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RESEARCH ARTICLE

EVALUATION OF ANTIOXIDANT ACTIVITY OF SUCCESSIVE EXTRACTS OF CLERODENDRUM MULTIFLORUM STEM

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ABSTRACT

The successive extracts of *Clerodendrum multiflorum* stem, a traditional medicinal plant used in India. It possessed asignificant amount of total phenolic and flavonoid contents with potent antioxidant activities in scavenging DPPH, ABTS radicals,and good total antioxidant capacity. On the other hand, the ethylacetate and ethanol extracts showed the highest inhibition ofDPPH, ABTS radicals and phosphomolyb date assay. This studyverified that the ethyl acetate and ethanol extracts have strong antioxidant activities which might be due to high level of phenolics and flavonoids. Thus results confirms its traditionalclaims can be used as a source of potential antioxidant.

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INTRODUCTION

During normal physiological functions, reactive oxygen species (ROS) such as hydrogen peroxide, singlet oxygen, superoxide anion radical, hydroxyl radical are generated from the auto-oxidation of lipids, as well as reactive nitrogen species (RNS) by UV irradiation. These ROS and RNS damages several cellular components such as lipids, proteins, nucleic acid, and DNAs through oxidation or nitration processes results into various degenerative diseases, including cancer, ageing, arteriosclerosis, and rheumatism [1]. It has been observed that natural antioxidants are safer than synthetic antioxidants. Therefore, there is an increasing interest amongst scientific communities in identifying natural source of antioxidants derived from plant origin. *Clerodendrum multiflorum* (Burn.f.) O.Kuntze is a common shrub of arid plains, low hills and tropical deserts. They are distributed throughout the drier parts of India. *Clerodendrum phlomids* Linn, (Syn. *Clerodendrum phlomidis*, *Volkameria multiflorum* (Burn.f). (Verbenaceae) is an important and well known medicinal plant extensively used in Ayurveda and Siddha system of medicine for treatment of various ailments. The popular therapies include on inflammation, diabetic, nervous disorders, asthma, rheumatism, digestive disorders, and urinary disorders [2]. Decoction of its root which is slightly aromatic and astringent is used as a demulcent in gonorrhea. It is also given to children during convalescence from measles [3].It has been reported that pectolinaringenin, scutellarein, 7-hydroxy flavones, clerodin, clerodendrin A, clerosterol, ceryl alcohol has been isolated from this plant.

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The leaves reported to posses significant hepatoprotective, anti-diabetic anti-diarrhoeal, mild tranquilizing properties [4, 5, 6, 7]. It also exhibited antifungal and anthelmintic activity in in-vitro studies [8]. Despite of strong recommendation of herb in oxidative stress induced diseases. The stem of plant has not explored for antioxidant potential, thus present study was undertaken to evaluate the in-vitro antioxidant potential in series of assay.

EXPERIMENTAL WORK

Stem of *C. multiflorum* were collected at Mahur- Kinwat region of Nanded district, Maharashtra. The plant was identified by Prof. Vishal R. Marathe, Science College, Nanded, and herbarium voucher specimen was deposited. The air-dried stem of *C. multiflorum* were powdered and exhaustively extracted with different polarity solvent, successively. The petroleum ether (60-80º) (PE-CM), chloroform (CH-CM), ethyl acetate (EA-CM), and methanol (ME- CM) extracts were filtered, evaporated under reduced pressure to obtain a viscous dried extracts. The extracts were preliminary investigated for presence of secondary metabolites using standard qualitative chemical tests.

Total Phenolic content

Total phenolic content was estimated using the Folin-Ciocalteu method [9]. 100 µl of test extracts were mixed thoroughly with 2 ml of 15% Na_2CO_3. After 2 min to this mixture, 200 µl of Folin-Ciocalteu reagent was added and incubated at room temperature for 30 min. The absorbance was measured at 760 nm against a blank. The standard calibration curve was prepared using gallic acid in place of test extract. Total phenolic content was expressed

milligram of gallic acid equivalents (GAE)per gram of dried extract. It was calculated by the following formula:

$$T = \frac{C \times V}{M}$$

Where, T = Total phenolic compounds (mg/g of plant extract) in GAE; C = Concentration of gallic acid established from the calibration curve (mg/ml); V = Volume of extract (ml); M = Weight ofplant extract (g)

Total Flavonoids content: The different concentrations (20-100 µg/ml) of standard quercetin solutions (0.5 ml) were separately mixed with 1.5 ml of ethanol, 0.1 ml of 10% aluminium nitrate, 0.1 ml of 1 M sodium acetate and 2.8 ml of water. The resultant mixture was kept at ambient temperature for 40 min. The absorbance of reaction mixture was measured at 415 nm; calibration curve was plotted for concentration against absorbance. Same procedure was followed for the extracts. In the blank solution,the volume of 10% aluminium nitrate was substituted with the same volume of distilled water [10]. The total flavonoid content in the extract expressed as milligram per gram of quercetin equivalents (QE) with formulae as mentioned for total phenolic content.

Antioxidant activity

DPPH radical-scavenging activity: The DPPH radical-scavenging activity was performed as per Ebrahimzadeh with minor modifications [11]. Different concentrations of test extracts mixed with an equal volume, methanolic DPPH (100mM) solution and added separately in wells of the micro-titre plate. After 20 min incubation at room temperature, the absorbance was measured at 517 nm using microplate spectrophotometer (BIO-Tek, USA. Model-96 well micro plate). Same procedure was followed for control by using methanol in place of extract. The percentage inhibition was estimated based on the percentage of DPPH radical scavenged using thefollowing formula:

% Inhibition = [(Control absorbance – Sampleabsorbance) / (Control absorbance)] × 100

ABTS radical cation scavenging activity: ABTS radical cation scavenging activity was performed using the method reported by Fellegrin with slight modifications [12]. In brief, ABTS solution (7 mM) was reacted with potassium persulfate (2.45 mM) solution and kept overnight in the dark to yield a dark colored solution containing $ABTS^{\cdot+}$ radical cation. Prior to use in the assay, the ABTS radical cation was diluted with 50% methanol for an initial absorbance of about 0.700 at 734 nm.After the addition of 1.0 ml of diluted $ABTS^{\cdot+}$ to 10 µl of sample, the absorbance was measured after 5 min of initial mixing. The percentage inhibition was calculated according to the formula used for DPPH activity. The antioxidant potential of extracts was expressed as IC_{50}, the concentration necessary for a 50% reduction of DPPH and $ABTS^{\cdot+}$ radicals.

Total antioxidant capacity by phosphomolybdenum method: The total antioxidant capacity of CMS extracts was determined as ascorbic acid equivalent [13]. The standard curve for total antioxidant capacity was plotted using ascorbic acid standard solution (20- 100 µg/ml). An aliquot of 100 µl of extract solutionswere combined with 1 ml of reagent (0.6 M sulfuricacid, 28 mM sodium phosphate and 4 mM ammonium molybdate). All tubes were capped and incubated in a boiling water bath at 95°C for 90 min. Tubes were allowed to cool at room temperature. Absorbance of the test and standard solutions was measured at 695 nm against blank containing 0.1 ml of distilled water and 1 ml of reagent. An antioxidant capacity was expressed as milimolar equivalents of ascorbic acid.

RESULTS AND DISCUSSIONS

Plant phenolics or polyphenols are very important secondary metabolites recognised for their potent their antioxidant activity. They act by different mechanism such as chelating redox-active metal ions, inactivating lipid free radical chains and preventing hydroperoxide conversions into reactive oxyradicals. The total phenolic content of successive CMS extracts was expressed as gallic acid equivalents (GAE), varied between 2.12 ± 0.52 mg and 36.36 ± 0.91 mg/g dry weight of fraction. The ethyl acetate extract exhibited the highest total phenolics content (36.36 ± 0.91 mg gallic acid equivalent/g of extract), whereas the contents obtained with Ethanol was much smaller (25.15 ± 2.10 mg gallic acid equivalent/g).

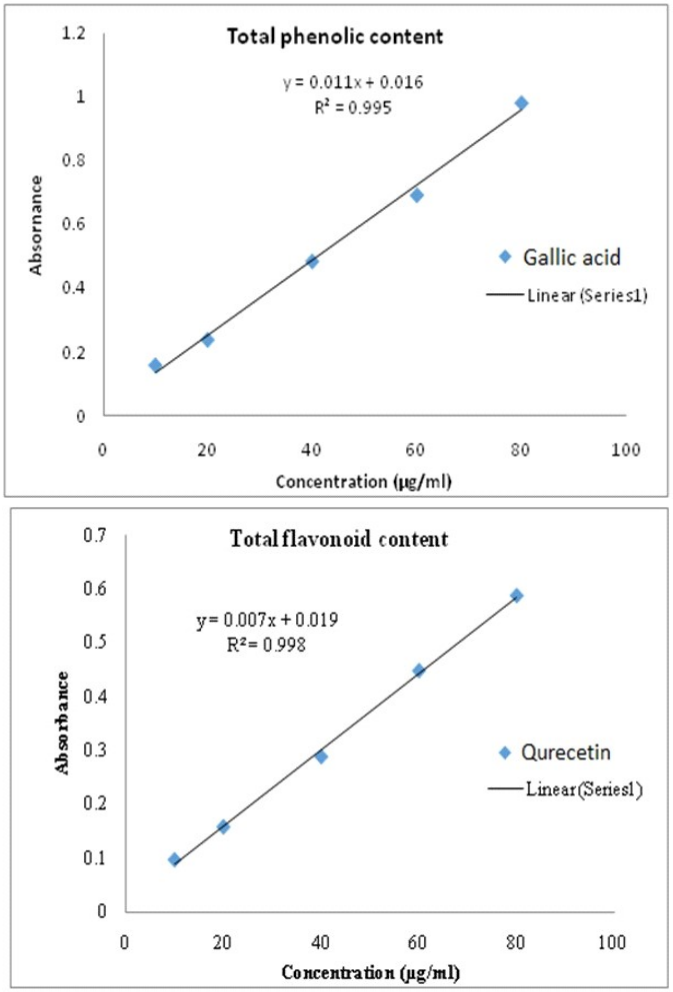

Figure 1. Calibration curve of standard gallic acid and quercetin

The content of total flavonoids expressed as quercetin equivalents, varied from 2.86 ± 1.43 to 27.62 ± 0.82 mg as quercetin equivalent/g of extract. Phenolic acids and flavonoids have been reported to be the main phytochemicals responsible for the antioxidant capacity of plant drug. It is well documented that polyphenolics could be a good source of antioxidants and having ability to protect against lipid peroxidation [14, 15].

Table 1. Total phenolic and flavonoid content of *C. multiflorum* leaves extracts

Extracts	Total phenolic (mg/g gallic acid)	Total flavonoid (mg/g quercetin)
PE- CMS	2.12 ± 0.52	2.86 ± 1.43
CH-CMS	8.48 ± 2.29	8.10 ± 3.33
EA- CMS	36.36 ± 0.91	20.62 ± 2.18
EO- CMS	25.15 ± 2.10	27.62 ± 0.82

(n=3)

DPPH is a stable free radical at room temperature and accepts an electron or hydrogen radical to become a stable diamagnetic molecule [16]. It has been widely accepted as a tool for estimating free radical

scavenging activities of antioxidants [17].The reduction capability of DPPH was determined bythe decrease in its absorbance at 517 nm, which is induced by antioxidants. Positive DPPH test suggests that the samples were free radical scavengers. The scavenging effect of CMS extracts and ascorbic acid on DPPH radical was compared. On the DPPH radical, CMS had significant scavenging effects with increasing concentration in the range of 40–200 µg/ml; when compared with that of ascorbic acid. The DPPH activity of CMS extracts were found to increase in dose dependent manner. Ethanol extracts had the highest DPPH radical scavenging activity, shown by the lowest value of IC_{50} values (154.77 µg/ml). A higher DPPH radical scavenging activity is associated with a lower IC_{50} value. It was evident that the extracts did show the hydrogen donating ability to act as antioxidants.

activity of the EA-CMS and EO-CMS extract revealed highest antioxidant activity, respectively. The possible reason might be the different contents and sorts of bioactive compounds including phenolics and other compounds responsible for antioxidant capacity. These results were consistent with the findings of many research groups, who reported such correlations between total phenolic content and free radical scavenging activity [20, 21]. Total antioxidant capacity based on the reduction of Mo (VI) to green colour Mo (V) complex by the antioxidant compounds, which results in the formation of a green coloured Mo (V) complex having a maximum absorbance at 695 nm. A high absorbance value indicated that the sample possesses high antioxidant activity [22, 23]. The results were expressed as mg/g of ascorbic acidequivalent.

Table 2. Antioxidant effect (IC_{50}) on free DPPH radicals of *C. multiflorum* stem extracts

Conc. of Sample(µg/ml)	PE-CMS	CH-CMS	EA-CMS	EO-CMS	As. Acid	Conc. of Std (µg/ml)
40	16.74	21.86	26.51	29.30	20.47	10
80	24.19	28.37	34.88	37.67	26.51	20
120	26.98	33.95	42.33	43.72	36.74	40
160	33.49	37.67	49.77	52.56	51.63	60
200	38.60	40.93	52.56	56.74	62.33	80
IC_{50} (µg/ml)	287.20	268.47	172.99	154.77	73.45	

Table 3. Antioxidant effect (IC_{50}) on ABTS radicals of *C. multiflorum* stem extracts

Conc. of Sample (µg/ml)	PE- CMS	CH-CMS	EA- CMS	EO- CMS	As. Acid	Conc. of Std(µg/ml)
40	24.40	30.13	38.27	39.07	37.60	10
80	31.47	39.20	49.60	46.93	46.93	20
120	40.40	45.60	54.00	54.40	57.20	40
160	46.40	48.80	56.80	59.73	67.33	60
200	48.13	51.87	58.93	62.80	69.47	80
IC 50 (µg/ml)	195.90	172.73	107.77	103.33	46.52	

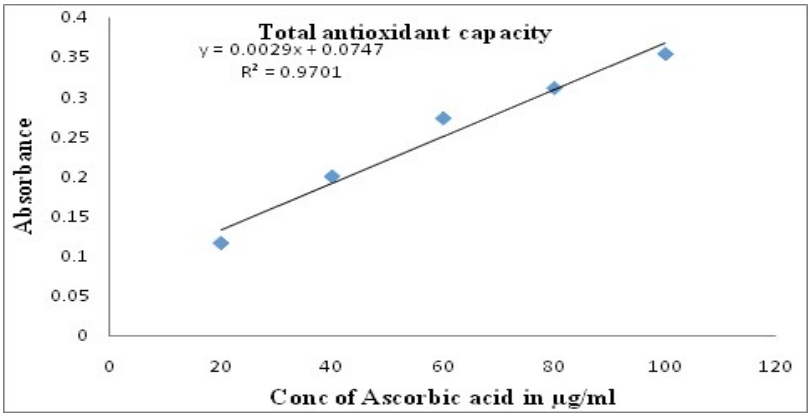

Figure 2. Standard calibration curve of ascorbic acid

Table 4. Antioxidant effect (IC_{50}) on total antioxidant capacity of *C. multiflorum* stem extracts

Extracts	Total antioxidantcapacity
PE-CMS	1.78 ± 1.02
CH-CMS	3.78 ± 1.02
EA-CMS	15.11 ± 1.02
EO-CMS	13.44 ± 2.00
(n=3)	

ABTS radical scavenging assay is based on the ability of the antioxidant compound to scavenge the protonated radical cation $ABTS^{+\bullet}$. This scavenging produces a decrease in the absorbance at 734 nm [18]. The extracts of CMS showed significant dose dependant ABTS radical scavenging activity. Although the IC_{50} values of the extracts were higher than that of ascorbic acid. Among all ethanol extract of CMS extracts showed potent ABTS radicals scavenging ability (103.33 µg/ml). The result was shown in Table 3. The DPPH and ABTS radicals are soluble in water and organic solvent, thus enabling the determination of antioxidant capacity of both hydrophilic and lipophilic compounds[19]. The DPPHand ABTS radical scavenging

The EA-CMS extract showed highest total antioxidant capacity when compared with other FMB extracts (15.11 ± 1.02 mg/g ascorbic acid equivalent) (Table 4). The results of present antioxidant study of CMS extracts established their correlation with polyphenolic content of selected plants, since many studies have proposed that polyphenols are responsible for their antioxidant properties [24,25]. The polyphenolic compounds contain hydroxyl groups in their structure and have electron donating ability which allow them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers and responsible for antioxidant property [26]. The total polyphenolic contents in EA-CMS and EO-CMS extracts were significantly higher than remaining.

Therefore, it can be presumed thatthe major polyphenolic compounds present in these extracts are responsible for free radical scavenging ability.

CONCLUSION

The results of present work revealed that ethyl acetate and ethanol extract of *C. multiflorum* stem have good antioxidant activity. The activity of these extracts is attributed to the phenolic and flavonoid contents. Consequently, our results suggested that the extract can be utilized as an effective and safe and accessible source of natural antioxidants with consequent health benefits.

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