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Phytochemical screening and *in-vitro* antioxidant activities of *Colubrina asiatica* Brong

Desai Nivas[1] and *Gaikwad D. K.[2]

[1]*Department of Botany, Shri Pancham Khemraj Mahavidyalaya, Sawantwadi, India*
[2]*Department of Botany, Shivaji University, Kolhapur, India*

ABSTRACT

The accumulation of free radicals in the body result in oxidative stress related diseases in humans. Recent era is directed towards finding natural antioxidants of plants origins. Present study was aimed to evaluate in vitro antioxidant activities, phytochemical constituents and antimicrobial activity of essential oil of Colubrina asiatica Brong [Family Rhamnaceae]. The antioxidant potential and phytochemical constituents of crude aqueous extract of leaves and stems involving DPPH, FRAP, Reducing power was assessed. The flavonoid and phenolic contents of the extract were also determined using standard phytochemical reaction methods. Composition of seed oil and their antimicrobial activity were also determined. Phytochemical analyses revealed the presence of flavonoids, saponins and total polyphenols. The total phenolic content of the aqueous leaf extract was 12.499 mg gallic acid equivalent/g of extract powder in leaves & 0.867 in stem. The total flavonoid contents in leaves were 6.8 ± 0 & 2.8 ± 0.1 mg gallic acid equivalent/g of extract in stem respectively. The percentage inhibition of DPPH of oxidation showed antioxidant activity of 57% in leaves and 43% in stem compared to those of BHT (84.6%) and gallic acid (96%). Also, the reducing potential in stem and leaves was found significant. The total antioxidant activity measured by FRAP assay in leaves was 137 ± 4.8 and 170 ± 38 μM Fe2+ in stem. Our findings provide evidence that the crude aqueous extract of C. asiatica is a potential source of natural antioxidants, some pharmaceutically important compounds in oil.

Keywords: Antioxidant activity, essential oil, Phytochemical screening, *Colubrina asiatica*

INTRODUCTION

Colubrina asiatica Brong. is a glabrous, scandent or sprawling shrubs of Rhamhanaceae family [1]. This species is reported to occur in Tropical America, Southeastern Asia, Malesia, Tropical Australia and Polynesia (to Hawaii), Coastal east Africa, extreme Southeastern India, southern Burma, Andaman and Nicobar Islands, Sumatra, Australia and Hawaii[2-3]. In India the species is wide spread in littoral scrub forests, tidal forests of Orissa and Ghats of Konkan[4-5]. The plant has some economic value, leaf contains saponin like principle which is used as soap substitute, can be used to prepare bowels, used to wash and whiten textile kilts and garments made from *Cyphlophus heterophvllus* in Samoa [6]. It can be used for food, medicine, as a fish poison, used as chewstick, tooth cleaners. The fruit is used to produce a soft drink[7]. Salinity is a harsh environmental factor that has the major effect on plant quantity and quality[8] (Zhu, 2002). In order to survive in salt stress condition, plants develop the network responses of physiological and biochemical defense mechanisms to protect themselves against stress[9] (Sutee et al., 2009). Since *C. asiatica* occurs in forested areas and brush along the coast grows in coastal sites adjacent to ocean beach

dunes, coastal strand tidal marshes and tidal mangrove swamps, it is included in the list of halophytes[10] by Aronson (1989).

Perusal of the previous literature revealed that this medicinal plant is unexplored; hence, it is thought worthwhile that the detailed study on analysis of major chemical constituents will add some important information for the commercial utilization of this promising medicinal plant.

EXPERIMENTAL SECTION

Plant material

Leaves stem and seeds of *C. asiatica* were collected in December 2011 from coastal area of Goa, India. The Plant materials were later confirmed with taxonomist. The leaves, stems and seeds were picked and washed with water to remove all unwanted plant materials and sand, dried under sunlight (27°C-30°C for 7 days), pulverized using Willys grinder and stored in an airtight container for further use.

Preparation of extract

The powdered plant material (200 g) was extracted thrice in distilled water (5.5 L; 27°C-30°C) on Orbital Shaker, for 48 hours. The extract was filtered using a Buchner funnel and Whatman No.1 filter paper. The filtrate of aqueous extract obtained was quickly frozen at -40°C and dried for 48 h using a freeze dryer to give a yield of 30 g of dry extract. The resulting extract was reconstituted with distilled water to give desired concentrations used in this study.

Chemicals

All chemicals were of highest purity (≥ 99.0%). Ferric chloride, HCl, Dragendorff's reagent, magnesium metal strips, methanol, gallic acid, commercial saponins were purchased from Sigma, chloroform, H2SO4, Folin-Ciocalteu reagent, Na2CO3, vanillin, aluminium chloride, potassium acetate, phosphate buffer, K3Fe(CN)6, trichloroacetic acid (TCA), 2-thiobarbituric acid (TBA), thiocyanate (FTC), butylated hydroxyl toluene (BHT), 2, 2-diphenyl-1-picrylhydrazyl (DPPH), 2, 2'-azino-bis (3- ethylbenzthiazoline-6-sulphonic acid (ABTS), potassium persulphate, sodium nitroprusside, hydrogen peroxide, sulfanilic acid, glacial acetic acid, naphthylethylenediamine dichloride, potassium metabisulphite (PMS), NADH were all purchased from Merck.

Phytochemical screening of the plant extract

A small portion of the dry extract was used for the phytochemical tests for compounds which include, flavonoids, saponins with the methods of [11,12] with little modifications. Exactly 1.0 g of plant extract was dissolved in10 ml of distilled water and filtered (using Whatman No 1 filter paper) About 0.2 g of the extract was dissolved in 2 ml of methanol and heated. A chip of magnesium metal was added to the mixture followed by the addition of a few drops of concentrated HCl. The occurrence of a red or orange colouration was indicative of the flavonoids. Freshly prepared 7% blood agar plate was used and wells were made in it. The crude extract dissolved in 10% methanol was used to fill the wells bored in the blood agar plates. Ten percent methanol was used as a negative control while commercial saponin solution was used as a positive control. The plates were incubated at 35°C for 6 h. complete haemolysis of the blood around the extract was indicative of saponin.

Determination of total phenol

The amount of phenol in the aqueous extract of *C. asiatica* was determined with Folin-Ciocalteu reagent method [13] with slight modification. 2.5 ml of 10% Folin-Ciocalteu reagent and 2 ml of Na2CO3 (2% w/v) was added to 0.5 ml of each sample (3 replicates) of plant extract solution (1 mg/ml). The resulting mixture was incubated at45°C with shaking for 15 min. The absorbance of the samples was measured at 760 nm using UV/visible light. Results were expressed as milligrams of Gallic acid (0-0.5 mg/ml) issolved in distilled water.

Estimation of total flavonoids

Aluminum chloride colorimetric method[14] was used for flavonoids determination. One millilitre (1 ml) of sample (1 mg/ml) was mixed with 3 ml of methanol, 0.2 ml of 10% aluminum chloride, 0.2 ml of 1 M potassium acetate and 5.6 ml of distilled water and remains at room temperature for 30 min. The absorbance of the reaction mixture was measured at 367 nm with UV visible spectrophotometer. The content was determined from extrapolation of calibration curve which was made by preparing gallic acid solution (0-0.8 mg/ml) in distilled water. The concentration of flavonoid was expressed in terms of mg/ml.

Determination of reducing power

The reducing power of the extract was evaluated according to the method of Oyaizu [15]. The mixture containing 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of K3Fe(CN)6 (1%w/v) was added to 1.0 ml of the extract dissolved in distilled water. The resulting mixture was incubated at 50°C for 20 min, followed by the addition of 2.5 ml of TCA (10% w/v). The mixture was centrifuged at 3000 rpm for 10 min to collect the upper layer of the solution (2.5 ml), mixed with distilled water (2.5 ml) and 0.5 ml of FeCl3 (0.1%, w/v). The absorbance was then measured at 700 nm against blank sample.

2, 2-Diphenyl-1-Picrylhydrazyl (DPPH) assay

The method of Liyana-Pathiana and Shahidi [16] was used for the determination of scavenging activity of DPPH free radical. One ml of 0.135 mM DPPH prepared in methanol was mixed with 1.0 ml of aqueous extract ranging from 0.2-0.8 mg/ml. The reaction mixture was vortexed thoroughly and left in dark at room temperature for 30 min. The absorbance was measured spectrophotometrically at 517 nm. The scavenging ability of the plant extract was calculated using this equation;

DPPH Scavenging activity (%) = [(Abscontrol − Abssample)]/(Abscontrol)]×100

where Abscontrol is the absorbance of DPPH + methanol; Abssample is the absorbance of DPPH radical + sample (i.e. extract or standard).

Nitric oxide scavenging activity

The method of Garratt [17] was used to determine the nitric oxide radical scavenging activity of aqueous extract of *H. longifolium*. Sodium nitroprusside in aqueous solution at physiological pH spontaneously generate nitric oxide which interacts with oxygen to produce nitrite ions determined by the use of Griess reagents. Two milliliter of 10 mM sodium nitroprusside dissolved in 0.5 ml phosphate buffer saline (pH 7.4) was mixed with 0.5 ml of plant extract at various concentrations (0.2-0.8 mg/ml). The mixture was incubated at 25°C. After 150 min, 0.5 ml of incubation solution was withdrawn and mixed with 0.5 ml of Griess reagent [(1.0 ml sulfanilic acid reagent (0.33% in 20% glacial acetic acid at room temperature for 5 min with 1 ml of naphthylethylenediamine dichloride (0.1% w/v)]. The mixture was incubated at room temperature for 30 min. The absorbance was measured at 540 nm. The amount of nitric oxide radical was calculated following this equation:

% inhibition of NO = [A0 − A1]/A0 ×100

Where A0 is the absorbance before reaction and A1 is the absorbance after reaction has taken place.

Scavenging activity of superoxide anion

The scavenging activity of superoxide anion was determined by the method of Yen and Chen [18]. The reaction mixture consists of 1 ml of plant extract (1 mg/ml), 1 ml of PMS (60 μM) prepared in phosphate buffer (0.1 M pH 7.4) and 1 ml of NADH (phosphate buffer) was incubated at 25°C for 5 min, the absorbance was read at 560 nm against blank samples.

Hydrogen peroxide scavenging activity

Scavenging activity of hydrogen peroxide by the plant extract was determined by the method of Ruch et al. [19]. Plant extract (4 ml) prepared in distilled water at various concentration was mixed with 0.6 ml of 4 mM H2O2 solution prepared in phosphate buffer (0.1 M pH 7.4) and incubated for 10 min. The absorbance of the solution was taken at 230 nm against blank solution containing the plant extract without H2O2.

Extraction of oil and GC MS analysis

Extraction of essential oil

Hydrodistillation of the plant material was performed in a Clevenger-type apparatus for 3h and half. The oil obtained was light yellow, liquid at room temperature and its odor was agreeable.

After its isolation, the essential oil was collected and stored in steeled glass vials in refrigerator at 4-5°.

The samples were analysed by GC-MS (Schimadzu) using capillary column. The GC-MS conditions were as follows; injection volume (1 mL), temperature programme 80°C to 160°C for 5 min at 10°C/min; 160°C to 235°C

for 5 min at 5°C/min and 235°C to 290°C for 5 min at 50°C/min.; injector temperature (280°C), MS transfer line (290°C), ion source (200°C) spit ratio (1:10) and mass range at 50-450. Data was analysed by by compared to a SI (standard index) from the NIST library available.

RESULTS

Phytochemical screening.
The phytochemical analysis conducted on *C. asiatica* extract revealed the presence of flavonoids and saponins. The total phenol content of the aqueous leaf extract was 0.387 and 0.178 mg gallic acid equivalent/g of extract power in stem. The total flavonoid contents were 0.225 in leaves and 0.008 mg gallic equivalent/g of extract powder in stem respectively with reference to standard curve (Y = 0.0067x+0.0132, r2 = 0.999) (Table 1).

These phytochemical compounds are involved in most of bioactive activities in medicinal plants and thus they are responsible for the antioxidant activities of this plant extract used in this study.

Free radical scavenging activities
The reducing power potentials of the extract
The reducing power potentials of the aqueous extract of the test plant in comparison with a standard BHT at 700 nm is shown in Fig.1. The reducing capacity of the extract, is found to be significant in leaves as well as stem extract. In the reducing power assay, the presence of antioxidants in the sample would result in the reduction of Fe3+ to Fe2+ by donating an electron. The results show that the reducing power potential is dose dependent, increase in reducing power of the plant extract as the extract concentration increases.

Free Radical Scavenging Potential
The percentage inhibition of scavenging activities of the aqueous extract of *C. asiatica* leaves and stem for DPPH, hydrogen peroxide, nitric oxide and superoxide anion radical were shown in Table 2. The nitric oxide radical scavenging activity of the extract at 0.4mg/ml, which was the highest concentration of the extract tested, was 63.83% and 36.18% in leaves and stem respectively. The leaf extracts showed appreciable free radical scavenging activities on hydrogen peroxide, superoxide anion radical and DPPH. The percentage inhibitions at 0.4 mg/ml are 71.47%, 63.83% and 72.32% for hydrogen peroxide, DPPH and superoxide anion radicals respectively (Table 2).

Composition of essential oil
The essential oil content quantified showed presence of 10 compounds (Tab. 3) in which, dodecamethylcyclohexasiloxane has showed the highest (17%) and decamethylcyclopentasiloxane showed the lowest percentage (1.6%).α-cubebene, comprised of 10% .

Discussion
The phytochemical analysis conducted on *C. asiatica* extract revealed the presence of flavonoids, and saponins. Flavonoids can either directly scavenge superoxides, or can scavenge the highly reactive oxygen derived radical called peroxynitrite[21]. Flavonoids acts as health promoting compound as a results of its anion radicals [22]. The presence of saponin in the plant extract reveals its medicinal potential, as saponins are known to to produce inhibitory effect on inflammation [23] and thus responsible for certain biological effects [24]. The presence of these two important phytochemical constituents in *C. asiatica* justify its use in traditional medicine. These compounds can contribute for their antioxidative properties and thus the usefulness of these plants in herbal based medicines. Phenolic compounds have been found to useful in the formulations of antimicrobial substances.

The results of free radical scavenging activity assay of this plant shows its potential against caused of free radicals, which indicates that the extract contains phytochemical constituents are capable of donating hydrogen to a free radical in order to remove odd electron which is responsible for radical's reactivity. The result of DPPH scavenging activity implies that the plant extract may be useful for treating radical related pathological damage [25].

The Superoxide anion radical scavenging activity of this radical by the plant extract compared with gallic acid suggests that the plant is also a potent scavenger of superoxide radical.

The study of activity of Hydrogen peroxide is important because of its ability to penetrate biological membranes. Hydroxyl radicals are highly reactive[26] and can induce severe damage to adjacent molecules[27]. Scavenging of

H2O2 by the plant extracts may be attributed to their phenolics, which donate electron to H2O2, thus reducing it to water. The extract was capable of scavenging hydrogen peroxide in a concentration dependent manner.

The NO_ scavenging activity of the extract is of potential health interest as it has been proposed that NO_ plays an important role in the progression of many diseases and pathological conditions such as septic shock, atherosclerosis, ischaemia reperfusion, neurodegenerative disorders like Alzheimer's and Parkinson's diseases, cancer and diabetes [28]. The level of nitric oxide was significantly reduced in this study by the crude extract. Since NO plays a crucial role in the pathogenesis of inflammation [29], this may help for the further use of *C. asiatica* in the treatment of inflammation and for wound healing.

Free radicals are known as major contributors to several clinical disorders such as diabetes mellitus, cancer, liver diseases, renal failure and degenerative diseases as a result of deficient natural antioxidant defence mechanism [30]. Thus the potent activity of the extract will direct towards the further use of *C. asiatica* in herbal based biomadicines.

Essential oil composition

In the present study, the components present in the essential oil are identified using NIST library. The composition of essential oils showed dodecamethylcyclohexasiloxane the highest (18%) and decamethylcyclopentasiloxane showed the lowest percentage (1.6%). These compounds were reported to be in many personal care products such as toiletries. volatile compounds is essential to determine the predominant components and their composition in order to investigate their bioactivity including antioxidant and antibacterial activities. A number of reports have shown that plant volatile compounds exhibited potent antioxidant and antibacterial activities[31]. α-cubebene was reported to show potent antibacterial properties[32] and antioxidant properties[33].

Table 1. The phytochemical components of *C. asiatica* based on the preliminary aqueous crude leaf extract screening

Phytochemical compounds		Presence	Extract equivalent of Gallic (mg/g)
Flavonoids	Leaves	++	ND
	Stem	+	ND
Saponins	Leaves	+++	ND
	Stem	++	ND
Total Polyphenols	Leaves	+++	0.387
	Stem	++	0.178
Total Flavonoids	Leaves	+++	0.225
	Stem	++	0.008

+++ = appreciable amount (positive within 5 mins.); ++ = moderate amount (positive after 5 mins. but within 10 mins); + = trace amount (positive after 10 mins. but within 15 mins); - = completely absent.

Table 2: Radical scavenging activities of aqueous crude leaf extract of *C. asiatica* at different concentrations
Percentage inhibition (% I) of radical scavenging

Extract concentration (mg/ml)		Superoxide anion	Nitric oxide	DPPH	Hydrogen peroxide
Leaves	0.2	68.18	61.28	54.58	68.57
	0.4	72.32	63.83	64.39	71.47
Stem	0.2	57.15	21.58	38.29	47.18
	0.4	52.39	36.18	43.73	54.92

Table 3. Composition of essential oil in *C. asiatica* leaves

Peak	Rt	Compound name	%
1.	13.4	Dodecamethylcyclohexasiloxane	**19**
2.	20.1	Tetradecamethyl-cycloheptasiloxane	14.3
3.	16.2	α-Cubebene	14
4.	2.5	2,4-Dimethylhexane	13
5.	5.3	6,6-Methylenebicyclo[3.1.1]heptane	12
6.	21.9	Cadina-1(10),4-diene	6.2
7.	27.1	Hexadecamethyl-cyclooctasiloxane	4.9
8.	6.6	Octamethylcyclo-tetrasiloxane	4.1
9.	18.1	Isocaryophillene	3.2
10.	6.9	Dehydro-N-[4,5-methylenedioxy-2-nitrobenzylidene]-tyramine	2.3

The antibacterial efficacy of essential oil was evaluated in the present study. The oil revealed weak to good antimicrobial activity against Staphylococcus aureus,Bacillus cereus , Escherichia coli. Gram-positive bacteria are

more sensitive to plant oils and extracts than Gram-negative bacteria [34]. The findings suggest feasibility of application of C. asiatica oil in treatment of the infections caused by those microorganisms.

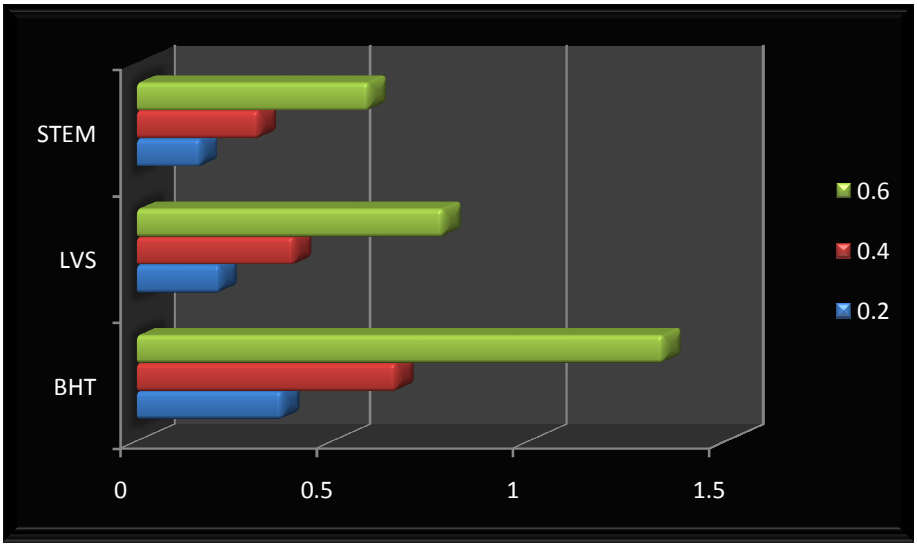

Fig.1. Reducing power activities of the aqueous extract of *Leaves and stem of C. asiatica* in comparison with a standard (BHT) at λ = 700 nm. BHT: Butylated hydroxyl toluene

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