Phytochemical Screening and *In-vitro* Antioxidant Profiling of Solvent Fractions of *Canna edulis* Ker Gawler

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ABSTRACT

Introduction: *Canna edulis* Ker Gawler, grown in the sub Himalayan region containing starchy rhizome are used by the tribal people as food and herbal medicine. **Methods:** Investigations were done to find out 2,2-diphenyl-1-picryl hydrazyl (DPPH) radical scavenging activity, total flavonols and total proanthocyanidin contents, nitric oxide (NO) scavenging activity, hydroxyl radical scavenging activity of different solvent fractions of aquous extract of *C. edulis* rhizome. Among the cellular molecules, lipids containing unsaturated fatty acids with more than one double bond are particularly susceptible to action of free radicals. The resulting reaction, known as lipid peroxidation and anti lipid peroxidation activity has been performed using goat liver homogenate. **Results:** Six out of 29 fractions showed DPPH free radical inhibition above 75% and were used for further phytochemical screening. Diethyl ether : ethyl acetate (1:3) fraction showed the maximum inhibition percent. Highest amount of total flavonol and total proanthocyanidins were recorded in diethyl ether : ethyl acetate (1:3) fraction. Maximum NO scavenging activity and hydroxyl radical inhibition activity were observed in bioactive diethyl ether : ethyl acetate (1:1) fraction. Inhibition of lipid peroxides was maximum in ethyl acetate fraction. Data from thin layer chromatography (TLC) revealed the presence of phenolic compounds in all fractions, whereas flavonoid glycoside compounds were restricted in ethyl acetate and diethyl ether : ethyl acetate (1:3). **Conclusions:** Thus it can be concluded that the antiradical scavenging activity of *Canna* rhizome may be due to the presence of polyphenolic compouns like phenols, flavonoids, proanthocynidins etc.

Keywords: Anti-lipid peroxidation, *Canna*, column chromatography, DPPH, polar.

INTRODUCTION

Oxygen plays a vital role in the survival of life on earth. However, during it's utilization in various life processes, a tiny amount of oxygen gets reduced to different free radicals like hydrogen peroxide, superoxide, nitric oxide, hydroxyl radicals etc.[1,2] All these reactive oxygen radicals are accountable for various health hazards to human life causing the development of degenerative diseases.[3] Antioxidant molecules, on the other hand, can slow down or cease the activity of these harmful oxygen species by being oxidizing themselves. Plants often contain good amount of natural antioxidants.[4,5] Different phytochemicals like phenols, flavonols, carotenoids, proanthocyanidins, vitamin C and E can be utilized to scavenge the excess free radicals from human body.[6]

Canna edulis Ker Gawler, is a medium sized perennial flowering plant found in the north eastern Himalayan regions of India.[7] The plants bear broad, flat, blackish green leaves with spectacular red flowers. Besides, it also tickles our test buds as it is known to be edible and mainly consumed by the Indian tribes like Lepchas, Bhutias and Nepalis.[8] Different parts of the plants like rhizomes, leaves and seeds are used as medicines by the tribal communities of North eastern India.[9] Amongst various plant parts the extracts of rhizomes are rich source of polyphenolic compounds like phenols and flavonoids,[10] which in turn provide the evidences of antioxidative properties of the plant.

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DOI: 10.5530/ax.2012.2.5

The antioxidants like polyphenol and flavonoid contents of methanolic seed extracts of *Canna indica* were investigated to find out their inhibitory effects on oxidation of phosphatidylcholine (PC) liposomes and their aggregation,[11] where as comparative analysis of total phenols, flavonoids and antioxidant activity by 2,2-diphenyl-1-picryl hydrazyl (DPPH) and trolox equivalent antioxidant capacity (TEAC) assay of methanolic extracts of red and yellow varieties of *Canna indica* was done to find out their role as potential source of natural antioxidants.[12] Further the *in vitro* free radical scavenging activities of methanolic extract of aerial parts of *Canna indica* was investigated[13] to indicate them as powerful antioxidants. Correlation of *in vitro* antioxidant properties and radical scavenging activity was analysed between the cold and hot aquous extracts of *C. edulis* rhizome.[10] However, no information on the separation of phytochemicals in different solvent fractions i.e from less polar solvent (hexane) to highly polar solvent (water) have been done. Taking this into consideration, in the present study, total flavonol, total proanthocyanidins, nitric oxide scavenging activity, hydroxyl radical scavenging activity and anti lipid peroxidation activity of different fractions of rhizome extract of *Canna edulis* Ker Gawler found in the sub Himalayan West Bengal and Darjeeling hills have been taken up. Detection of compound of bioactive fractions through thin layer chromatography (TLC) has also been performed.

MATERIALS AND METHODS

Chemicals Used

DPPH (2,2-diphenyl-1-picryl hydrazyl), ferric chloride, vanillin, sulphanilamide and naphthylethylene diamine dihydrochloride were procured from HiMedia Laboratories Pvt. Ltd, Mumbai, India. Ethylenediamine tetra acetic acid (EDTA), methanol, potassium dihydrogen phosphate (KH_2PO_4), di-hydrogen potassium phosphate (K_2HPO_4), potassium hydroxide, sodium hydroxide, ascorbic acid, 2-deoxy-D-ribose, trichloroacetic acid (TCA), quercetin, hydrogen peroxide, butylated hydroxytoluene (BHT), aluminium chloride ($AlCl_3$), potassium chloride, ferrous sulphate, sodium acetate, sodium nitroprusside, butanol, ethanol, ethyl acetate, ammania, iodine reagent, glacial acetic acid and hydrochloric acid (HCl) were purchased from Merck, Mumbai, India. Orthophosphoric acid, hexane, benzene, chloroform, diethyl ether, ethyl acetate, acetone etc. were procured from SD fine chem Limited, Mumbai, India. Thiobarbituric acid (TBA) was obtained from Loba Chemical, Mumbai, India. Polyethylene glycol was procured from Qualigens, Mumbai, India. Trizma base was procured from Sigma chemicals. All chemicals and solvents are of analytical grade.

Animal material

Goat liver, which was used for anti lipid peroxidation assay, was collected from slaughter house immediately after slay. Experiment was conducted within one hour after collection.

Plant material

C. edulis germplasm was collected from hilly regions of Darjeeling (Latitude 27.03° N, Longitude 88.18° E), West Bengal, India in the fall of 2008. The authentication of the plant material was done at the Taxonomy and Environmental Biology Laboratory, University of North Bengal. The material has been deposited in the "NBU Herbarium" against the accession no. 9588.

Rhizome extracts

Ten grams of rhizome of *C. edulis* were taken and washed properly and dried. These were then crushed in 40 ml of double distilled water (DDH_2O) using mechanical grinder to get the required concentration of 1:4 (biomass:H_2O, w/v). The extract was soxhleted exhaustively at boiling temperature for a period of 12 hours. The aquous extract thus obtained was evaporated at 45 °C to make a final volume of 3 ml. It was then subjected to Silica gel (SD fine chem Limited, 200-300 mesh size) column chromatography. Different solvents (base on their polarity level) like hexane, benzene, chloroform, diethyl ether, ethyl acetate, acetone, methanol and water were eluted in the column to separate different phytochemicals in order of their affinity towards these solvents. The above series of less polar to highly polar solvents were passed through the silica column in various combinations like 25, 50, 75 and 100% to get different solvent fractions. The fractions were air dried and dissolved in 5 ml of methanol and stored in amber glass bottle at 4 °C until further use.

DPPH radical scavenging activity

The free radical scavenging capacity of all the solvent fractions were determined using 2,2-diphenyl-1-picryl hydrazyl (DPPH).[14,15] DPPH solution (0.006% w/v) was prepared in 95% methanol. One thousand eight hundred µl of freshly prepared DPPH solution was added to 200 µl of the fractions (200 mg/l), to make a final volume of 2 ml. Discoloration of these extracts was measured at 517 nm after incubation for 30 min in dark at room temperature with Themo UV1 spectrophotometer. In case of –ve control same ingredients have been used except 200 µl of methanol

in place of extract. DPPH scavenging activity (%) was measured using the following formula:

$$I = \frac{A_0 - A_1}{A_0} \times 100 \quad \textbf{(1)}$$

where A_0 was the absorbance of the control and A_1 was the absorbance in the presence of the sample (different fractions of *Canna*), whereas 'I' stood for %age inhibition of DPPH. The actual decrease in absorption induced by the test compounds was compared with the positive controls.

The fraction showing maximum antiradical responses was diluted to different concentrations (100 µg to 1000 µg) and their antiradical activities were observed. IC_{50} value was calculated from graphical presentation of concentration verses radical scavenging activity.[16]

Determination of total flavonol content

Total flavonol content was determined using aluminium chloride ($AlCl_3$) method with quercetin as a standard.[17] The bioactive fractions (2 ml each) were mixed with equal volume of 2% $AlCl_3$ (ethanolic solution) followed by the addition of 3 ml of CH_3COONa (50 gm/l). The mixture was incubated at 20°C for 2.5 hours. The absorbance was measured at 440 nm. The flavonol content was calculated from a quercetin standard curve.[17]

Determination of total proanthocyanidins

Total proanthocyanidins was determined according to Sun et al., 1998,[18] using catechin as a standard. The reaction mixture contains 0.5 ml of extract, 3 ml of 4% methanolic solution of vanillin and 1.5 ml of conc. HCl and incubated for 15 min at room temperature. The absorbance was measured at 500 nm. The proanthocyanidin content was calculated from catechin standard curve.

Inhibition of nitric oxide production

Nitric oxide scavenging activity of bioactive fractions of *Canna* rhizome was determined as per standard protocol.[19,20] Briefly, sodium nitroprusside solution (1 ml of 10 mM) was mixed with 1 ml of different concentrations of bioactive fractions of *C.edulis* in PO_4 buffer (pH-7.4). The mixture was incubated at 25°C for 150 min. To 0.5 ml of incubated solution, 1 ml of Griess reagent (1 ml of 1% sulphanilamide, 0.5 ml of 2% orthophosphoric acid and 1 ml of 0.1% naphthyl ethylene diamine dihydrochloride) was added and the reaction mixture was incubated at 25°C for 30 min. The absorbance of the pink chromophore formed by the diazotization of nitrite with sulphanilamide and subsequent coupling with naphthyl ethylene diamine dihydrochloride was measured at 540 nm. Control sample was prepared containing the same volume without any extract. Nitric oxide scavenging activity (%) was calculated using formula 1, where 'I' stood for %age inhibition of NO.

Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity was measured according to Kunchandy and Rao (1990)[21] with some modifications, by studying the competition between test extract and deoxyribose for hydroxyl radical generated by Fenton's reaction. The reaction mixture containing 0.2 ml of 20 mM phosphate buffer (pH-7.4), 0.2 ml $FeCl_3$ (10 mM), 0.1 ml ascorbic acid (0.1 mM), 0.1 ml EDTA (1 mM), 0.1 ml H_2O_2 (10 mM), 0.2 ml of 2-deoxy-D-ribose (10 mM) and different concentration of bioactive fractions (1 ml each) was incubated at room temperature for 60 min. To this 1 ml each of 1% TBA and 2.8% TCA were added. The final mixture was kept in boiling water bath for 30 min to get pink chromophore. The damage imposed due to free radicals was determined colorimetrically by measuring the thiobarbituric acid reactive substances (TBARS) at 532 nm. Control sample was prepared containing the same volume without any extract. Hydroxyl radical scavenging activity (%) was measured using formula 1, where 'I' stood for %age inhibition of OH^-.

Inhibition of lipid peroxidation activity

Lipid peroxide inhibition activity was determined *in vitro* according Kumar et al., 2000.[22] Freshly collected goat liver homogenate was used as the source of polyunsaturated fatty acids for determining the extent of hepatocyte protective activity. Goat liver (5%) was homogenized with 40 mM tris HCl buffer and centrifuged at 1000Xg for 10 min to get a clear supernatant. Reaction mixture containing 0.5 ml supernatant, different concentrations of bioactive fractions (1 ml each), 100 µl of .15 M KCl, 1 ml of 15 mM $FeSO_4$, 1 ml of 6 mM ascorbic acid was incubated at 37 °C for 1 hr. TCA (1 ml; 10%) was added to the mixture and the samples were centrifuged at 4000Xg for 20 min at 4 °C to remove insoluble proteins. Supernatant was removed and 1 ml of TBA (0.8%) was added to this fraction followed by heating at 90 °C for 20 min in a water bath. A pink chromophore was produced after cooling which was extracted with organic solvent (2 ml ice cold butanol) and absorbance was measured at 532 nm. Control sample was prepared containing the same volume without any extract. Inhibition of lipid peroxidation activity (%) was calculated from formula 1, where 'I' stood for %age inhibition of lipid peroxide.

Detection of compounds through thin layer chromatography (TLC)

The bioactive fractions of *C. edulis* rhizome extract were analyzed through thin layer chromatography (TLC) using the method of Wagner and Bladt, 1996.[23] About 10 µl of extract (2 mg/ml) of all the bioactive fractions were loaded on TLC plates (Merck, India 10 × 10 cm^2). The plate was air dried and developed in Hexane: Diethyl ether (2:3) for 30 min. The plate was dried in a hot oven (at 80 ºC for 10 min) and detected under UV light (365 nm) and ammonia vapor. For spraying iodine solution, Natural Product/Polyethylene Glycol reagent (5% NP/PEG in ethanolic solution), ferric chloride (1% solution in 50% aquous methanol) were used in the experiment.

All the tests were performed in triplets.

Statistical Analysis

Results are expressed as mean ± S.E.M. of triplets. The groups were compared by two-way ANOVA using Graph Pad Prism, Version 5.0 (Graph Pad Software, San Diego, CA, USA). P-values < 0.001 were considered significant.

RESULTS

Identification of bioactive fraction

In the present study, 29 solvent fractions were generated. Out of which 6 fractions showed inhibition above 75%. Rest of the fractions showed inhibition less than 25%, except one fraction [ethyl acetate: acetone (3:1)] which showed activity little over 50% (figure 1). These six fractions are chloroform: diethyl ether (1:3) [Fr-A], diethyl ether [Fr-B], diethyl ether : ethyl acetate (3:1) [Fr-C], diethyl ether : ethyl acetate (1:1) [Fr-D], diethyl ether : ethyl acetate (1:3) [Fr-E], ethyl acetate [Fr-F] and identified as bioactive fractions of *C. edulis* rhizome. Interestingly these fractions are moderately polar and nonpolar. Six fractions which showed scavenging activity more than 75% were used for further phytochemical screening.

Fr-E [Diethyl ether: ethyl acetate (1:3)] showed the maximum inhibition percent (93.08%) for DPPH scavenging activity. The above fraction was diluted to different concentration (100 µg to1000 µg) and subjected to DPPH assay. The most bioactive fraction showed a concentration dependent DPPH antiradical activity with IC_{50} of 658 µg/ml fresh weight basis.

Extractive yield of the bioactive fractions

Yield of these bioactive fractions were in the range of 5 mg/ml to 12 mg/ml. These fractions were diluted to 1 mg/ml for further use.

Total flavonol and proanthocyanidin contents

The determination of total flavonol contents of the above six fractions showed flavonols ranging from 37.12 mg/ml to 9.92 mg/ml quercetin equivalent per 100 mg rhizome extract [figure-2(a)]. As of DPPH antiradical activity, Fr-E also showed highest flavonol contents (37.12 mg/ml quercetin equivalent per 100 mg rhizome extract) followed by Fr-F (25.12 mg/ml quercetin equivalent per 100 mg rhizome extract). We found that total flavonol was found to be decreased with decrease in concentration.

We also studied total proanthocyanidin contents of above six fractions of *Canna* rhizome extract. [figure-2(b)]. Similar to flavonol contents, Fr-E had highest total proanthocyanidins (i.e. 0.012 mg/catechin/g dry weight) among all the bioactive fractions. Total proanthocyanides content showed a dose dependent increase with increase in concentration. Fr-F and Fr-A were recorded to contain least amount of total proanthocyanides (0.0005 mg/catechin/g dry weight) at 0.25 mg/ml.

Free radical inhibition activities

In the present study, we observe the bioactive fractions of *Canna* rhizome inhibit the generation of NO radicals. Figure-3(a) indicates that *Canna* rhizome has nitric oxide scavenging activity. Maximum scavenging activity observed in bioactive Fr-D was 78.41% at a concentration of 1 mg/ml. Here we found that NO scavenging activity decreased with decrease in concentration.

Hydrogen peroxide reacts with ferrous salt to form hydroxyl radical via Fenton's reaction.[24] The hydroxyl radical (OH^-) thus produced may attack the sugar of DNA base causing sugar fragmentation, base loss and DNA strand breakage.[25] In the present study, this hydroxyl radical scavenging activity was shown by all the six bioactive factions [figure-3(b)]. This radical scavenging activity showed a dose dependent increase with increase in concentration. The highest inhibition percent was observed in Fr-D (i.e. 39.04% at a concentration of 1 mg/ml).

Free radicals induce lipid peroxidation in polyunsaturated lipid rich area like brain and liver.[26] We analysed that different *Canna* rhizome fractions showed protection against damage due to lipid peroxides [figure-3(c)]). Maximum inhibition

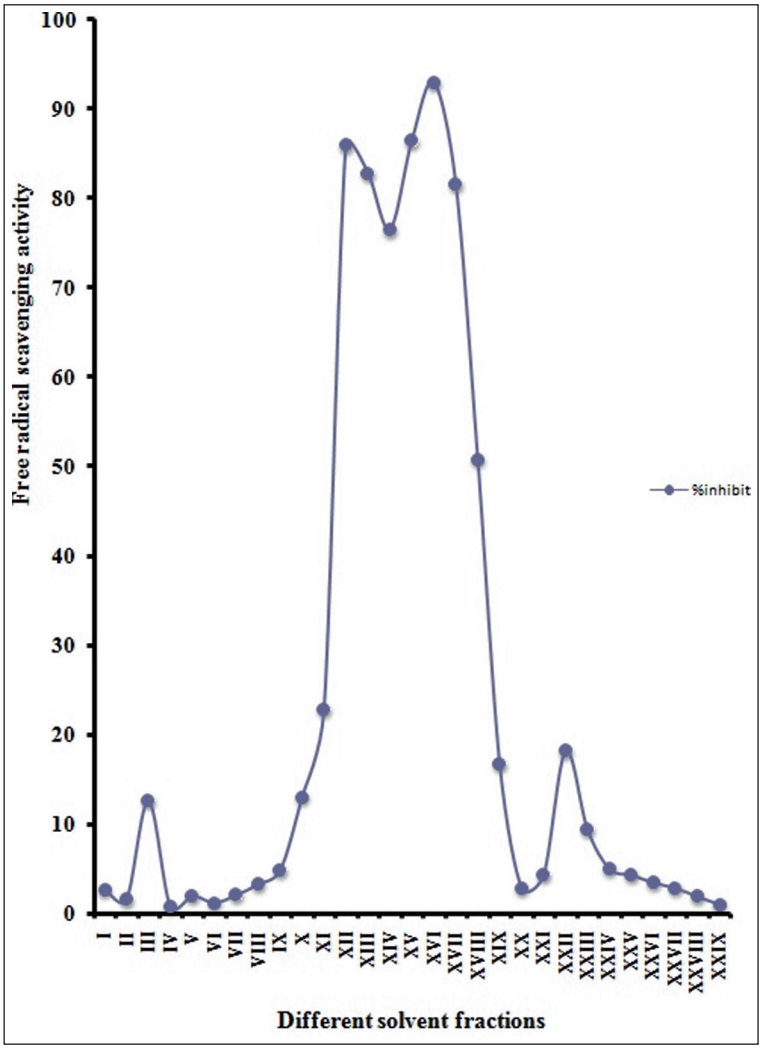

Figure 1: DPPH radical scavenging activities (%) of different solvent fractions of *C. edulis* : I- Hexane, II- Hexane : Benzene (3:1), III-Hexane : Benzene (1:1), IV- Hexane : Benzene (1:3), V- Benzene, VI- Benzene : Chloroform (3:1), VII- Benzene : Chloroform (1:1), VIII- Benzene : Chloroform (1:3), IX- Chloroform, X- Chloroform : Diethyl ether (3:1), XI- Chloroform : Diethyl ether (1:1), XII- Chloroform : Diethyl ether (1:3), XII- Diethyl ether, XIV- Diethyl ether : Ethyl acetate (3:1), XV- Diethyl ether : Ethyl acetate (1:1), XVI- Diethyl ether : Ethyl acetate (1:3), XVII- Ethyl acetate, XVII- Ethyl acetate : Acetone (3:1), XIX- Ethyl acetate : Acetone (1:1), XX- Ethyl acetate : Acetone (1:3), XXI- Acetone, XXII- Acetone : Methanol (3:1), XXIII- Acetone : Methanol (1:1), XXIV- Acetone : Methanol (3:1), XXV- Methanol, XXVI- Methanol : Water (3:1), XXVII- Methanol : Water (1:1), XXVIII- Methanol : Water (1:3), XXIX- Water.

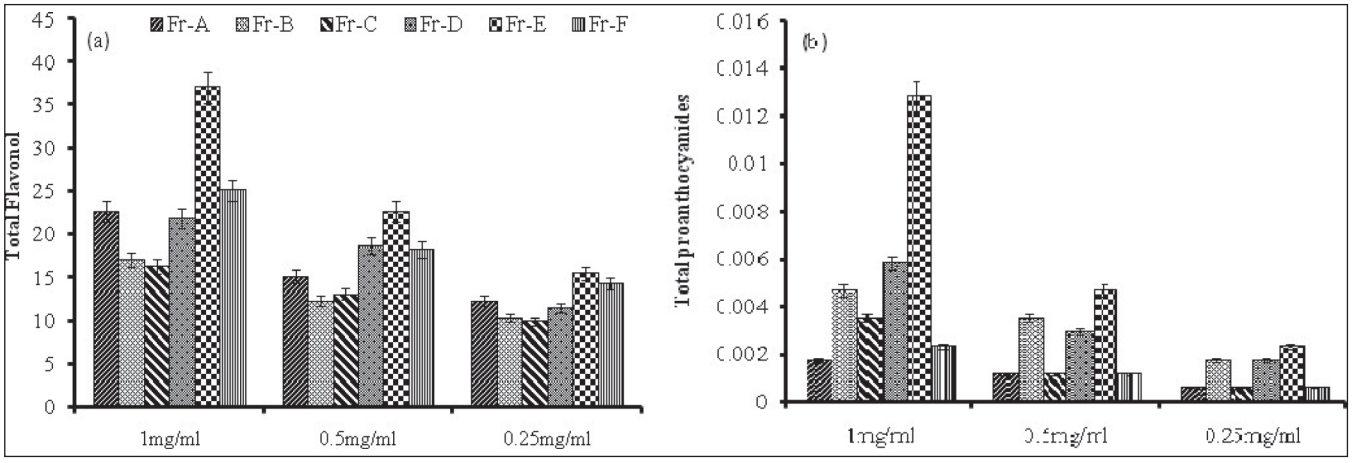

Figure 2: (a) Total flavonols of bioactive solvent fractions of *Canna* **(b)** Total proanthocyanidins of bioactive solvent fractions of *Canna*. Here X and Y axes represent Conc. of solvent fractions and Inhibition percent respectively.

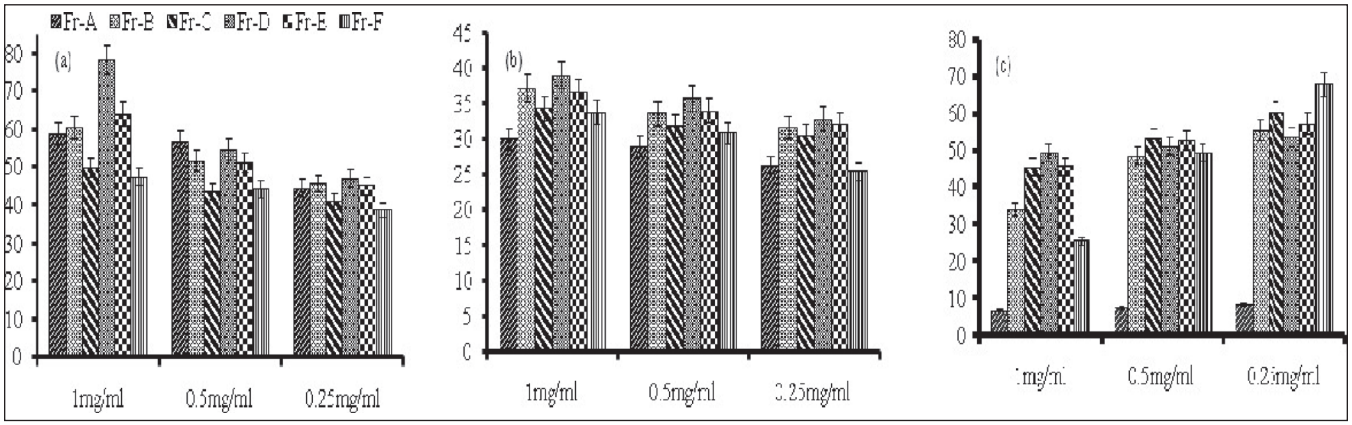

Figure 3: (a) Nitric oxide scavenging activity of bioactive solvent fractions of *Canna* **(b)** Hydroxyl radical scavenging activity of bioactive solvent fractions of *Canna* **(c)** Lipid peroxidase activity. Here X and Y axes represent Conc. of solvent fractions and Inhibition percent respectively.

Table 1: List of some qualitative characters of spots visualized on TLC plate after chromatographic development with different reagents. Within parenthesis is the band intensity.

Reagents / Sol frac[a]	Ammonia (NH_3)	Iodine (I_2)	Ferric chloride ($FeCl_3$)	NP/PEG[b]
Fr-A	Violet (+)	Brown (+)	Yellow (++)	–
Fr-B	Violet (+++)	Brown (++)	Yellow (++)	–
Fr-C	Violet (+)	–	Yellow (+)	–
Fr-D	Violet (+++)	Brown (++)	Yellow (++)	Orange (+)
Fr-E	Violet (+++)	Brown (++)	Yellow (+++)	Orange (+++)
Fr-F	Violet (+)	Brown (++)	Yellow (++)	Orange (+++)
Probable phytochemical group	Polyphenolic compounds	Unsaturated aromatic compounds	Phenolic compounds	Flavonoid glycoside i.e. triterpene glycoside compound

[a]*Sol frac- Solvent fraction* [b] *NP/PEG- Natural Product/ Polyethylene glycol*

was recorded in Fr-F (67.89%) and lowest in Fr-A (8.27%). Here we observed hepatocyte protective activity increases with decrease in concentration.

Compound detection through TLC

Highly fluorescent bands were viewed in three bioactive fractions like Fr-B, Fr-D and Fr-E under UV light (365 nm). The compounds were separarated in the TLC plate and viewed as described in table I.

DISCUSSION

Total flavonol and proanthocyanidin contents

Canna edulis rhizome extracts have been considered to be a source of natural antioxidants as it contains higher amount polyphenolic compounds.[27] In this study, we found, elevated amount of total flavonols and proanthocyanidins in bioactive Fr-E. The release of high extent of flavanols may be due to the affinity of free hydroxyl group of flavonoid compounds towards ethyl acetate and diethyl ether fractions while, the nucleophilic character of proanthocyanidins, which have more attraction towards ethyl acetate and diethyl ether for carbocation (an ion with a positively-charged carbon atom) formation may be the cause of its elevation. The results are in line with Termentzi et al., 2008.[28] and Salas et al., 2004.[29]

Free radical inhibition activities

Nitric oxide (NO) is an important chemical mediator which involves in the regulation of various biochemical and physiological processes. Excess concentration of NO is associated with several diseases.[30] Oxygen reacts with excess amount of nitric oxide to generate nitrite and peroxynitrite anions, which act as free radicals.[31] In this experiment, fraction containing diethyl ether and ethyl acetate showed strong scavenging and preventive capacity against NO because of the presence of higher amount of polyphenolic compounds in those fractions.[32,33]

In case of inhibition of hydroxyl radicals, fractions containing diethyl ether and ethyl acetate showed maximum activity, may be because of their higher ability to bind iron ion and potential for direct scavenging activity on OH^-, which revealed the same results as Verma et al., 2010.[34]

In our study *in vitro* hepatocyte protective activity was induced in goat liver by using $FeSO_4$ and ascorbic acid and it was observed that generation of lipid peroxides were prevented by bioactive fractions of *Canna* rhizome extract. Maximum inhibition in Fr-F i.e. 67.89% is same as the results obtained by Kang et al., 2011.[35] Lowest antilipid peroxidation activity was observed in Fr-A because of lowest level of polyphenolic compounds among the bioactive fraction.[36] Thus among the bioactive solvent fractions of *Canna* rhizome lipid peroxidase activity was lower in the less polar chloroform fraction and higher in more polar ethyl acetate fraction.

Compound detection through TLC

We analysed the distribution of polyphenols in different bioactive fractions. It was found that phenolic compounds were distributed from less polar to more polar solvent i.e. chloroform to ethyl acetate fraction where as mixture of flavonoid glycoside or probably triterpene glycoside related compounds were restricted to Fr-E and Fr-F as these bioactive fractions showed their sensitivity against NP/PEG.[22] Therefore, it may be concluded that the polyphenols present in *Canna* rhizome may be responsible for their antiradical scavenging activity. Phenols and flavonoids have already been reported to act as potent antioxidant in *Alpinia nutants*,[37] *Citrus sinensis*[38] etc.

CONCLUSION

We undertook the present study with an aim to make a profile of antioxidant activity of an important edible ornamental plant of sub Himalayan West Bengal and Sikkim which is also used widely as medicinal plants among tribal. We also had an aim to establish this important plant as a medicinal one, scientifically. In conclusion, the result of the present work indicated that, among the different solvent fractions derived from *Canna edulis* rhizome, the more polar fractions like Fr-D, Fr-E and Fr-F possessed highest antioxidant activity and free radical scavenging activity. It was analysed that high scavenging activity of *Canna* rhizome may be due to the presence of some polyphenolic compounds like phenols, flavonoids, proanthocyanides, glycosides etc. This result also indicated that the antioxidant rich fractions of *Canna* may probably be used in preventing the oxidative deterioration of food. The present findings appear useful in leading to further experiments on the isolation, identification, characterization and structural elucidation of the active constituents that are responsible for relatively high antioxidant activities.

ACKNOWLEDGEMENT

We are thankful to the Department of Biotechnology, Government of India for providing financial support to carry out our research.

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