was measured using the aluminum chloride method with some modifications [31]. Aliquots of the extracts were added to volumetric flasks containing 4 mL of distilled water. Then 0.3 mL $NaNO_2$ (5%) was added. After 5 min, 0.3 mL of 10% $AlCl_3$ and 2 mL of 1M NaOH were added and the volume was made upto 10 mL. The absorbance was measured at 510 nm. A standard calibration plot was generated using known concentrations of quercetin. The concentration of total flavonoid content was calculated as quercetin mg/g.

DPPH Radical Scavenging Method

In order to evaluate the free radical scavenging activity of the test samples, the change in optical density by DPPH radical was assessed [32]. The sample extracts were diluted with methanol to give different concentrations of the plant extracts (3, 9, 20, 40, 60, 80, 100, 110 µg/mL). Then 0.2 mL of DPPH was added to 2.8 mL of the extracts at various concentrations and incubated at 37ºC for 30 min. Absorbance was measured at 517 nm. Ascorbic acid was used as reference standard. Percentage inhibition was calculated as:

$$\text{DPPH Scavenged (\%)} = [(Abs_{control} - Abs_{test}) / Abs_{control}] \times 100$$

Where $Abs_{control}$ is the absorbance of the control reaction and Abs_{test} is the absorbance in the presence of the sample.

Nitric Oxide Scavenging Activity

Free radical scavenging activity by nitric oxide scavenging test using sodium nitroprusside generating NO was compared with their parent compound [33]. Sodium nitroprusside was mixed with 1 mL plant extract of different concentrations (3, 9, 20, 40, 60, 80, 100, 110 µg/mL) prepared in phosphate buffer. The mixture was subsequently incubated at 25ºC for 150 min. This was followed by addition of 1 mL of Griess reagent to the incubated mixture. Absorbance was measured at 546 nm. Ascorbic acid was used as reference standard. Percentage inhibition was calculated.

Hydroxyl radical scavenging activity

The scavenging activity for hydroxyl radical was measured by studying the competition between deoxyribose and the test extracts for the hydroxyl radical generated by Fenton's reaction [8]. The reaction mixture contained 0.2 ml plant extract of different concentrations (3, 9, 20, 40, 60, 80, 100, 110 µg/mL), 0.2 mL of EDTA (1.04 mmol/L), 0.2 mL of $FeCl_3$ (0.2 mmol), 0.2 mL of 2-deoxyribose (2.8 mmol), 0.2 mL of ascorbic acid (1 mmol) and 0.2 mL of H_2O_2 (10 mmol). After incubation at 37 °C for 1 h, 1 mL of cold TBA (2.8%) was added to the reaction mixture followed by 1 mL TCA (1%). The mixture was heated at 100 °C for 15 min and then cooled. Absorbance was measured at 512 nm.

Superoxide radical scavenging activity

Superoxide anion scavenging activity was measured according to Robak & Gryglewki with some modifications [34]. The assay is based on the inhibition of the production of nitroblue tetrazolium formazon of the superoxide ion. The reaction mixture contained 1 mL NBT (156 µM), 1 mL NADH (468 mM) and 3 mL plant extracts of different concentrations (3, 9, 20, 40, 60, 80, 100, 110 µg/mL). The reaction was started by adding 0.1 mL PMS (60 mM). The mixture was incubated at 25ºC for 5 min and absorbance measured at 560 nm.

Reducing power assay

Reducing power of plant extract is based on the ability of antioxidants to form coloured complex with potassium ferricyanide, TCA and $FeCl_3$ [35]. In this test, 1 mL of different concentration of the plant extracts (100, 200, 300, 400, 500, 600 µg/mL) were added to 2.5 mL K_4FeCN_6 (1%) and 2.5 mL phosphate buffer (pH6.6). The mixture was then incubated at 50ºC for 20 min. The reaction was stopped by adding 2.5 mL TCA (1%) and centrifuged at 3000 rpm. 2.5 mL supernatant was added to 2.5 mL distilled water and 0.5 mL $FeCl_3$ (0.1%). Absorbance was measured at 700 nm.

Ferric reducing antioxidant power (FRAP) assay

FRAP assay was carried out according to Benzie and Strain with certain modifications [36]. FRAP solution (2.8 mL) containing 300 mM acetate buffer, 10 mM TPTZ solution and 20 mM $FeCl_3$ solution were added to 0.15 mL of different concentrations (3, 9, 20, 40, 60, 80, 100, 110 µg/mL) of the plant extracts. The mixture was incubated at 37ºC for 30 min in the dark. Absorbance of the colored product was measured at 593 nm. The results were expressed in µM $FeSO_4.7H_2O$ equivalent from a standard calibration curve.

Estimation of minimal inhibitory concentration (IC_{50}) value

IC_{50} represents the amount of sample (µg extract/ml) necessary to scavenge free radicals by 50%. The IC_{50} values were calculated by extrapolation from linear regression analysis.

Analysis of extracts by HPTLC

The primary phytochemical analysis touted the presence of flavonoid and polyphenols in the ethanolic, hydro-ethanolic and aqueous extract of the plant. Hence, all the above stated fractions were subjected to HPTLC study to detect and quantify the presence of the gallic acid and quercetin with the aid of CAMAG HPTLC System. Plant samples were dissolved in methanol to prepare a concentration of 10 mg/ml. Standards were prepared in the same solvent as 0.5mg/ml. 3 µl of each samples and standards were spotted on the 10×10 Silica Gel F_{60} using CAMAG LINOMAT 5 applicator. Mobile phase was applied as methanol:hydro-ethanol:formic acid (2:6:0.2) and spotted plate was subjected to develop in the CAMAG ADC2 Automated Developing Chamber. After drying the plate, it was scanned in CAMAG TLC Scanner wielding long UV wavelength, 366 nm.

Statistical Analysis

Triplicate analyses were performed on all the samples. All numeric data were expressed as mean ± S.E.M. The graphs were prepared using Graph Pad Prism 6 (version 6.03). The effects of ethanolic, hydro-ethanolic and aqueous extracts of *A. calamus*, both treatment and concentrations wise, on the activities exhibited were analyzed by two-way analysis of variance (ANOVA) followed by LSD using PASW 18.0 statistics (SPSS Inc.). $p<0.05$ was considered to be statistically significant.

RESULTS

Phytochemical screening

Table I shows the phytochemical screening of the ethanolic, hydro-ethanolic and aqueous extracts of *A. calamus*.

Pharmacologically active compounds such as steroids, phenolics, tannins, flavonoids, glycosides, diterpenes, triterpenes and alkaloids were found to be present in the ethanolic, hydro-ethanolic and aqueous extracts of *A. calamus*.

Table I. Screening of phytochemicals present in ethanol, hydro-ethanol and aqueous extracts of *A. calamus*

Tests	Ethanol	Hydro-ethanol	Aqueous
Steroids	+++*	++[§]	+
Phenolics	+++	+++	+
Tannins	+[#]	+	+
Flavonoids	+++	++	+
Glycosides	+++	+++	+
Diterpenes	+++	++	+
Triterpenes	+++	++	+
Alkaloids	+	+	+

Note: sufficiently present (*), moderately present ([§]), present in trace amount ([#]).

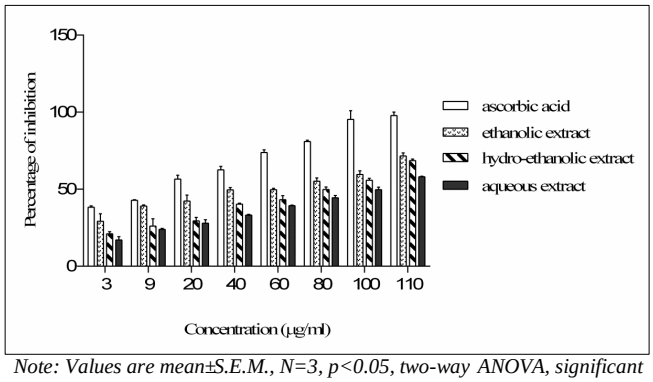
Total phenol and flavonoid content

Total phenolic content was found to be 29.3, 27.5, 10.02 in ethanolic, hydro-ethanolic and aqueous extracts, respectively expressed as gallic acid/g dry weight. Total flavonoid content was 18 mg for ethanolic extract, 13 mg for hydro-ethanolic extract and 8 mg for aqueous extract, all of which were expressed in terms of quercetin/ g dry weight.

DPPH radical scavenging activity

The radical scavenging activity of *A. calamus* and standard based on DPPH assay is depicted in Fig. I. Ethanol, hydro-ethanol and aqueous extracts showed IC_{50} values of 54.82, 74.248 and 93.066 µg/mL. IC_{50} value of ascorbic acid was found to be 18.36 µg/mL. As lower IC_{50} values indicate higher scavenging activity, thus, it was seen that the ethanol extract exhibited higher scavenging potential followed by the hydro-ethanol extract and the aqueous extract. However, ascorbic acid displays significant (p<0.05) scavenging activity over the ethanol extract.

Fig. I. DPPH radical scavenging activity of ethanol, hydro-ethanol and aqueous extracts of *A. calamus*

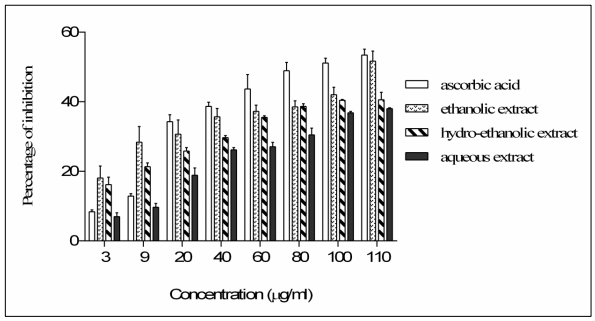


Note: Values are mean±S.E.M., N=3, p<0.05, two-way ANOVA, significant

Nitric oxide scavenging activity

Fig. II. illustrates the nitric oxide scavenging potential of *A. calamus* and ascorbic acid The IC_{50} values were found to be 118.802, 140.35, 148.11 µg/mL for ethanolic, hydro-ethanolic and aqueous extracts respectively. The IC_{50} value of ascorbic acid was found to be 88.54 µg/mL.

Fig. II. Nitric oxide scavenging activity of ethanol, hydro-ethanol and aqueous extracts of *A. calamus*

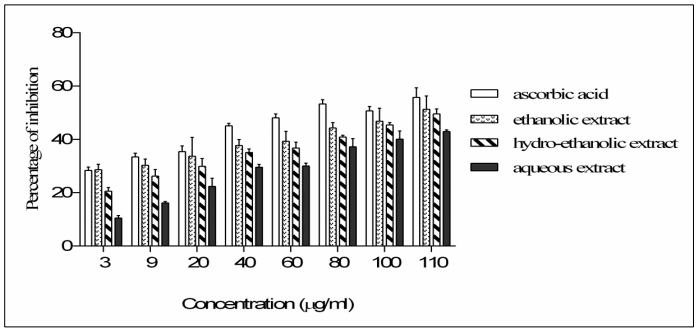


Note: Values are mean±S.E.M., N=3, p<0.05, two-way ANOVA, significant

Hydroxyl radical scavenging activity

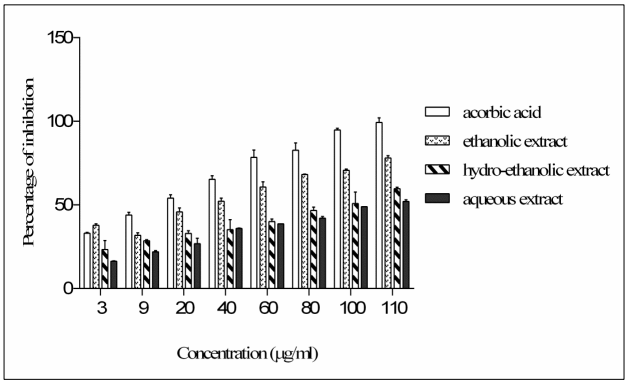
The antioxidant activity of *A. calamus* based on hydroxyl radical scavenging activity is illustrated in Fig. III. Ethanol extract exhibited the lowest IC_{50} value (109.45 µg/mL) indicating the highest scavenging activity of hydroxyl radical followed by the hydro-ethanol extract (114.89 µg/mL) and the aqueous extract (131.229 µg/mL). However, IC_{50} of ascorbic acid was found to be lower than the ethanol extract at 79.49 µg/mL.

Fig. III. Hydroxyl radical scavenging activity of ethanol, hydro-ethanol and aqueous extracts of *A. calamus*



Note: Values are mean±S.E.M., N=3, p<0.05, two-way ANOVA, significant

Fig. IV. Superoxide radical scavenging activity of ethanol, hydro-ethanol and aqueous extracts of *A. calamus*



Note: Values are mean±S.E.M., N=3, p<0.05, two-way ANOVA, significant

Superoxide radical scavenging activity

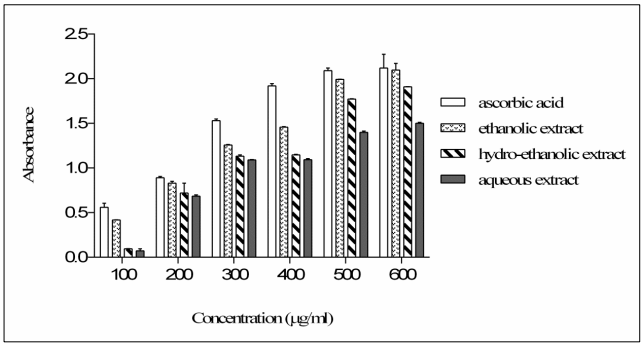
Fig. IV summarizes the superoxide radical scavenging activity of the extracts compared to ascorbic acid as standard. The extracts showed potent superoxide scavenging activity in a concentration dependent manner when compared with ascorbic acid at similar concentrations. The ethanol extract exhibited a low IC_{50} value at 38.3 µg/mL. Hydro-

ethanol and aqueous extracts showed an IC_{50} value of 88.575 and 101.136 µg/mL respectively. Ascorbic acid showed an IC_{50} value of 19.58 µg/mL.

Reductive ability

The reductive ability of the extracts was found to increase with the increasing concentrations as depicted in Fig. V. The absorbance for reducing capacity of *A. calamus* at the concentration of 600 µg/mL was found to be 2.096, 1.91, 1.50, 2.12 for ethanol, hydro-ethanol, aqueous extracts and ascorbic acid respectively.

Fig. V. Reductive ability of ethanol, hydro-ethanol and aqueous extracts of *A. calamus*

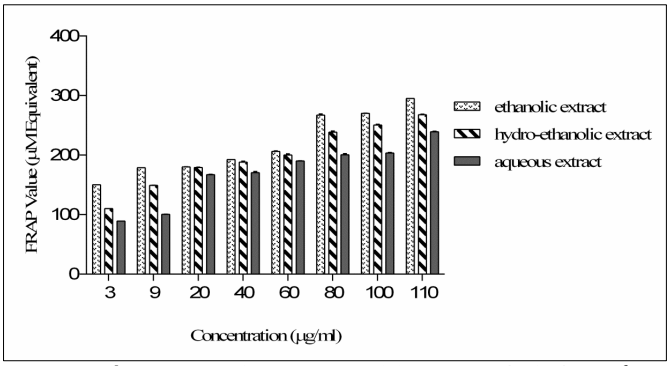


Note: Values are mean±S.E.M., N=3, p<0.05, two-way ANOVA, significant

FRAP assay

Fig. VI. illustrates the ferric reducing antioxidant power of the extracts. Ethanol extract showed the highest activity with 295 µM FRAP equivalents followed by the hydro-ethanol and aqueous extracts (267 µM and 239 µM FRAP equivalents respectively) at 110 µg/mL concentration.

Fig. VI. FRAP assay of ethanol, hydro-ethanol and aqueous extracts of *A. calamus*



Note: Values are mean±S.E.M., N=3, p<0.05, two-way ANOVA, significant

Table II. IC_{50} values of the ethanol, hydro-ethanol, aqueous extracts of *A. calamus* with ascorbic acid as standard

Model	Ethanol extract (µg/mL)	Hydro-ethanol extract (µg/mL)	Aqueous extract (µg/mL)	Ascorbic acid (µg/mL)
DPPH radical scavenging activity	54.82	74.24	93.066	18.36
Nitric oxide scavenging activity	118.802	140.35	148.11	88.54
Hydroxyl radical scavenging activity	109.45	114.89	131.229	79.49
Superoxide radical scavenging activity	38.3	88.57	101.136	19.58

N=3, p<0.05, two-way ANOVA, significant

HPTLC analyses

HPTLC analyses of different extracts of the plant, *A .calamus* showed the profiles of gallic acid and quercetin. The

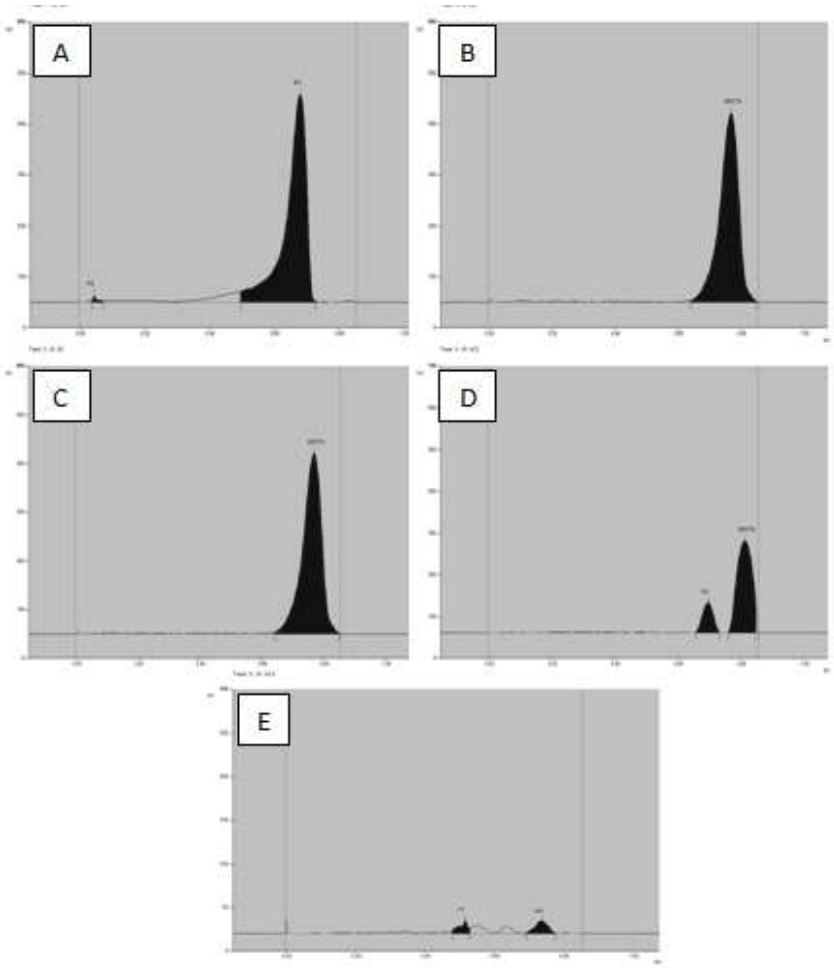
ethanol extract exhibited the presence of both gallic acid and quercetin with R_f values and areas of 0.72, 0.85 and 256.5 AU, 10240.4 AU respectively. The Rf values and areas of the gallic acid and quercetin were found to be 0.73, 0.85 and 1906.7 AU, 8224.6 respectively in the hydro-ethanolic extract of the plant. The aqueous extract showed the presence of only gallic acid with R_f value and area of 0.78 and 426.7 AU. The R_f values and areas of the standard gallic acid and quercetin were 0.73, 0.85 and 19146.4 AU, 17293.7 AU respectively.

Table III. HPTLC profile of standards and ethanol, hydro-ethanol and aqueous extracts of *A. calamus*

	Rate of flow (R_f value)		Area	
	Gallic acid	**Quercetin**	**Gallic acid**	**Quercetin**
Standard	0.73	0.85	19146.4AU	17293.7AU
Ethanolic extract	0.72	0.85	256.5AU	10240.4AU
Hydro-ethanolic extract	0.73	0.85	1906.7AU	8226.4AU
Aqueous extract	0.78	-	426.7AU	-

Fig. VII. Chromatogram of standards and *A.calamus*

a. Chromatogram of gallic acid at 366 nm
b. Chromatogram of quercetin at 366 nm
c. Chromatogram of ethanol extract of A. calamus at 366 nm
d. Chromatogram of hydro-ethanol of A. calamus at 366 nm
e. Chromatogram of aqueous extract of A. calamus at 366 nm



DISCUSSION

There has been an increasing interest in the role of plants as therapeutic agents as they are easily available and are devoid of harmful side-effects as opposed to their synthetic counterparts. The medicinal properties of plants can be attributed to the presence of secondary metabolites. Polyphenols are an important group of secondary metabolites which have a number of beneficial effects including their role as antioxidants. Flavonoids are a group of polyphenolic compound with known free radical scavenging activity [37]. A positive correlation between total phenolic content and free radical scavenging activity has been reported earlier [38]**.** Thus, it can be inferred that the polyphenolics present in *A. calamus* contribute to the antioxidant property. Ethanol extract of *A. calamus* showed high value of total phenolics and flavonoids indicating good antioxidant activity which was coherent in the subsequent assays.

DPPH is a stable free radical and accepts electron or hydrogen radical to become a stable diamagnetic molecule. Freshly prepared DPPH solution exhibits purple coloration. When an antioxidant is present in the medium, it donates an electron or hydrogen atom to the radical resulting in scavenging of the radical by hydrogen atom rendering the formation of a colourless complex [39]. The degree of discolouration is measured to evaluate the antioxidant activity. The effect has been found to be dose dependant as higher concentration of the extracts showed higher percentage inhibition of free radical. As lower IC_{50} value indicates higher scavenging activity, it was seen that the ethanol extract exhibited higher scavenging potential followed by the hydro-ethanol extract and the aqueous extract. Nevertheless, ascorbic acid displays significant scavenging activity over ethanol extract of *A. calamus*.

Nitric oxide is spontaneously generated from sodium nitroprusside in aqueous solutions which reacts with oxygen (O) to produce nitrite (NO) which can be estimated by Griess reagent. Scavengers of NO compete with O leading to a decreased production of NO [40]. It was seen that the generation of NO was suppressed in a dose dependent manner by the extracts. The ethanol extract of *A. calamus* showed the highest activity and had an IC_{50} value almost comparable to the reference standard indicating a good nitric oxide scavenging potential.

Hydroxyl radical is an extremely reactive and highly damaging free radical species which has the capacity to join nucleotides and cause strand breakage in DNA leading to possible carcinogenesis, cytotoxicity and mutagenesis [41]. Ferric-Ascorbate-H_2O_2-EDTA according to Fenton reaction forms hydroxyl radical which on generation reacts with deoxyribose to form thiobarbituric acid reactive substance (TBARS). On heating with TBA a pink chromogen is formed. Free radical scavengers compete with deoxyribose for hydroxyl radicals and thus cause a reduction in the color formation. The extracts showed inhibition of deoxyribose degradation in an increasing dose dependent manner. The ethanol extract showed better scavenging activity with the lowest IC_{50} value.

Superoxide anion is an initial free radical and a weak oxidant which ultimately produces stronger oxidative species such as singlet oxygen species and hydroxyl radicals [42]. The extracts showed a potent superoxide scavenging activity in a concentration dependent manner when compared with ascorbic acid standard at similar concentrations. Ethanol extract exhibited the lowest IC_{50} value and hence showed the best superoxide radical scavenging activity.

The extracts of *A. calamus* showed reductive ability by reducing Fe^{3+}ferricyanide complex to Fe^{2+} [43] in a dose dependent manner but reductive ability of the extracts increased with increasing concentration. Assessment of the reductive ability serves as an important indicator of the antioxidant potential. The reductive ability of the extracts might be due to the presence of reductones. Although, the three extracts showed potent reductive ability the ethanol extract showed the highest reductive property.

FRAP assay measures the ability of the plant extracts to reduce ferric to ferrous at low pH causing the formation of ferrous-tripyridyltriazine complex. It is a simple and effective assay for assessment of antioxidant reducing potential. The antioxidant ability of the plant extracts were presented in terms of mM Fe^{2+}. The ethanol extract exhibited better ferric reducing property than the other two extracts under study.

HPTLC analyses of the extracts showed the presence of quercetin and gallic acid in both ethanol and hydro-ethanol extracts. Quercetin was found to be present in a greater concentration in the ethanol extract. On the contrary, gallic acid was present in a lesser concentration in the ethanol extract as compared to the hydro-ethanol extract. Quercetin was absent in the aqueous extract but showed the presence gallic though in a lesser concentration as compared to the ethanol and hydro-ethanol extract. The better antioxidant potential exhibited by the ethanol extract in comparison to

the hydro-ethanol and aqueous extracts can be possibly attributed to the high polyphenolic content in the extract. But gallic acid, a polyphenol was detected in a higher concentration in the hydro-ethanolic extract than in the ethanolic extract. Therefore, the superior activity shown by the ethanol extract could be due to the presence of other phenolic compounds which were not assessed and also due to a greater amount of quercetin detected compared to the hydro-ethanol and aqueous extracts.

According to previous findings, ethanolic and hydro ethanolic extracts of both rhizome and root exhibited a good DPPH scavenging activity, though the ethanol extract showed maximum antioxidant activity (59.13±18.95 % inhibition) as compared to the hydroethanolic extract (56.91±19.14 % inhibition) [25]. Our study corroborates with this finding that the ethanol extract of the whole plant exhibited maximum antioxidant activity. It was reported that the methanol extracts of leaves of *Acorus calamus* exhibited striking DPPH scavenging activity (at 20 µg/mL), ferric ion chelating potential (at 18.8 µg/mL) and reductive ability (concentration dependant) whereas, methanol extract of rhizome exhibited better superoxide radical scavenging potential (at 30.5 µg/mL) [44]. We have observed that all the three extracts under study exhibited concentration dependent free radical scavenging activity. It was also reported that at 100 µg/mL concentration methanol extract of *Acorus calamus* rhizome exhibited low DPPH radical scavenging activity [45] whereas in our study all the extracts exhibited considerable DPPH scavenging activity.

CONCLUSION

The objective of the study was to determine the antioxidant and free radical scavenging potential of the whole plant, *Acorus calamus* and also to provide a comparative analysis between the ethanol, hydro-ethanol and aqueous extracts of the plant as a free radical scavenger to specify the extract with a better scavenging potential. According to the findings, the ethanol extract showed the highest amount of phenolics, flavonoids and free radical scavenging activity as compared to the hydro-ethanol and the aqueous extracts. Studies on the whole plant also revealed that antioxidant property of the plant is in no way lesser than its root, rhizomes or leaves. Since use of roots or rhizomes results in uprooting of the whole plant from *in situ*, hence their use can be replaced by the whole plant itself without uprooting. As we know that free radicals are important contributors to several severe pathological conditions, the findings suggest that the extracts of the whole plant is equally useful as a source of natural antioxidants with subsequent health benefits. Our study on its anti oxidant profile shows that instead of using its root and rhizomes, whole plant can be a better substitute and further investigation of its medicinal properties using whole plant can be undertaken. Moreover most of its medicinal properties as mentioned above have been reported for rhizomes, whereas we have found promising anti ulcer and anti arthritic activity in the whole plant in an ongoing study, which is a direct evidence of our claim.

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