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ANTIOXIDANT EFFECT AND CHARACTERIZATION OF BIOACTIVE CONSTITUENTS ISOLATED FROM *ECHINOCHLOA COLONA* (POACEAE).

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ABSTRACT

Aim: *Echinochloa colona* family Poaceae commonly known as awnless barnyard grass is a type of wild grass originating from tropical Asia and distributed throughout India. This study was designed to investigate antioxidant properties of some fractions obtained from ethanol extract and isolate some bioactive constituents. The ethanolic extract of whole plant was prepared by continuous hot extraction and then fractionated to get various fractions. Chloroform, ethyl acetate and ethanol fractions were screened for antioxidant property using *in vitro* models. In conclusion various fractions obtained from ethanol extract exhibited antioxidant capacity but chloroform fraction has shown significant antioxidant potential and hence was chromatographed to isolate some chemical constituents. Three bioactive compounds were isolated, characterized and structures were elucidated using various spectroscopic techniques. Therefore the studied plant composes a principal source of presumed antioxidants.

KEYWORDS: Echinochloa colona, DPPH, soxhlet apparatus, pharmacological, nitric oxide.

INTRODUCTION

One of the important mechanisms involved in the cell damage is oxidative stress. Extremely reactive oxygen atom is capable to do so. Some highly reactive species are generated in the presence of oxygen namely reactive oxygen species (ROS) and reactive nitrogen species (RNS).These highly reactive species are by- products of ATP biosynthesis in the mitochondria and cellular oxidation reduction process. ROS are hydroxyl ($OH^{\bullet}$), superoxide ($O_2^{\bullet-}$), lipid peroxyl ($LOO^{\bullet}$), peroxyl ($ROO^{\bullet}$), alkoxyl ($RO^{\bullet}$) radicals, where as RNS are nitric oxide ($NO^{\bullet}$) and nitrogen dioxide ($NO_2^{\cdot}$). These free radicals are able to convert to other non-radical reactive species which are capable to attack the healthy cells and tissues of the body to lose their structure and function. Overall, free radicals have been proved as a concern of pathogenesis many of diseases.[1] Human body has been gifted with some endogenous antioxidants which neutralize these free radicals and maintains the radox balance and hence protect the damage to the body.[2] In addition to endogenous antioxidant like superoxide dismutase, glutathione reductase, glutathione peroxidase and metal binding proteins like ceruloplasmin, ferritin, lactoferrin, and albumin, exogenous antioxidants also work synergistically for the neutralization of free radicals which may be the component of our diet or plant derived bio active phytoconstituents like Vitamin E, vitamin C, which have proved their free radical scavenging and antioxidant potency.[3]

The dietary sources like; fruits,[4] vegetables,[5] legumes,[6] spice,[7] beverages[8] etc. have shown their potential towards antioxidation. Antioxidant bioactive compounds like Glycosinolates,[9] derivatives of Gallic acid,[10] Quercetin and Kaempferol[11] etc. has been isolated and proved to be fruitful for anti oxidation. Taking into consideration the importance of medicinal plants in curing elements, the present study includes isolation of some bioactive compounds.

MATERIALS AND METHODS

Plant material

Echinochloa colona (Poaceae) is a terrestrial, tufted and erect grass commonly known as Jungle rice in India. This species propagates mostly by seeds but also vegetatively, widely distributed in tropics and subtropics, including South and Southeast Asia and tropical Africa. Leaves are alternate spiral, sessile linear, more than 2 cm long/wide, apex acute, base clasping, parallel-veined. It is Annual, culms geniculately ascending, or decumbent; 10-100 cm long.

Collection and authentication of the plant

The plant material of *Echinochloa colona* was collected from Dharmapuri, Tamilnadu, India and was authenticated from Botanical survey of India. The whole plant material was cleaned to remove earthy matters with the aid of water and then dried under shade avoiding direct drying in sunrays.

Extraction of plant

The shade dried whole plant approximately 5 Kg was coarsely powdered and extracted with ethanol using soxhlet apparatus for 72 hrs. The ethanol extract was then concentrated using rotary evaporator and fractioned successively using petroleum ether, hexane, chloroform, ethyl acetate and ethanol and were used for and phytochemical studies (Table 1). The chloroform, ethyl acetate and ethanol residues obtained were formulated as 25, 50, 75 and 100 mg/mL and investigated for their antioxidant activity. The powdered drug was utilized for determination of ash values and extractive values.

Table 1: Phytochemical investigation on fractions of *Echinochloa colona.*

Phytoconstituents	Petroleum ether	Hexane	Chloroform	Ethyl acetate	Ethanol Residue
Alkaloids	-	+	+	-	-
Carbohydrates	-	-	-	+	+
Tannins & Phenols	-	-	+	-	-
Glycosides	-	-	-	+	+
Flavones	+	-	+	-	-
Steroids	-	+	+	-	-

Physicochemical parameters

Percentage of total ash, water soluble ash, acid insoluble ash and loss on drying were calculated as per Indian pharmacopoeia (Table 2). Extracts of the powdered drug were prepared with different solvents for the study of extractive values. The total ash of the powdered drug was tested for different inorganic constituents [12]. Fluorescence analysis of the extracts was carried out by standard methods [13, 14] (Table 3).

Table 2: Ash values of powdered drug of *Echinochloa colona*.

Physical Constants	Ash value (%) w/w			Average (%)w/w
	I	II	III	
Total Ash	10.50	12.57	12.59	11.88
Acid insoluble ash	1.16	1.15	1.13	1.14
Water soluble ash	4.46	4.48	4.50	4.48
Loss on drying	8.45	8.47	8.48	8.46

Table 3: Consistency and fluorescence analysis of various fractions of *Echinochloa colona.*

Fractions	Consistency	Day light	UV light
Petroleum ether	Sticky mass	Dark green	Green fluorescence
Hexane	Semisolid	Dark green	Green fluorescence
Chloroform	Resinous	Brown green	Brown fluorescence
Ethyl Acetate	Semisolid	Dark green	Green fluorescence
Ethanol Residue	Solid	Brown	Brown fluorescence

***In vitro* Antioxidant Studies**

Reducing power assay[15]

The basic mechanism involved in the reducing power assay is reduction of Fe (III) to Fe (II) in the presence of the solvent fractions which can be estimated by measuring the absorbance at 700 nm using UV spectrophotometer. The concentrations of chloroform, ethyl acetate and ethanol residues were made as 25, 50, 75 and 100 mg/mL using phosphate buffer (0.2 *M*, pH 6.6). To the above each sample, 2 mL of potassium ferricyanide (10 mg/mL) was added and incubated (50°C for 20 min) followed by addition of 2 mL of trichloroacetic acid (100 mg/L) and were centrifuged at 3000 rpm for 10 min. A volume of 2 mL from each upper layer of the solution was collected and mixed with 2 mL of distilled water and 0.4 mL of 0.1% (w/v) fresh neutral ferric chloride. The absorbance was measured at 700 nm after 10 minutes. Higher reducing power is indicated by increase in absorbance of the reaction mixture. The concentrations of standard ascorbic acid was prepared and treated similarly. Reducing power is given in ascorbic acid equivalent (ASE mL^{-1}).

DPPH assay method[16, 17]

In- vitro free radical scavenging activity of chloroform, ethyl acetate and ethanol residues was performed using DPPH assay method, measured as a decrease in the absorbance of methanolic solution of DPPH. The stock solution of DPPH was prepared by dissolving 33 mg in 1L methanol and stored at 20°C until necessary. The absorbance of stock solution was obtained 0.47 at 517 nm using the UV spectrophotometer. Each sample was prepared at concentrations of 25, 50, 75 and 100 mg/mL using methanol as solvent. A 5 mL of DPPH stock solution was added to 100 µL of above each sample. After shaking the reaction mixture well, all the reaction mixture was incubated in the dark for 30 min at room temperature and then absorbance was taken at 517 nm along with the standards (25-100 mg/mL Ascorbic acid). The control was prepared devoid of any sample as above. The scavenging activity was estimated depending upon the percentage of DPPH radical scavenged using equation:

Percent scavenging effect =Absorbance of Control - (Absorbance of Sample) x 100
(Absorbance of Control)

Nitric oxide radical method[18]

Each sample of chloroform, ethyl acetate and ethanol residues were prepared at concentrations of 25, 50, 75 and100 mg/mL in standard phosphate buffer solution (pH 7.4). Likewise standard Ascorbic Acid solution was prepared. Sodium nitroprusside was added (5 μ M) in each tube; and incubated at 25^0C for 5 hrs. Control experiments without sample were carried out with identical conditions. After 5 hrs, 1mL of incubated solutions were removed and diluted with 1 mL of Griess reagent. The absorbance of the color developed after diazotization of nitrite with sulphanilamide and its coupling with napthylethylenediamine hydrochloride was observed at 546 nm using UV spectrophotometer. Scavenging of Nitric oxide was calculated as "inhibition percentage" as per the following equation.

Inhibition percent =Absorbance of Control - (Absorbance of Sample) x 100
(Absorbance of Control)

IC_{50} value

Inhibition Concentration (IC_{50}) parameter was used for the interpretation of the results from DPPH and nitric oxide method. It is defined as the amount of sample necessary to decrease the absorbance by 50 %.

Statistical analysis

Tests were carried out in triplicate. The amount of sample needed to inhibit free radicals concentration by 50%, IC_{50}, was graphically determined by linear regression method using MS- window based graph pad instat software. Results are expressed as graphically/ mean± standard deviation. Dunnett test was performed. Experimental groups were compared with the standard.

Isolation and characterization of the compound

Chloroform fraction has shown better antioxidant effect comparing to other ethyl acetate and ethanol residues hence selected for column chromatography and was then eluted with solvent mixtures of increasing polarity. While elution, the chromatographic fractions were collected and monitored on TLC. All the fractions showing single spot were pulled together, purified and observed for its R_f value using TLC. The presence of elements other than carbon was detected by usual method. The isolated compounds were characterized with the aid of UV,

IR, 1HNMR and MS. The three compounds were isolated and found to be β-sitosterol, 2, 3, 4-trihydroxy-6-methylbenzoic acid and ethyl- 3, 4, 5trihydroxy benzoate. (Fig. 1)

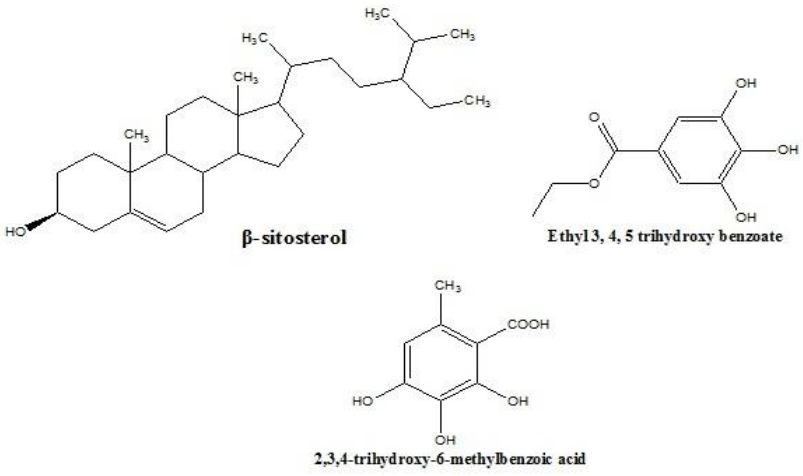

Fig. 1: Isolated bioconstituents from *Echinochloa colona*.

RESULTS AND DISCUSSION

In vitro antioxidant assay

Reducing power assay: The antioxidant activity can be indicated by measurement of the reducing ability. More the formation of Fe (II) more the reducing capability of a sample compound. It has been found that the reducing power of extracts was increased with increasing concentration. The results suggested that chloroform fraction has shown higher reducing power as compared to other extracts (Table 4).

Table 4: Reducing power assay

Concentration (mg/mL)	Absorbance			
	Ascorbic acid	**Chloroform**	**Ethyl acetate**	**Ethanol**
25	0.51 ± 0.05**	0.40 ± 0.03**	0.21 ± 0.01**	0.31 ± 0.01**
50	0.70 ± 0.06**	0.55 ± 0.02**	0.35 ± 0.02**	0.47 ± 0.02**
75	0.81 ± 0.02**	0.72 ± 0.01**	0.50 ± 0.03**	0.60 ± 0.03**
100	1.41 ± 0.04**	1.08 ± 0.03**	0.89 ± 0.02**	0.92 ± 0.02**

*Values are given as mean ± S. D. (n=3). **Significant at $p < 0.01$, p-value was calculated by comparing with control by ANOVA followed by Dunnett's test, values are expressed as ± SEM.*

DPPH free radical method: DPPH free radical scavenging activity of chloroform, ethyl acetate and ethanol residues against standard antioxidant ascorbic acid was carried out. The inhibition of DPPH free radical was calculated as percent inhibition and was observed as 41.49 (±0.31), 63.64 (±0.02), 77.53 (±0.06) and 91.22 (±0.46) for ascorbic acid at 25, 50, 75 and 100 mg/mL respectively. Chloroform fraction has shown better inhibition of DPPH free radical as compare to other at the same concentration. The IC_{50} values of standard ascorbic acid and chloroform, ethyl acetate and ethanol residues were 58, 62, 72, and 71 respectively (Table 5).

Table 5: DPPH free radical scavenging assay

Concentration (mg/mL)	% Inhibition			
	Ascorbic acid (IC_{50}-58 mg/mL)	**Chloroform** (IC_{50}-62 mg/mL)	**Ethyl acetate** (IC_{50}-72 mg/mL)	**Ethanol** (IC_{50}-71 mg/mL)
25	41.49 (±0.31)	31.27 (±0.56)	23.66 (±0.49)	25.46 (±0.02)
50	63.64 (±0.02)	45.20 (±0.02)	37.23 (±1.11)	40.86 (±0.56)
75	77.53 (±0.06)	63.04 (±0.31)	54.99 (±0.52)	58.81 (±0.46)
100	91.22 (±0.46)	69.06 (±0.33)	59.79 (±0.48)	64.81 (±0.52)

Nitric oxide radical method: In this method sodium nitroprusside generates nitric oxide which reacts with oxygen to form nitrite; which is then inhibited by antioxidants by competing with oxygen to react with nitric oxide. Sulfanilamide reacts with nitrite in acidic conditions (5% phosphoric acid) and quantitatively converted to a diazonium salt. This diazonium salt formed coupled with N- (1- naphthyl)-ethylenediamine (NED) to form an azo dye which can be measured quantitatively at 542 nm. The results showed that the chloroform fraction has higher % inhibition and lowest IC_{50} value as compared to other fractions. The IC_{50} values of standard ascorbic acid, chloroform, ethyl acetate and ethanol residues were 55.12, 65.41, 72.57, and 77. 51 at concentration of 25-100mg/mL (Table 6).

Table 6: Nitric oxide radical assay

Concentration (mg/mL)	% Inhibition			
	Ascorbic acid (IC_{50}-55.12 mg/mL)	**Chloroform** (IC_{50}-65.41mg/mL)	**Ethyl acetate** (IC_{50}-72.57 mg/mL)	**Ethanol** (IC_{50}-77. 51mg/mL)
25	34.39 (±0.21)	30.27 (±0.56)	20.66 (±0.49)	26.46 (±0.02)
50	55.54 (±0.01)	48.20 (±0.02)	34.13 (±1.10)	39.94 (±0.56)
75	67.43 (±0.05)	64.04 (±0.31)	55.89 (±0.51)	58.80 (±0.45)
100	79.12 (±0.45)	69.03 (±0.33)	59.89 (±0.46)	59.71 (±0.52)

Characterization of the compound

Compound1: The compound is white crystalline powder with steroidal nature. The compound 1 was isolated at 30:70 in % ratio of chloroform ethanol mixture. The R_f value and

melting point was found to be 0.74 and 137-139^{0}C respectively. In the spectral evidences λmax, 210 nm, Ethanol, FTIR (KBr, cm^{-1}) 3545 (OH), 2931 (CH_2), 2860 (CH), 1637 (C=C), 1033 (C-O), 1HNMR ($CDCl_3$, 400 MHz) signals at δ 1.01, 1.04, 1.06, 1.04, 1.17, 1.21(-CH_3), 1.57, 1.98, 1.13, 1.79, 1.24, 1.27, 1.35, 1.34, 1.25, 1.29, (-CH_2-), 3.25(-CH-), 5.37 (H-cyclohexene) and m/z at 414.7 (molecular mass) corresponding to $C_{29}H_{50}O$ with other characteristic fragmentations of m/z: 414, 396, 381, 330, 290, 273, 255, 212, 199 and 173 were observed. The above spectral data and those reported in the literature supports the proposed structure is β-sitosterol.[19, 20, 21]

Compound 2: The compound is white amorphous powder with phenolic nature. The compound 2 was isolated at 40:60 in % ratio of chloroform ethanol mixture. The R_f value 0.48 and melting point 145-147^{0}C was observed. In the spectral evidences, UV spectrum showed typical aromatic bands confirming a substituted aromatic benzoic acid. FTIR (KBr, cm^{-1}) signal at 1688 (COOH), 2935 (Ar-H), 3400 (OH), 1HNMR ($CDCl_3$, 400 MHz) signals at δ 11.80 (COOH), 6.5 (Ar-H), 5.10 (Ar-OH), 2.33 (Ar-CH_3) and molecular ion peak at *m/z* 184 corresponding to $C_8H_8O_5$ and main peak at 166 [184-(H_2O)] $^+$ with other fragments at m/z: 120, 84, 42, 27 were observed. The above spectral data suggested isolated compound is 2, 3, 4-trihydroxy, 6-methyl benzoic acid.

Compound 3: A third bioactive compound was isolated at 10:90 in % ratio of chloroform ethanol mixture. The isolated compound has confirmed test for ester with R_f value 0.68 and melting point 150- 152^{0}C. The FTIR (KBr, cm^{-1}) band at 1736 (RCOOR) and 3400 (Ar-OH), 1HNMR ($CDCl_3$, 400 MHz) at δ 1.25 (-CH_3), 4.65 (-CH_2-), 4.58 (Ar-OH) and molecular ion peak at m/z 198 corresponding to $C_9H_{10}O_5$ with other fragments at 183, 149[183-CH_3] $^+$, 129, 111, 97, 83, 69, 57, 43 were observed. The above spectral data suggested isolated compound is ethyl 3, 4, 5 trihydroxy benzoate.

CONCLUSION

The chloroform, ethyl acetate and ethanol fractions were obtained from ethanol extract of *Echinochloa colona* and screened by *in vitro* antioxidant methods using reducing power assay, DPPH free radical and nitric oxide free radical assay. All these fractions shown antioxidant potential but chloroform fraction has shown good antioxidant activity comparing other two. The chloroform fraction exhibiting better antioxidant potential was undergone chromatographic separation and three compounds β-sitosterol, 2, 3, 4-trihydroxy, 6-methyl benzoic acid and ethyl 3, 4, 5 trihydroxy benzoate was isolated and characterized using

various spectroscopic techniques. For the compound 1 the melting point and R_f value was found to be 137-139 ^{0}C and 0.74 respectively. The spectral and physical data of isolated compound 1 has matched with earlier reported β-sitosterol. The compound 2 and 3 was found to be newly isolated and chemical structures were elucidated as 2, 3, 4-trihydroxy, 6-methyl benzoic acid and ethyl 3, 4, 5 trihydroxy benzoate respectively. In conclusion *Echinochloa colona* may be composed of principal source of presumed antioxidants.

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