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Antioxidant Potential and Xanthine Oxidase Inhibitory Activity of *Croton sparsiflorus*

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Croton sparsiflorus (traditional treatment for cuts, ulcer, fever, inflammation, hypertension, analgesic and as antivenom) extracts were examined for xanthine oxidase inhibitory (XOI) potential, antioxidant activity ($DPPH^{\bullet}$ and phosphomolybdenum assays) and total phenolics. Dichloromethane (CSD), chloroform (CSC), ethyl acetate (CSEA), acetone (CSA), ethanol (CSE) and *n*-butanol (CSB) extracts inhibited xanthine oxidase > 50 % at 100 µg/mL concentration with IC_{50} values of 23.82, 17.34, 9.31, 11.35, 43.25 and 87.50 µg/mL, respectively. CSEA, CSM (methanolic extracts) and CSE extract exhibited significant $DPPH^{\bullet}$ free radical scavenging potential. Total phenolics; estimated as gallic acid equivalents (mg/g of the extract); ranged from 28 to 483 (CSC, CSEA and CSA > 400 mg/g). A weak correlation of total phenolics with XOI and phosphomolybdenum assays ($R^2 = 0.5413$ and 0.4975, respectively) proved phenolics are not the only bioactive compounds responsible for these activities. Strong correlation between total phenolics and $DPPH^{\bullet}$ free radical scavenging ($R^2 = 0.8485$) marked phenolics as the major free radical scavengers.

Key Words: DPPH, Euphorbiaceae, *Croton bonplandianus*, Phosphomolybdenum, Antioxidant activity, Total phenols.

INTRODUCTION

Biological and chemical pro-oxidants are considered to be important for the provocation of free radical mediated diseases in an individual. Although free radicals are considered to be important for normal physiology but when produced in excess they cause cellular damage. The radicals initiate a chain reaction of lipid and protein peroxidation by attacking the double bonds of these molecules. About 40 diseases are now being considered as free radical-mediated. Most of them are metabolic, nervous or other old age diseases[1]. Recently the involvement of free radicals and other oxidants in ageing and several diseases has been investigated in detail. Active oxygen damages the skin directly and forms lipid peroxides which result in the formation of insoluble pigments such as eumelanins and phaeomelanins. Much

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physiological damage may be directly imputable to the hydroxyl radical, because it is highly reactive and so any hydroxyl radical produced *in vivo* would react at or close to its site of formation.

Xanthine oxidase (XO) is a cytosolic enzyme, widely distributed among different species (from bacteria to man) which catalyzes the hydroxylation of hypoxanthine to uric acid which regulates the antioxidant capacity of the blood[2]. Since significant increments of xanthine oxidase in the serum level are clinically observed in patients who suffered from hepatitis, brain tumour, hyperuricaemia and gout, xanthine oxidase has been reported as a key enzyme associated with the incidence of hyperuricaemia-related disorders[3,4]. Accordingly, one of the therapeutic approaches to treat gout is the use of XO inhibitors that block the production of uric acid. Allopurinol is the sole XO inhibitor under the clinical application in the past three decades[5,6]. However, this drug inevitably gives rise to the severe adverse effects such as hepatitis, nephropathy, allergic reactions and 6-mercaptopurine toxicity[7-9]. Therefore, there is an urgent need to search for new antioxidants which have medicinal applications[10] and useful as XO inhibitors.

The genus *Croton* belongs to the family Euphorbiaceae. Some of the species of this genus are reported to have beneficial medicinal effects *i.e.* analgesic, purgative, antiinflammatory, treatment of constipation, cough, fever, sprains, bruises, rheumatic swelling, alterative, cholagogue, chronic hepatitis, blood diseases, gout and lumbago[11,12]. One of the species of the genus *Croton* (*Croton tiglium*) is traditionally used in the treatment of gout, which is a metabolic disorder caused by over activity of xanthine oxidase[11,13]. Therefore, present study was carried out to evaluate xanthine oxidase inhibitory (XOI) activity of different solvent extracts of *Croton sparsiflorus*, which can be useful for the treatment of gout and other related disorders.

EXPERIMENTAL

Xanthine oxidase (bovine milk), xanthine, Folin-Ciocalteu reagent, 2,2′-diphenyl-1-picrylhydrazyl (DPPH), butylated hydroxy toluene (BHT), sodium phosphate, ammonium molybdate, Tween-80 and allopurinol were purchased from Fluka and Sigma-Aldrich. All the solvents were purchased from Panreac Chemicals.

Croton sparsiflorus was collected in Sep. 2007 from Rana town near Kala Shah Kaku, Muridke, Pakistan and was identified by Dr. Zaheer-ud-Din Khan, Head of Botany Department, GC University. A voucher specimen was submitted (GC-Herb-Bot-617) in Sultan Ahmad herbarium at Department of Botany, GC University, Lahore.

Preparation of plant extracts: The whole plant of *C. sparsiflorus* was dried in shade, grinded and extracted successively with *n*-hexane, dichloromethane, chloroform, ethyl acetate, acetone, methanol, ethanol, *n*-butanol and water by Soxhlet apparatus to get CSH, CSD, CSC, CSEA, CSA, CSM, CSE, CSB and CSW extracts of *C. sparsiflorus*, respectively. Solvents were removed under reduced pressure by rotary evaporator and dried extracts were stored in refrigerator for further study.

Determination of total phenols: Total phenolics of all *Croton sparsiflorus* extracts were estimated using Folin-Ciocalteu reagent[14]. 0.1 mL of extract solution was mixed with 2.8 mL of 10 % Na_2CO_3 and 0.1 mL of 2 N Folin-Ciocalteu reagent. After 40 min, absorbance was recorded at 725 nm by UV-visible spectrophotometer. The results were determined as milligram gallic acid equivalent (mg GAE)/g of extract by computing with standard calibration curve obtained from different concentrations of gallic acid.

Antioxidant activities: Variety of antioxidants are present in plants, therefore several methods have been developed to evaluate their antioxidant potential. DPPH free radical scavenging and phosphomolybdenum methods are relatively fast spectroscopic methods to evaluate the antioxidant activity. DPPH is widely used as an *in vitro* model system to investigate the scavenging activity of several natural compounds and plant extracts. When DPPH is scavenged, the colour of the reaction mixture changes from purple to yellow with decrease of absorbance at 517 nm.

DPPH free radical scavenging assay: Antiradical activity of CSEs was determined by the method of Lee *et al.*[15]. 1.0 mL of the extracts (100 µg/mL in methanol) was mixed with 2.0 mL methanolic solution of DPPH (10 mg/L).The mixture was shaken vigorously and allowed to stand at room temperature for 15 min. The absorbance was recorded at 517 nm against methanol as blank with a spectrophotometer. The % of DPPH discolouration of sample was calculated according to the formula.

$$\% \text{ age scavenging of DPPH} = 100 \times (1 - \text{Absorbance of sample / Absorbance of control})$$

Determination of total antioxidant activity: Total antioxidant activities of CSEs were evaluated by the formation of green phosphomolybdenum complex[16]. 0.1 mL solution of extract in methanol (1 mg/mL) was mixed with 1.9 mL of reagent solution (0.6 M H_2SO_4, 28 mM sodium phosphate and 4 mM ammonium molybdate). The blank solution contained 2 mL reagent solution only. The vials were capped and incubated in a water bath for 1 h at 95 °C. On cooling absorbance was recorded at 695 nm spectrophotometrically. Butylated hydroxytoluene (0.5 mg/mL) was used as standard reference.

***In vitro* Xanthine oxidase inhibitory (XOI) activity:** All of the nine extracts in different solvents were subjected to *in vitro* XOI activity. The assay was performed spectrophotometrically under aerobic conditions[17]. The assay mixture consisted of 1 mL of test solution (10- 100 µg/mL) in 0.1 % Tween 80, 2.9 mL of phosphate buffer (pH 7.5) and 0.1 mL of enzyme solution (0.01 units/mL in phosphate buffer, pH 7.5). The mixture was left for 15 min then the reaction was initiated by the addition of 2 mL of substrate solution (150 µM xanthine in the same buffer). The assay mixture was incubated at 25 °C for half an hour. The reaction was then stopped by the addition of 1 mL of 1 N hydrochloric acid and the absorbance was measured at 290 nm using a UV-visible spectrophotometer against a blank. XOI activity was expressed as the percentage inhibition of XO in the above assay system, calculated as:

$$\% \text{ inhibition of XO} = (Ab_{enzyme}\text{-}Ab_{blank})\text{-}(Ab_{enzyme+extract}\text{-}Ab_{extract})/(Ab_{enzyme}\text{-}Ab_{blank}) \times 100$$

where Ab_{enzyme} is the absorbance of enzyme and substrate solution without test extract, Ab_{blank} is absorbance of substrate solution without test extract and enzyme, $Ab_{enzyme+extract}$ and $Ab_{extract}$ are the absorbance of the test extract with and without enzyme, respectively. Allopurinol was used as a positive control. IC_{50} values were calculated from the mean value of data.

Statistical analysis: All the measurements were done in triplicate. Mean values were calculated and presented with ± SD (standard deviation). Correlation coefficient of determination (R^2) was calculated.

RESULTS AND DISCUSSION

Determination of total phenols: Phenolic compounds have been suggested to play important role in the protection against cancer and heart diseases[18]. Total phenols of CSEs were estimated as mg GAE/g of the extract by Folin-Ciocalteu reagent. The results ranged from 483 to 28 mg GAE (Table-1). CSC (415 mg), CSEA (483 mg), CSA (404 mg) and CSM (393 mg) extracts of *C. sparsiflorus* contained higher phenolic contents than other extracts.

TABLE-1
RESULTS OF % AGE YIELD, TOTAL PHENOLS, DPPH$^{\bullet}$ ASSAY AND XO INHIBITION OF *C. sparsiflorus* EXTRACTS

Extracts & Std.	Yield of extracts (%)	Total phenols (mg GAE/g of extract)	DPPH$^{\bullet}$ assay (% SC) at 100 µg/mL	XOI at 100 µg/mL (%)	XO IC_{50} (µg/mL)
CSH	1.85	28.0 ± 15.2	16.7 ± 0.5	7.1 ± 0.1	–
CSD	2.15	107.0 ± 10.3	26.5 ± 1.0	73.2 ± 0.3	23.82
CSC	1.29	415.0 ± 15.5	49.6 ± 0.1	75.1 ± 0.5	17.34
CSEA	0.6	483.0 ± 12.1	73.3 ± 0.3	74.2 ± 0.2	9.31
CSA	1.44	404.0 ± 25.2	62.6 ± 0.3	70.4 ± 0.3	11.35
CSM	9.57	393.0 ± 13.4	56.0 ± 0.2	45.3 ± 0.8	–
CSE	2.33	305.0 ± 5.6	58.2 ± 0.1	61.1 ± 1.0	43.25
CSB	0.14	273.0 ± 2.3	35.3 ± 0.1	55.8 ± 0.2	87.51
CSW	4.37	36.0 ± 0.5	29.3 ± 1.0	8.5 ± 0.5	–
BHT	–	–	72.6 ± 0.6	–	–
Allopurinol	–	–	–	91.3 ± 0.2	8.73

Values are mean of three parallel measurements with ± SD.

Antioxidant activities: DPPH$^{\bullet}$ scavenging activity of CSEs (100 µg/mL) was measured by the method of Lee *et al.*[15] as percentage scavenging of DPPH (Table-1) at 100 µg/mL concentration. Among all the extracts of *C. sparsiflorus* CSEA, CSA, CSM and CSE showed significant free radical scavenging activity (DPPH$^{\bullet}$), 73.3, 62.2, 56.0 and 58.2 %, respectively.

Total antioxidant activity of CSEs was measured by their ability to reduce Mo(VI) complex into greenish Mo(V) complex. The phosphomolybdenum assay responds to a variety of antioxidants: ascorbic acid, phenols, tocopherols and carotenoids[16].

The observed order of total antioxidant activity was CSEA > CSA > CSC > CSE > CSD > CSB > CSM > CSW > CSH. Except *n*-hexane, methanol, *n*-butanol and water extracts all other extracts of *C. sparsiflorus* showed higher total antioxidant activity than BHT (Fig. 1).

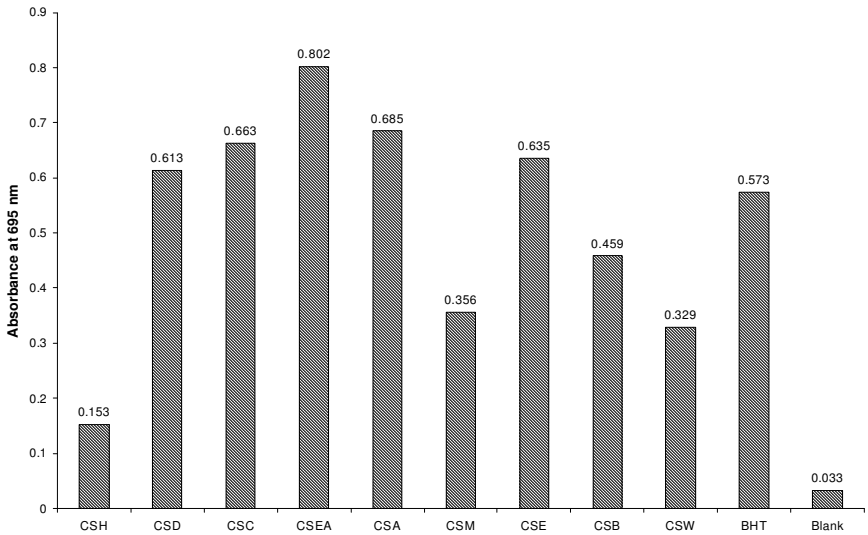

Fig. 1. Total antioxidant activity of CSEs (1 mg/mL) and BHT (0.5 mg/mL) by phosphomolybdenum method

***In vitro* Xanthine oxidase inhibitory activity:** *In vitro* XO inhibitory activity of CSEs was assayed by the method of Owen *et al.*[17]. Concentration dependant assay was carried out (10 to 100 µg/mL) to determine IC_{50} of CSEs (Table-1). Six out of nine extracts of *C. sparsiflorus*, (CSD, CSC, CSEA, CSA, CSE and CSB) showed more than 50 % XO inhibition at 100 µg/mL concentration. Chloroform (17.34 µg/mL), ethyl acetate (9.31 µg/mL) and acetone (11.35 µg/mL) extracts showed least IC_{50} values which are comparable with allopurinol (Table-1).

Correlation studies: Total phenols correlated strongly with DPPH scavenging activity of CSEs ($R^2 = 0.8485$), suggesting that phenolics are the main free radical scavenging moieties present in the active extracts. While a weak correlation was observed between the results of phosphomolybdenum assay ($R^2 = 0.4975$) and XO inhibition ($R^2 = 0.5413$) with total phenols.

Conclusion

Gout is one of the most common metabolic disorders affecting human. The over activity of xanthine oxidase (XO) is one of the major causes of this disease[13]. XO also serves as an important biological source of oxygen derived free radicals that contribute to oxidative damage to living tissues involved in many pathological processes such as inflammation, atherosclerosis, cancer, diabetes and ageing[19].

Previous investigations of *Croton sparsiflorus* Morong. (syn. *Croton bonplandianus* Baill.) revealed the presence of alkaloids and flavonoids[20-23]. Acharya and Pokhrel[24] reported the ethno-medicinal use of this plant for the treatment of cuts and ulcer. In India leaf extract of this plant is used for the treatment of snake bite[25]. Palanivelu and Murugesan[26] reported analgesic activity in methanolic extract of *C. sparsiflorus* leaves.

The significance of CSEs as XO inhibitors and their antioxidant potential was evaluated in this study. Concentration up to 100 µg/mL of extracts was used to check their suitability as XO inhibitor. % age XO inhibition of CSEs ranged from 7.1 to 75.1 %. The results of IC_{50} values of CSD, CSC, CSEA, CSA and CSE indicated that these extracts inhibited XO more than 50 % at less than 50 µg/mL concentration. CSEA, CSA, CSM and CSE extracts of *C. sparsiflorus* showed significant inhibition of DPPH free radical at 100 µg/mL concentration (73.3, 62.6, 56.0 and 58.2 %, respectively). Correlation between total phenols and XOI activity (R^2 = 0.5413) suggested that the compounds other than phenols are involved in the XO inhibition. It is evident from the literature that certain alkaloids, flavonoids, saponins and terpenoids show XO inhibition[27-30]. Total phenolics showed strong correlation with DPPH scavenging activity of CSEs (R^2 = 0.8485) concluding that phenolics are dominant free radical scavengers.

Present results suggested that further investigation on active extracts of *C. sparsiflorus* could provide new targets for the design of xanthine oxidase inhibitors.

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