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Pharmacological Study

In vitro evaluation of antioxidant activity of *Cordia dichotoma* (Forst f.) bark

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Abstract

Cordia dichotoma Forst. f. bark, identified as botanical source of *Shleshmataka* in Ayurvedic pharmacopoeia. Present investigation was undertaken to evaluate possible antioxidant potential of methanolic and butanol extract of *C. dichotoma* bark. *In vitro* antioxidant activity of methanolic and butanol extract was determined by 1,1, diphenyl–2, picrylhydrazyl (DPPH) free radical scavenging assay. The extracts were also evaluated for their phenolic contents and antioxidant activity. Phenolic content was measured using Folin–Ciocalteu reagent and was calculated as Gallic acid equivalents. Antiradical activity of methanolic extract was measured by DPPH assay and was compared to ascorbic acid and ferric reducing power of the extract was evaluated by Oyaizu method. In the present study three *in vitro* models were used to evaluate antioxidant activity. The first two methods were for direct measurement of radical scavenging activity and remaining one method evaluated the reducing power. The present study revealed that the *C. dichotoma* bark has significant radical scavenging activity.

Key words: Antioxidant, anti–radical, *Cordia dichotoma*, diphenyl–2, picrylhydrazyl, reducing power, *Shleshmataka*

Introduction

Oxidative stress has been implicated as a primary factor in the progression of many degenerative diseases, such as cancer, hyperlipidemia, gastric ulcer, and diabetes.[1] Antioxidants are vital substances which possess the ability to protect body from damage caused by free radical–induced oxidative stress.[2] Radicals and other reactive oxygen species (ROS) are formed constantly in the human body and are removed by the enzymic and non–enzyme antioxidant defense systems.[3] There is an increasing interest in natural antioxidants, for example, polyphenols, present in medicinal and dietary plants, which might help prevent oxidative damage.[4] ROS including free radicals, such as superoxide anion radicals (O_2), hydroxyl radicals (OH^-) andnon–free radicals, such as H_2O_2 andsinglet oxygen (O_2) along with various forms of active oxygen are involved in various physicochemical processes in the body and aging derivatives of oxygen, attributed as ROS, are continuously generated inside the human body. The generated ROS are detoxified by the antioxidants present in the body. However, overproduction of ROS and/or inadequate antioxidant defence can easily affect and persuade oxidative damage to various bimolecular substances, including proteins, lipids, lipoproteins, and DNA.[5] This oxidative damage is a critical etiologic factor implicated in several chronic human diseases, such as diabetes mellitus, cancer, atherosclerosis, arthritis, and neurodegenerative diseases and also in the aging process. Moreover, knowledge and application of such potential antioxidant activities in reducing oxidative stresses in vivohas prompted many investigators to search for potent and cost–effective antioxidants from various plant sources.[6-9] Antioxidants are micronutrients that have gained importance in recent years due to their ability to neutralize free radicals or their actions.[10] The majority of the antioxidant activity is due to the flavones, isoflavones, flavonoids, anthocyanin, Coumarines, lignans, catechins, and isocatechins.[11] These research activities have contributed to new or renewed public interests worldwide in Phytomedicines.

Cordia dichotoma Forst. f. (Boraginaceae) is tree of tropical and subtropical regions, commonly known as *Lasura* in Hindi and *Shleshmataka* in Sanskrit. It is a medium-sized tree with short crooked trunk, leaves simple, entire and slightly dentate, elliptical–lanceolate to broad ovate with round and cordite base, flower white, fruit drupe, yellowish brown, pink or nearly black when ripe with viscid sweetish transparent pulp surrounding a central stony part.[12] It grows in sub–Himalayan tract and

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outer ranges, ascending up to about 1500 m elevation.[13] It is used as immunomodulator, antidiabetic, anthelmintic, diuretic, and hepatoprotective in folklore medicine. *C.dichotoma* seeds have disclosed the presence of α–Amyrin, betulin, octacosanol, lupeol–3–rhamnoside, β–sitosterol, β–sitosterol–3–glucoside, hentricontanol, hentricontane, taxifolin–3, 5–dirhmnoside, and hesperitin–7–rhamnoside.[14] Preliminary phytochemical analysis of *C. dichotoma* bark indicated the presence of relatively high levels of alkaloids, flavonoids, terpenoids, and steroids. Hence the present investigation was undertaken to determine the antioxidant potential of *C. dichotoma* bark.

Materials and Methods

Plant material

C. dichotoma bark was collected by scholar from its natural habitat, Jamnagar, Gujarat, India, in the month of April–May 2009. The plant was authenticated in the Pharmacognosy Laboratory, Institute for Post Graduate Teaching and Research in Ayurveda, Gujarat Ayurved University, Jamnagar, Gujarat, India.

Preparation of plant extract

The bark was shade dried and crushed to make coarse powder. The powder (303 g) was successively extracted by Soxhlet extraction with solvents of increasing polarity beginning with 2 L petroleum ether (60°C-80°C) and then extracted with 3 L of methanol (95%v/v) by continuous extraction method for 48 h. In this methanolic extract, solvent was distilled off and the extract was concentrated and dried under reduced pressure, which yielded a brownish green mass. The extract was preserved at 2°C-4°C and Butanol (BuOH) extracts were obtained after successive partition from Methanol (MeOH) extracts. MeOH and BuOH extracts were used for this study, which were used for further isolation study. This crude extract of methanol and butanol was used for further investigation for potential of antioxidant properties.

Preliminary phytochemical screening of extract

The methanolic extract was testing to detect for the presence of different chemical groups of compounds as per the methods described in Ayurvedic Pharmacopoeia of India. Preliminary phytochemical screening shows the presence of relatively high levels of alkaloids, flavonoids, coumarins, terpenoids, tannins, and steroids.

Chemicals and instruments

Chemicals

The chemicals 2,–2–Diphenyl–1–picrylhydrazyl (DPPH, Lancaster–, UK) andgallic acid (Loba,–India) were purchased from Krishna Scientific Traders, Rajkot, Gujarat, India. Folin–Ciocalteu reagent, sodium carbonate, ascorbic acid, hydrogen peroxide, potassium ferricyanide, trichloroaceticacid, ferric chloride, and all other reagents of analytical grade were obtained from the Pharmaceutical Chemistry Laboratory of I.P.G.T and R.A., Jamnagar, Gujarat, India.

Instrument

UV spectrophotometer (Model–UV–1601, Shimadzu Europa, Gmbh, Germany) Centrifuge (Model–TC–4100D, Eltek Equipment Pvt. Ltd., Vasai (E), Thane, India).

Antioxidant assay

The antioxidant activity of plant extracts were determined by different *in vitro* methods, such as the DPPH free radical scavenging assay, phenolics content by Folin–Ciocalteu reagent and reducing power methods using Oyaizu method. All the assays were carried out in triplicate and average values were considered.

2,2-Diphenyl-1-picrylhydrazyl assay

The free radical scavenging capacity of the methanolic and butanol extracts of bark was determined using DPPH method.[15] It was measured by a decrease in absorbance at 516 nm of a solution of colored DPPH in methanol brought about by the sample.[16-18] A stock solution of DPPH (1.3 mg/mL in methanol) was prepared. The concentration of methanolic and butanol extracts solution was 10 mg/10 mL. From this solution, 1mLwas taken in test tubes and diluted with the same solvent up to 10 mL. This is stock solution. From stock solution 0.10, 0.15, 0.25, 0.50, and 0.60 mL was taken in different test tubes, whose concentration was then 10, 15, 25, 50, and 60 µg/mL, respectively. Freshly prepared DPPH solution 75 µL (1.3 mg/mL) was added in each of these test tubes containing methanolic and butanol extracts and kept in dark for 30 min, the absorbance was taken at 517 nm using a spectrophotometer (Systronics UV–Visible Spectrophotometer 2201) IC_{50} was calculated from % inhibition. Ascorbic acid was used as a reference standard and dissolved in distilled water to make the stock solution with the same concentration of methanolic and butanol extracts of *C. dichotoma*. Control sample was prepared containing the same volume without any extract and reference ascorbic acid; 95% methanol was used as blank and % scavenging of the DPPH free radical was measured using the following equation:

% inhibition = {(A control – A sample)/(A control)} ×10

A control = absorbance of DPPH alone

A sample = absorbance of DPPH along with different concentrations of extracts.

Total phenolic content

The total phenolics content of the extract was estimated according to the method described by Singleton and Rossi.[19] The concentration of methanolic and butanol extracts solution was 10 mg/10 mL. From this solution, 1mLwas taken in test tubes and by dilution with same solvent up to 10 mL. This is stock solution. From stock solution different concentrationswere taken in different test tubes. This same procedure was used for standard. Gallic acid (Loba Chemie Pvt. Ltd., Mumbai) was used as a standard; 1 mL of Folin–Ciocalteu reagent was added in this concentration and the content of the flask was mixed thoroughly and 5 min later 4 mL of 20% sodium carbonate was added, and the mixture was allowed to stand for 30 min with intermittent shaking. The absorbance of the blue color that developed was read at 765 nm in UV spectrophotometer.

Reducing power

The Fe^{3+}–reducing power of the extract was determined by the method of Oyaizu[20] with a slight modification. Different concentrations (10, 25, 50, 75, 100 µg/mL) of the extract were mixed with 2.5 mL phosphate buffer (0.2 M, pH 6.6) and 2.5 mL potassium hexacyanoferrate (1%), followed by incubation at 50°C in a water bath for 20 min. After incubation, 2.5 mL of TCA (10%) was added to terminate the reaction and centrifuged at 3000 rpm for 10 min. The upper portion of the solution (2.5 mL) was mixed with 2.5 mL distilled water and 0.5 mL $FeCl_3$ solution (0.1%) was added and the absorbance

was measured at 700 nm against an appropriate blank solution. Ascorbic acid at various concentrations (10-100 µg/mL) was used as standard. Increased absorbance of the reaction mixture indicated increased reducing power.

Results

Several concentrations ranging from 10 to 250 µg/mL of the *C. dichotoma* bark were tested for antioxidant activity in different *in vitro* models.

2,2-Diphenyl-1-picrylhydrazyl activity

C. dichotoma exhibited a comparable antioxidant activity with that of standard ascorbic acid at varying concentrations tested (10, 15, 25, 50, 60 µg/mL). There was a dose–dependent increase in the percentage antioxidant activity for all concentrations tested. Ascorbic acid was used as the standard drug for the determination of the antioxidant activity by DPPH method. The concentration of ascorbic acid varied from 1 to 60 µg/mL. Ascorbic acid at a concentration of 10 µg/mL exhibited a percentage inhibition of 52.74% and for 60 µg/mL 99.86% [Table 1]. The IC50 value of ascorbic acid was less than 10 approx to be 6.1 µg/mL. IC50 value was observed 28 µg/mL for the MeOH extract and 36 for BuOH extract. From Figure 1 and Table 1, it is observed that both extracts show significant DPPH radical scavenging property.

Total phenolic content

The total phenolics content of MeOH and BuOH extract of *C. dichotoma* calculated as gallic acid equivalent of phenols was detected. The total phenol content shows good linear relation in both standard and sample extract. Figure 2 and Table 2 show that the *C. dichotoma* contains a considerable amount of phenols (gallic acid equivalent).

Reducing power

Reducing power assay method is based on the principle that substances, which have reduction potential, react with potassium ferricyanide (Fe^{3+}) to form potassium ferrocyanide (Fe^{2+}), which then reacts with ferric chloride to form ferric–ferrous complex that has an absorption maximum at 700 nm. Figure 3 shows how the reducing power of the test extracts increases with the increase in amount of sample [Table 3]. The reducing power shows good linear relationship in both standard (R^2 = 0.950) andMeOH extract (0.975) and BuOH extract (R^2 = 0.978).

Discussion

ROS are involved in the pathogenesis of various diseases. Uncontrolled oxidation is caused by free radicals. Free radicals oxidize all major classes of biomolecules. The products of these oxidation reactions diffuse from the original site of attack and spread the damage all over the body and produces serious damage

Table 1: Percentage inhibition of standard (ascorbic acid) and test drug

Concentration (µg/ml)	% inhibition MeOH	% inhibition BuOH	% inhibition STD
10	31.25	28.12	52.74
15	40.25	30.56	62.33
25	49.05	41.25	78.57
50	71.34	59.24	98.79
60	85.25	80.21	99.86

Table 2: Absorbance at various concentrations (µg/mL) of standard (gallic Acid) and test drug in total phenolicscontain

Concentration (µg/mL)	Absorbance-MeOH	Absorbance-BuOH	Absorbance-STD
25	0.08	0.091	0.194
50	0.150	0.131	0.395
75	0.270	0.219	0.652
100	0.392	0.339	0.790
200	0.58	0.52	1.192
250	0.829	0.616	

Table 3: Absorbance of various concentrations (µg/mL) of standard (ascorbic acid) and test drug in ferric reducing power

Concentration (µg/mL)	Absorbance-MeOH	Absorbance-BuOH	Absorbance-STD
10	0.081	0.061	0.09
25	0.095	0.078	0.122
50	0.189	0.132	0.240
75	0.210	0.201	0.352
100	0.291	0.242	0.471

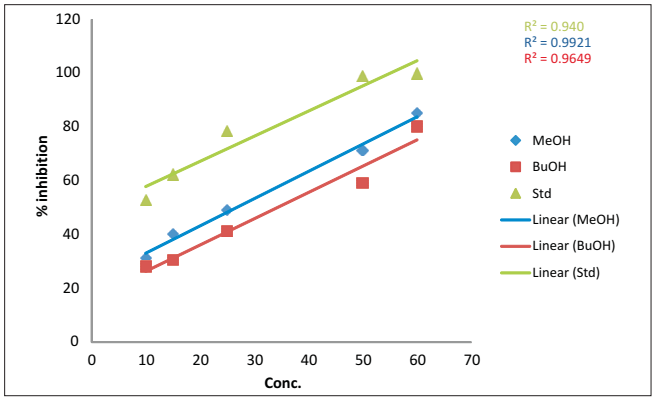

Figure 1: DPPH free radical scavenging activity (% inhibition vs concentration graph for standard and test drug)

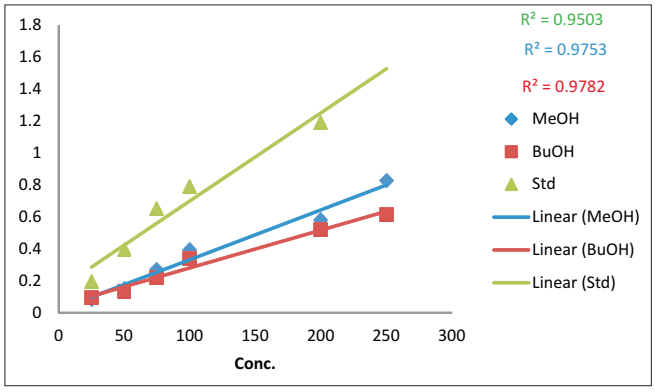

Figure 2: Effect of MeOH and BuOH extract on OH radical scavenging activity

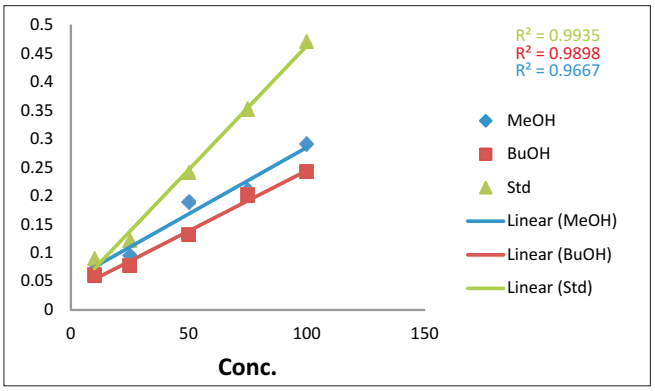

Figure 3: Effect of MeOH and BuOH extract on reducing power

to almost all the cells. Natural antioxidants that are present in herbs and spices are responsible for inhibiting or preventing the deleterious consequences of oxidative stress. Spices and herbs contain free radical scavengers, such aspolyphenols, flavonoids, and phenolic compounds. The effect of antioxidants on DPPH radical scavenging is thought to be due to their hydrogen donating ability. DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule. The reduction capability of DPPH radicals was determined by the decrease in its absorbance at 517 nm induced by antioxidants. Many antioxidants that react quickly with peroxyl radicals may react slowly or may even be inert to DPPH.[21]

Phenolic compounds are known as powerful chain breaking antioxidants. Phenols are very important plant constituents because of their scavenging ability due to their hydroxyl groups. The phenolic compounds may contribute directly to antioxidant action. It is suggested that polyphenoliccompounds have inhibitory effects on mutagenesis and carcinogenesis in humans, when ingested up to 1g daily from a diet rich in fruits and vegetables.[22]

Ferric reducing antioxidant power measures the ability of antioxidants to reduce ferric 2,4,6-triperidyl–s–triazine complex to intensively blue colored ferrous complex in acidic medium. Hence any compound which is having redox potential lower than that of redox pair Fe (III)/Fe (II) can theoretically reduce Fe (III) to Fe (II).[21]

Conclusion

C. dichotoma showed strong antioxidant activity by inhibiting DPPH, and reducing power activities when compared with standard l–ascorbic acid. In addition, both the extractswere found to contain a noticeable amount of total phenols, which play a major role in controlling oxidation. The results of this study show that the extract can be used as an easily accessible source of natural antioxidant. However, the chemical constituents present in the extract such as alkaloids, flavonoids, proteins, reducing sugars, steroids, terpenoids, and tannins present in the extract may be responsible for such activity. The phytoconstituents responsible for the antioxidant activity of *C. dichotoma* are currently unidentified. Therefore, it is suggested that activity–guided isolation study should be performed.

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हिन्दी सारांश

कॉर्डिया डायकोटोमा (फ़ोर्स्ट.एफ़.) के काण्डत्वक् की एण्टीऑक्सिडण्ट क्रियाशीलता का प्रयोगशालीय परीक्षण

पंकज बी. नारिया, नयन आर. भालोडिया, विनय जे. शुक्ला, रबिनारायण आचार्य, मुकेश बी. नारिया

कॉर्डिया डायकोटोमा (फ़ोर्स्ट.एफ़.) की काण्डत्वक् को आयुर्वेदिक फ़ार्माकोपिया द्वारा 'श्लेष्मातक' वनस्पति के वनस्पतिशास्त्रीय स्त्रोत के रूप में निर्धारित किया गया है । इस द्रव्य की सम्भाव्य एण्टीऑक्सिडण्ट क्रियाशीलता को परखने हेतु प्रस्तुत अध्ययन किया गया है। मिथेनॉल एवं ब्युटेनॉल विद्राव्य सत्त्वों की प्रयोगशालीय एण्टीऑक्सिडण्ट क्रियाशीलता डीपीपीएच फ्री रेडिकल स्केवेंजिंग परीक्षण एवं फ़ेरिक रिड्युसिंग एण्टीऑक्सिडण्ट पॉवर विधियों द्वारा निर्धारित कर उसकी एस्कॉर्बिक एसिड के साथ तुलना की गई । साथ ही फ़ॉलिन–सिओकेल्ट्यु विधि द्वारा टोटल फ़िनॉल कण्टेण्ट भी गेलिक एसिड के समकक्ष निर्धारित किया गया । प्रस्तुत अध्ययन यह सिद्ध करता है कि कॉर्डिया डायकोटोमा के काण्डत्वक् में लक्षणीय एण्टीऑक्सिडण्ट क्रियाशीलता है ।