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IN VITRO ANTIOXIDANT ACTIVITY OF METHANOL EXTRACT OF BARK OF *CORDIA DICHOTOMA* FORST

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ABSTRACT: In this study, the methanol extract of bark of *Cordia dichotoma* Forst., belonging to the family Boraginaceae was prepared by successive extraction procedure (petroleum ether, ethyl acetate, methanol) in soxhlet apparatus and subsequently, the extract was evaluated for its phytoconstituents and pharmacological activity. Comparatively, higher yield (7.11%) of methanol extract was obtained. Phytochemical tests revealed the presence of alkaloid, glycosides, proteins, amino acids, phenolic compounds, flavonoids, tannins and saponins in the extract. The methanol extract was evaluated for its *in vitro* antioxidant property by 1, 1-diphenyl-2-picryl hydrazyl (DPPH) radical assay; sodium nitroprusside generated nitric oxide (NO), hydrogen peroxide (H_2O_2).Total phenolic content and total flavonoid contents were also carried out. The extract showed IC_{50} values of 62.46 µg/mL, 48.75µg/mL, 63.5 µg/mL for DPPH free radical, total phenolic content and flavonoid content, respectively. The current study is carried out to investigate the free radical scavenging potential of methanolic extract of bark of *Cordia dichotoma* using *in-vitro* models. These models demonstrate antioxidant activity in a concentration dependent manner.

INTRODUCTION: Antioxidants are a group of substances, which inhibit or delay oxidative processes. Most of the potentially harmful effects of oxygen are due to the formation and activity of a number of chemical compounds, known as reactive oxygen species (ROS), which have the tendency to donate oxygen to other substances. Many such reactive species are free radicals. Type of free radicals include the hydroxyl radical (OH), superoxide radical (O_2), the nitric oxide radical (NO), and the lipid peroxy radical (LOO).

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This oxidative damage is a decisive etiological factor concerned in quite a lot of chronic human diseases such as diabetic mellitus, cancer and also in the aging course [1].

Free radicals due to environmental pollutants, radiation, chemicals, toxins, deep fried and spicy foods as well as physical and mental stress, cause depletion of immune system antioxidants, change in gene expression and induce abnormal proteins. Due to these problems natural antioxidants as free radical scavengers may be necessary [2, 3, 4].

It has been mentioned the antioxidant activity of plants might be due to their phenolic compounds [2]. Flavanoids are a group of polyphenolic compounds with known properties, which include free radical scavenging, inhibition of hydrolytic and oxidative enzymes and anti-inflammatory action [5].

The biological action of these compounds is related to their antioxidant activity [6]. The rapid and sensitive for the antioxidant screening of plant extract is free radical scavenging assay using 1,1-diphenyl-2-picryl hydrazyl (DPPH) stable radical Spectrophotometrically (UV spectrophotometer, Shimadzu-UV-1601). Being presence of an antioxidant, DPPH radical obtains one more electron and the absorbance decreases [7]. The majority of the antioxidant activity is due to the flavones, isoflavones, flavonoids, anthocyanin, coumarins, lignans, catechin and isocatechin [8].

In an effort to expand the spectrum of antioxidant agents from natural resources, *Cordia dichotoma* Forst. belonging to Boraginaceae family has been selected. While, the whole plant has antidiabetic & antileprotic activity [9, 10, 11], *Cordia* genus is reported for other activities like wound healing [12], anthelmintic [13], anti-inflammatory [14], gastro-protective and antiulcer [15] and antioxidant [16]. Several research workers have reported the mentioned activities on the other plants belong to Boraginaceae family and other parts of plant except bark of *Cordia dichotoma* Forst. However, there is no scientific report on the bark of the plant *Cordia dichotoma* Forst. of same family .

Therefore, the objective of the present investigation was to explore its phytoconstituents and pharmacological activities, specifically for its antioxidant potential.

MATERIAL AND METHODS:

Plant Material: The plant *Cordia dichotoma Forst*. (Family: Boraginaceae) was collected from the local forest, Ghaziabad, Uttar Pradesh, India, in the month of May-June, 2012. The plant was identified from National Institute of Science Communication and Information Resources, New Delhi. A voucher specimen (NISCAIR/RHMD/ Consult/2012-13/2025/33) has been preserved in laboratory for further references. The bark was washed, dried at room temperature under shed and then grounded in a mill to a coarse powder.

Extraction of plant material: The powdered bark was subjected to successive soxhlet extraction using a series of solvents of increasing polarity starting from petroleum ether, ethyl acetate, and methanol respectively. The extracts were vacuum dried and the percentage yields of the extracts were 1.5%, 1.0%, 7.11%, respectively.

Preliminary phytochemical analysis: The phytochemical tests were performed using various reagents as described in **table 1**. The extract was tested for the presence of absence of alkaloids, glycosides, tannins, steroids, reducing sugars, proteins and amino acids, phenolic compounds and flavonoids (Table 1).

TABLE 1: PRELIMINARY PHYTOCHEMICAL SCREENING OF MECD EXTRACT

Phytoconstituents	Test performed/reagents used	Presence or absence
Alkaloids	Meyers test	+
	Dragendroffs test	+
	Hagers test	+
Steroids	Libermann-Burchard test	-
Flavonoids	Shinoda test	+
Tannins	Ferric chloride	+
	Lead acetate	+
Saponins	Test for stable foam	+
Glycoside	Borntrager test	+
Protein and amino acid	Ninhydrin test	-
Reducing sugar	Fehlings test	+
	Benedict test	+

Acute toxicity study: The acute toxicity of methanol extract of *Cordia dichotoma* (MECD) was studied on swiss albino mice (40-45g) "Fixed dose procedure"-OECD 420. The Institutional Animal Ethical Committee permitted the use of the animals for this purpose.

After fasting condition for overnight, the animals were administered a fixed maximum dose 2000 mg/kg BW intraperitoneally. No animals were found died after 24 hrs [17]. Hence, the dose 2000 mg/kg BW is safe and two doses would be used for further studies.

Evaluation of Antioxidant activity of Methanol (MECD) extract:

DPPH radical scavenging activity of MECD extract: The stable 1, 1-diphenyl-2-picryl hydrazyl radical (DPPH) was used for determination of free radical scavenging activity of the MECD extract [7] The MECD extract was added at different concentrations (10-200 µg/mL) with an equal volume, to methanol solution of DPPH (100 um). After incubation for 15 minutes at room temperature, the absorbance was recorded at 517 nm. The experiment was carried out in triplicate. The IC_{50} value i.e. the concentration of sample required to scavenge 50% of stable DPPH free radicals was determined from the % inhibition vs. concentration of MECD extract curve by comparing the absorbance values of control (Ao) and test compounds (At) (**table 2, fig. 1**).

$$\% \text{ Inhibition} = (Ao\text{-}At/Ao) \times 100$$

TABLE 2: DPPH FREE RADICAL SCAVENGING OF MECD EXTRACT

Conc. of MECD Extract (µg/mL)	Mean± SEM (Absorbance of Extract)	% Inhibition of Extract	% Inhibition of Std. (Ascorbic acid)
10	0.468± 0.0010	29.94	43.86
25	0.412± 0.0021	38.32	49.85
50	0.342± 0.0021	48.80	57.18
100	0.244± 0.0042	63.47	70.35
200	0.047± 0.0026	92.96	98.65

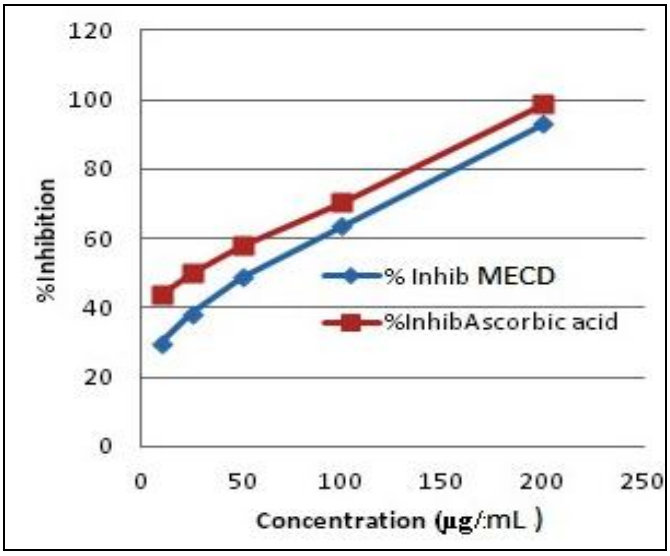

FIG. 1: DPPH SCAVENGING ACTIVITY OF MECD EXTRACT VS STD.

Nitric oxide scavenging activity of MECD extract: Nitric oxide scavenging activity was measured following the method reported earlier [18, 19]. The reaction mixture containing 1.5 mL sodium nitroprusside (10mM) in phosphate buffer saline and 1.5 mL of different concentration (10-200µg/ml) methanol extract was incubated at 25°C for 150 min. Following incubation, 0.5 mL of reaction mixture was mixed with 0.5 mL of Griess reagent (1% sulphanilamide, 2% H_3PO_4 and 0.1% naphthyl ethelene diamine dihydrochloride). The percentage inhibition of nitric oxide generated from sodium nitroprusside was measured by comparing the absorbance values of control (Ao) and test compounds (At) at 546 nm (**table 3, fig. 2**). The following equation was used;

$$\% \text{ Inhibition} = (Ao\text{-}At/Ao) \times 100$$

TABLE 3: NITRIC OXIDE (NO) SCAVENGING OF MECD EXTRACT

Conc. of MECD Extract (µg/mL)	Mean± SEM (Abs. of Extract)	% Inhibition of Extract	% Inhibition of Std. (Ascorbic acid)
10	0.376± 0.0029	6.93	24.75
25	0.360± 0.0013	10.89	28.21
50	0.342± 0.0035	15.34	38.12
100	0.273± 0.0056	32.42	50.49
200	0.252± 0.0035	37.62	73.52

Scavenging activity of Hydrogen peroxide radical: The hydrogen peroxide scavenging of methanol extract was determined according to the method reported elsewhere [20]. A solution of hydrogen peroxide (40mM) was prepared in phosphate buffer (pH 7.4) and concentration was determined spectrophotometrically at 230 nm. Different concentration of extracts (10-200 µg/mL) in distilled water was added to a hydrogen peroxide solution (0.6 mL, 40 mM) and the absorbance of hydrogen peroxide at 230 nm was determined after 20 min against a blank solution in phosphate buffer without hydrogen peroxide (**table 4, fig. 3**).

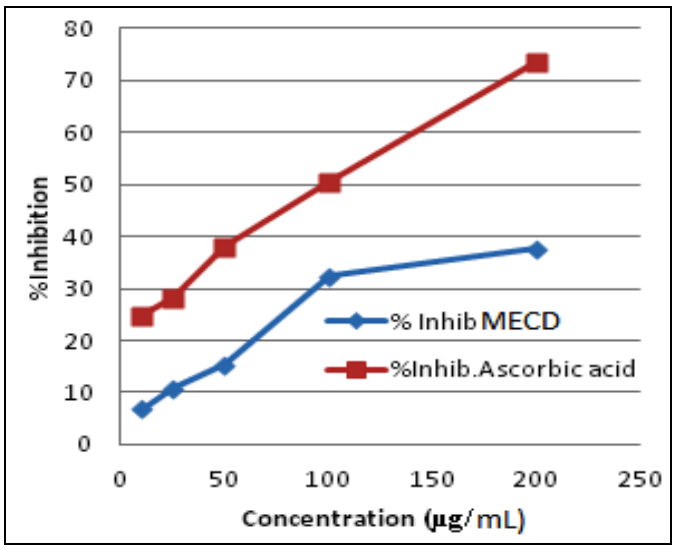

FIG. 2: NITRIC OXIDE (NO) SCAVENGING ACTIVITY OF MECD EXTRACT VS STD.

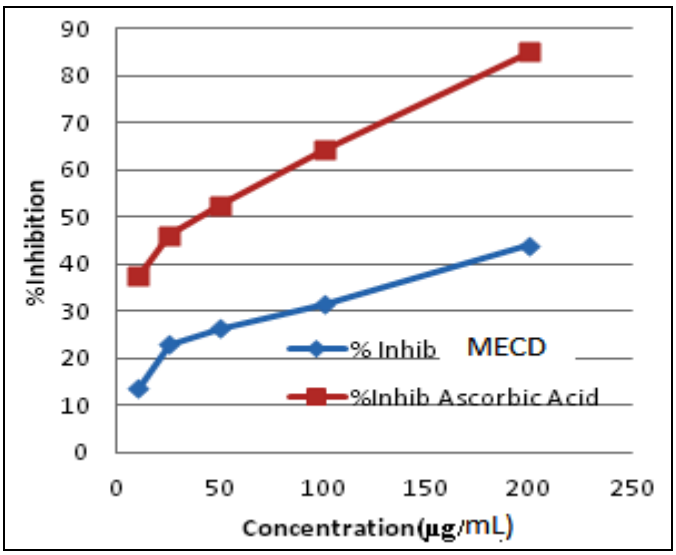

FIG. 3: HYDROGEN PEROXIDE (H_2O_2) SCAVENGING ACTIVITY OF MECD EXTRACT VS STD.

TABLE 4: HYDROGEN PEROXIDE (H_2O_2) SCAVENGING ACTIVITY OF MECD EXTRACT

Conc. of MECD Extract (µg/mL)	Mean± SEM (Abs. of Extract)	% Inhibition of Extract	% Inhibition of Std. (Ascorbic acid)
10	0.290± 0.0014	13.69	37.50
25	0.259± 0.0021	22.9	46.13
50	0.247± 0.0007	26.48	52.67
100	0.230± 0.0014	31.54	64.28
200	0.188± 0.0010	44.047	85.12

The percentage scavenging of hydrogen peroxide was calculated using the following equation:

% Scavenged (H_2O_2) =

$(A_{control} - A_{sample}/A_{control}) \times 100$

The concentration of the extract was plotted against the % inhibition and IC_{50} value was determined.

Total Phenolic Content determination: Total phenolic content was estimated by the Folin-Ciocalteu method, based on the procedure suggested by Pourmorad *et al* 2006 [21].

Then, 0.5 mL of plant extract or gallic acid (standard phenolic compound) was mixed with folin ciocalteu reagent (5mL) and aqueous Na_2CO_3 (4 mL, 1M). The mixture was allowed to stand for 15 min and the total phenols were determined by colorimeter at 765 nm. Gallic acid was used as a standard for calibration curve. Total phenol values were expressed in terms of mg equal gallic acid in 1 gm powder of dry plant (**table 5, fig. 4**).

TABLE 5: TOTAL PHENOLIC CONTENT OF MECD EXTRACT

Concentration (µg/mL)	Absorbance (Mean± SEM)
25	0.449±0.0056
50	0.712±0.0014
100	1.172±0.0016
150	1.590±0.0057
200	1.989±0.0060
250	2.321±0.0048
300	2.894±0.0036
Sample	0.664±0.0042

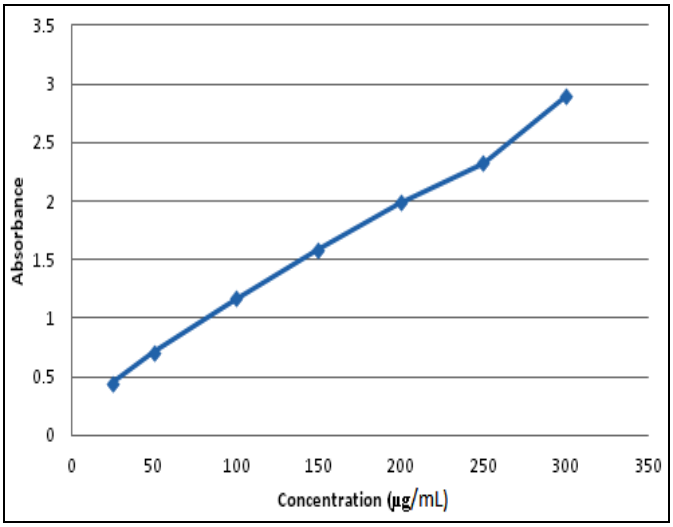

FIG. 4: TOTAL PHENOLIC CONTENT OF MECD EXTRACT VS STD.

Total Flavonoids determination: Total flavonoids content of extract was determined by aluminium chloride method [21]. Plant extract (0.5 mL) was mixed with 1.5 mL of solvent (methanol as MECD extract), 0.1 mL of 10% aluminium chloride, 0.1 mL of 1 M potassium acetate and 2.8 mL of distilled water. They were kept at room temperature for 30 min, the absorbance of the reaction mixture was measured at 415 nm with a spectrophotometer. Rutin was used as a standard for calibration curve. Total flavonoid values were expressed in terms of mg equal Rutin in 1 gm powder of dry plant (**table 6, fig. 5**).

TABLE 6: TOTAL FLAVONOID CONTENT OF MECD EXTRACT

Concentration (µg/mL)	Absorbance (Mean± SEM)
10	0.382±0.0084
20	0.469±0.0045
30	0.538±0.0056
40	0.601±0.0063
50	0.663±0.0049
60	0.701±0.0047
70	0.781±0.0013
80	0.849±0.0036
90	0.937±0.0090
100	1.012±0.0057
Sample	0.705±0.0042

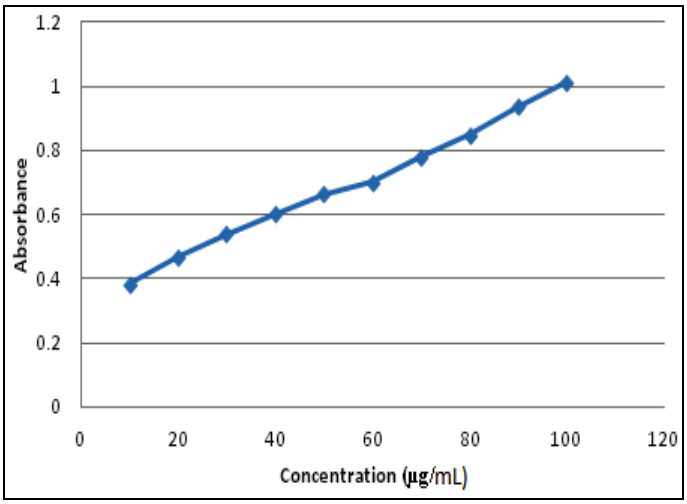

FIG. 5: TOTAL FLAVONOID CONTENT OF MECD EXTRACT VS STD.

RESULT AND DISCUSSION: The acute toxicity study did not show any mortality of the experimental animals upto dose of 2000 mg/kg BW and hence, the methanol extract of the bark of *Cordia dichotoma* could be safely used upto that dose. The flavonoids, phenolic compounds, glycoside, reducing sugar, tannins, triterpenoids in the extract were identified by chemical tests (Table 1).

Many reports on flavonoids, triterpenoids, polyphenols indicated that they possess antioxidant activity [5]. These phytoconstituents may exert multiple biological effects against tumor, heart disease, AIDS, and different pathologies due to their free radical scavenging activities [22].

A significant decrease in the concentration of DPPH radical was observed due to the scavenging ability of methanol extract. The antioxidant activity increased with increasing concentration of the extract, exhibited as an IC_{50} values (i.e the concentration of the extract that inhibit 50% free radicals) of 62.46 µg/mL. Whereas the reported IC_{50} value for standard (ascorbic acid) was 27.66 µg/mL [21]. The extract significantly inhibited nitric oxide in a dose dependent manner with the IC_{50} being 254.13 µg/mL. This nitric oxide antioxidant activity could be attributed the presence of phenolic and polyphenolic compounds in the extract.

As shown, the extract also demonstrated hydrogen peroxide decomposition activity in a concentration dependent manner with an IC_{50} of 236.5 µg/mL. Similarly, the phenolic content and flavonoid content of the extract increased with increasing concentration exhibiting values i.e 48.75 µg/mL and 63.5 µg/mL respectively.

CONCLUSION: The present study suggested that *Cordia dichotoma* bark could be a potential source of natural antioxidant and thus could be useful as therapeutic agents in preventing or slowing the progress of aging,age-associated oxidative stresses-related degenerative diseases. The presence of the phytoconstituents such as flavonoids, tannins and phenolic compounds etc were found to be effective in such diseases. Further studies are required on *Cordia dichotoma*, for evaluating the pharmacological actions such as anticancer, antidiabetic, degenerative disorders, antiulcer and anti-inflammatory activities.

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