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ANTIOXIDANT AND ANTIMICROBIAL POTENTIAL OF *CRATEVA MAGNA* (Lour.) DC

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ABSTRACT

In the present study, *Crateva magna* bark was extracted with various solvents in order to their standing in the polarity chart and the extracts were then subjected to preliminary phytochemical investigation. After that we have subjected the extracts to antioxidant assay using FTC, TBA, DPPH and Reducing Power assay as models and Antimicrobial assay. The Chloroform extract has shown potential antioxidant and antimicrobial activity among all the extracts under evaluation. The findings here justify the ethnomedicinal claim against the plant under consideration.

Keywords: *Crateva magna*, Antioxidant, Antimicrobial, Ethnomedicine.

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INTRODUCTION

Crateva magna plant is densely foliaceous, deciduous tree, up to 10 m tall, fairly smooth with horizontal wrinkles, the wood is yellowish white, close grained, branches are grayish brown when dry, lenticellate.[1] The flower pedicles are up to 6.5 cm with ascending sepals, petals are white with stamens which are 5-5.5 cm ovary is oblong elliosoid, up to 0.4cm long and the stigma are sessile. The berries are sub-spherical, 3.5 cm across, the seeds are dorsally crested and tuberculate.[2]

The bark and leaves are astringent, acrid, bitter, diuretic, lithotriptic stimulant, expectorant, demulcent depurative, astiperiodic tonic and detergent. They Leaves and bark are also useful in vitiated conditions of Vata and kapha (Ayur.), dyspepsia, colic, flatulence, helminthiasis, strangury, renal and vesical calculi, cough, bronchitis, asthma, pruritus, skin diseases, intermittent fevers, visceramegaly, scrofula, inflammations and hepatopathy. They leaf paste is applied on piles externally and leaf juice is used as a drink to get relief from bleeding piles.[3,4]

Ability to utilize oxygen helps us in metabolizing carbohydrates, fats and proteins; however it also costs us heavily. Oxygen is a highly reactive species and is a pat of potentially damaging molecule commonly known as "Free radicals". Free radicals can attack the healthy cells of the body and the cells in-turn may lose their structure and function.[5] Free radical production occurs continuously in the body as a part of normal cellular functions. But, excessive production of free radicals triggered by endogenous or exogenous source may lead to diseased conditions.[6]

A free radical may be defined as a molecule capable of independent existence having an unpaired electron in an atomic orbital. Many of these radicals are highly reactive and may either donate or accept an electron, hence acting as oxidants or reductants. Most important free radicals for a number of diseased conditions are oxygen derivatives like superoxide or hydroxyl radical.[6]

Plants and plant products contains a large number of free radical scavenging molecules, like phenolic compounds (e.g. Flavonoids, Coumarins, Quinones,

Lignans, Tannins and Phenolic acids,), nitrogen containing compounds (e.g. Amines and Alkaloids), Vitamins, Terpenoids etc. which are known for their antioxidant activity. Studies shows many of these compounds to possess other therapeutic activities like antitumor, anti-atherosclerotic, anti-mutagenic, anti-inflammatory, antibacterial, or antiviral activities to some extent.[7]

Natural antioxidants are found to be associated with lower risks of cancer, diabetes, and other diseases. Synthetic antioxidants like BHA (butylated hydroxyl-anisole) and BHT (butylated hydroxyl-toluene) are used these days due to their effectiveness and low cost. But, the safety of synthetic antioxidants is of concern. And so, considerable interest has grown for the use of antioxidants from natural source.[8]

MATERIALS AND METHODS

Plants Material

Benzene (BZ), Diethyl Ether (DE), Chloroform (CH), Ethylacetate (EA) and Methanol (MT) extracts of both *Crateva magna* bark were subjected to test in these experiments.

Preparation of Extract

Around 1 kg of both the powdered drug material were packed in two different soxhlet assembly and extracted using the successive extraction method using various solvents (Benzene, Diethyl Ether, Chloroform, Ethyl acetate and Methanol) in order of their increasing polarity. All through before extraction with a new solvent, the powdered materials were air dried to remove traces of the previous solvent.[9]

Each extract thus obtained was concentrated and dried under reduced pressure using Eyela Rotary Evaporator (Japan) at a temp. ranging between 42-45°C. The dried extracts were then stored in dessicator for future use.

Preliminary Phytochemical Analysis

The preliminary phytochemical test for the different extracts of the plant drugs was performed by standard methods.[10,11]

Antioxidant activity of different extracts of *Crateva magna* bark.

Chemicals

TBA (Thiobarbituric acid) was procured from Loba Chemie, India. DPPH (1,1-Diphynyl-2-picryl hydrazyl), NADH and nitroblue tetrazolium (NBT) were all procured from Sigma chemicals, St. Louis, USA., Ethylene diamine tetra acetic acid (EDTA), trichloroacetic acid, hydrogen peroxide, ferrous sulphate, dimethyl sulphoxide ascorbic acid, mannitol, potassium dihydrogen phosphate, potassium hydroxide used in the experiment were of analytical grade and procured from Ranbaxy fine chemicals.

Ferric Thiocyanate Assay (FTC)

By FTC method, we determined the amount of peroxide at the initial stage and secondary stage of lipid peroxidation. The FTC assay was carried out using methods as described by Kikuzaki and Nakatani.[7] Solutions of various concentration of extracts were added to 4 ml of 99.5% ethanol, 8.0 ml of 0.02 M phosphate buffer (pH 7.0), 4.1 ml of 2.51% linoleic acid in 99.5% ethanol, and 3.9 ml of distilled water, in a screw-capped vial (38 x 75mm) and were placed in an oven at 40 ^{0}C and incubated in the dark. For measuring the antioxidant activity, 0.1 ml of the reaction mixture was taken in a test tube (13 x 150 mm), to it added 9.7 ml of 75% (v/v) aqueous ethanol, followed by 0.1 ml of 30% aqueous ammonium thiocyanate and 0.1 ml of 0.02 M ferrous chloride in 3.5% hydrochloric acid. After three minutes of addition of ferrous chloride to the reaction mixture, the absorbance was measured spectrophotometrically (Shimadzu 1601 Double beam, Japan) at 500 nm where ascorbic acid was used as a positive control.[12]

Thiobarbituric Acid Method (TBA)

The Thiobarbituric Acid analysis was performed according to the method described by Chang *et.al.* [13] The samples prepared for FTC method were used here too. To the required quantities of the sample solution, 2.0 ml of aqueous thio-barbituric acid (TBA) solution and 1.0 ml of 20% aqueous trichloro-acetic acid (TCA) were added. The mixtures were placed on boiling water bath for 10 minutes, cooled and then centrifuged at 3000 rpm for around 20 minutes and absorbance of the supernatant liquid was measured at 532 nm[14] using Shimadzu 1601 Double beam spectrophotometer, Japan. Both the methods (FTC and TBA) has described antioxidant activity by percent inhibition;

$$\% \text{ inhibition } \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

Reducing Power Assay (RP)

Reducing power assay was performed according to the method described by Oyiazu (1986). The required concentrations of various extracts were suspended in distilled water, to them added 2.5 ml of 0.2 M-phosphate buffer (pH6.6), and 2.5 ml of $K_3Fe(CN)_6$ (1% w/v). The mixtures were then incubated at 50 ^{0}C for around 20 min. Then, 2.5ml of TCA (10% w/v) were added to each. The resultant mixtures were then centrifuged at 3000 rpm for around 10 min. and the upper layers of these solutions (2.5 ml) were mixed with distilled water (2.5 ml) and 0.5 ml $FeCl_3$ (0.1% w/v), and the absorbance of the resultant mixtures were measured at 700 nm against blank sample. Ascorbic acid was used as standard antioxidant compound for this study.[15]

Free Radical Scavenging Activity by 1,1-Diphenyl-2-picryl-hydrazil (DPPH)

The free radical scavenging activity of different extracts were measured by 1,1-diphenyl-2-picryl-hydrazil (DPPH), following the procedure described by Blois.[16] 0.1 M solution of DPPH· in ethanol was prepared, 1 ml of this solution was added to each of the extract solution (3 ml) in water at different concentrations. The mixtures were then shaken vigorously and were allowed to stand at room temperature for 30 min. Absorbance of the resultant mixtures were measured spectrophotometrically at 517 nm (Shimadzu 1601

Double beam, Japan). Higher free radical scavenging activity of the reaction mixtures were indicated by lower absorbance values.[17] Ascorbic acid was used as standard in this assay.

$$\text{DPPH}\cdot \text{ scavenging effect (\%)} = \frac{Ao - A1}{Ao} \times 100$$

Where,

A_o, is the absorbance of the control reaction mixture,

and A_1, is the absorbance of the samples at various concentrations.

Antimicrobial assay of Chloroform extract of *Crateva magna* bark.

Microorganisms

The microbial species were obtained from Microbiology Department of Jaduvpur University, Kolkata. The collected species were *S. aureus, E.coli, K. pneumoniae, S.typhi sp., V. cholera, P. aeruginosa, B. subtilis and S. faecalis.* The cultures of bacteria were maintained on nutrient agar slants at 4°C and sub-cultures were transferred to nutrient broth, 24 hours before testing.

Preservation of bacterial strains

Strains of *S. aureus, E.coli, K. pneumoniae, S.typhi , V. cholera, P. aeruginosa, B. subtilis and S. faecalis* were preserved using slant cultures at 4ºC temperature. The routine sub culture of Gram +ve strains were done using nutrient agar media and for Gram -ve strains bromothymol blue lactose agar media was used.[18]

Standard antibiotic

Lincomycin (Lyka Labs, India) was used as standard drug for the study. The pure sample of the drug was received as gift sample from M/S Lyka Labs, India.

Preparation of impregnated discs of extract and standard antibiotics

Discs with 7.25 mm diameter were prepared by punching of whatman no.1 filter paper and all the discs were sterilized by dry heat at 160ºC for an hour in batches in screw capped Bijou bottles. The chloroform extracts of *Crateva magna* bark was weighed and dissolved in sterile distilled water to make the required solutions of concentration 128-2000 µg/ml. Similarly the stock solution of the control antibiotic (lincomycin) within the range of 0-1000 µg/ml were prepared by dissolving the required amount of the drugs in sterile distilled water. All the prepared stock solutions were then kept at 4ºC. Now, for preparing antibiotic impregnated discs, 1.0 ml of the stock solutions of the antibiotic was added separately to sterile Bijou bottles containing 100 discs each. The same procedure was followed for preparing impregnated discs of the plant extracts and their isolated compounds. The discs were used while wet and can be stored for further use at 4ºC, as they can retain the moisture and potency for at least 3 months in the screw capped Bijou bottles. [19, 20]

Antimicrobial assay

The antimicrobial assay was performed by disc diffusion method. Nutrient agar plates with inoculum size of 10^5-10^6 cfu/ml of the microorganisms were used. Previously prepared discs (Concentration 128-2000 µg/ml) and antibiotic (concentration 0-1000 µg/ml) were placed aseptically on petridishes. All the petridishes were then incubated at 37°C±2°C for 18 hour. After 18 hours, the activity was recorded by measuring the zone of inhibition on the petridishes around the discs. The zone of inhibitions thus observed, were measured using a transparent ruler and recorded with reference to the zone of inhibition of the standard drug. Lincomycin was taken as standard drug for this study. [21, 22]

Minimum Inhibitory Concentration (MIC)

MICs were determined using standard agar dilution method.[23] The chloroform extract was dissolved in 0.5 ml of dimethyl sulphoxide and then further diluted using sterilized distilled water. The drug solution thus prepared was then added to the molten nutrient agar in different tubes to give a concentration in the range of 0 – 128 µg/ml and then subsequently increasing it two folds up to 2000 µg/ml. The pH of the tubes were adjusted to 7.2 to 7.4 and transferred to sterile petridishes. Bacterial cell suspensions (10 µl) were then inoculated on the petridishes using sterile bacterial planter. Number of cfu inoculated onto the petridishes was 10^5 for all the microbial strains. All the inoculated petridishes were then incubated for 18 hours at 37°C±2°C. The petridish with lowest concentration which did not show any visible growth of microorganism upon incubation was considered as MIC for that particular microbial strain. The petridishes containing sterile distilled water and Lincomycin solution, served as negative and positive control respectively.

RESULT AND DISCUSSION

Preliminary Phytochemical Investigation:

Preliminary phyto-chemical analysis of powdered drug and different extracts of *Crateva magna* bark shows the presence of various phyto constituents which are shown in Table 1.

Antioxidant activity of various extracts from the bark of *Crateva magna.*

The results of antioxidant potential of different extracts of *Crateva magna* are discussed below. While evaluating the antioxidant potential of various extracts of *Crateva magna* in comparison to the Standard drug, we have studied using four different models. **Reducing power assay** has shown an increase in the absorbance with increase in concentration of extracts and the compound. the chloroform extract has also shown good response in a dose dependent manner whereas the methanol extract has shown minimal activity, as shown in Table 2.

Results for **FTC Lipid peroxidation** study suggests a dose dependent increase in the peroxidation effect. Chloroform extract here also was a close competitor for the standard drug and among the extracts, Ethyl acetate extract was distant second followed by methanol extract, whereas, benzene extract has shown the least activity. The detailed reports are shown in Table 2.

In vitro free radical scavenging effect of various samples by **Thio-barbituric Acid Method** exhibited the good percentage inhibition for the chloroform extract. The free radical scavenging of Benzene, Diethyl ether, Chloroform, Ethyl acetate, Methanolic extracts and ascorbic acid was found to be 27.32%, 25.01%, 69.44%, 22.36%, 29.49 and 78.92% respectively. The detailed result is shown in Table 2.

The Free radical scavenging assay by **DPPH method** also exhibit good result for Chloroform extract in a dose dependent manner and at higher dose has shown 68.12. Here in this study the lowest activity was shown by Diethyl ether extract as shown in Table 2.

The results from all the studies and the **IC_{50} values** (Table 3) indicated that the Chloroform extract from *Crateva magna* has potential antioxidant property and supports the idea behind the work. The graphical representation of all the activities for various extracts is given in Fig.1, 2, 3 and 4 respectively.

Table 1: Phytochemical analysis of powdered bark and various extracts of *Crateva magna*

Bioactive Constituents	Powder	Benzene	Diethyl Ether	Chloroform	Ethyl Acetate	Methanol
Carbohydrate	+	+	+	-	+	+
Gums and Muscilages	+	-	-	-	-	+
Proteins	+	-	+	-	-	-
Alkaloids	+	+	-	+	+	_
Glycosides	-	-	-	-	-	-
Saponins	+	+	-	-	+	+
Steroids	+	+	-	+	+	-
Flavonoids	-	-	-	-	-	-
Tanins	-	-	-	-	-	-
Phenolics	_	-	-	-	-	-
Terpenoids	+	-	+	-	+	-

[+] denotes present; [-] denotes absent

Table 2: *In vitro* free radical scavenging activity of various extracts of *Crateva magna by* RP, TBA, FTC *and* DPPH methods

S.N.	Extracts	Activity	20µg/ml	40µg/ml	60µg/ml	80µg/ml	100µg/ml
1	BZ	RP	0.016±0.01	0.028±0.043	0.086±0.005	0.116±0.002	0.249±0.13
		FTC	8.44±0.05	10.85±0.001	12.28±0.05	14.71±0.01	16.26±0.05
		TBA	14.44±0.001	18.85±0.01	22.28±0.05	24.71±0.032	27.32±0.002
		DPPH	12.62± 0.001	15.82 ±0.043	18.21 ± 0.18	21.87±0.032	25.36± 0.001
2	DE	RP	0.028±0.001	0.037±0.005	0.089±0.002	0.172±0.001	0.268±0.043
		FTC	11.85±0.001	13.28±0.05	15.71±0.001	17.32±0.002	19.26±0.05
		TBA	11.28±0.05	16.71±0.001	19.32±0.02	23.95±0.05	25.01±0.014
		DPPH	09.38± 0.32	11.78±0.018	14.86± 0.23	18.96± 0.001	20.16± 0.13
3	CH	RP	0.039±0.05	0.159±0.002	0.317±0.001	0.598±0.01	0.971±0.001
		FTC	37.25±0.05	47.21±0.001	52.46±0.05	59.63±0.001	67.55±0.002
		TBA	31.93±0.001	46.85±0.05	53.87±0.001	62.93±0.002	69.44±0.002
		DPPH	28.23± 0.01 7	36.06±0.014	42.12± 0.032	59.28± 0.005	68.12± 0.043
4	EA	RP	0.018±0.43	0.098±0.05	0.126±0.002	0.259±0.01	0.281±0.001
		FTC	14.84±0.05	16.76±0.001	22.83±0.002	26.19±0.001	29.12±0.05
		TBA	9.45±0.002	12.85±0.014	18.52±0.43	20.47±0.002	22.36±0.05
		DPPH	10.47± 0.01	12.06±0.014	18.01±0.032	21.12±0.043	23.56±0.01
5	MT	RP	0.017±0.001	0.021±0.005	0.029±0.05	0.048±0.001	0.057±0.043
		FTC	13.44±0.001	15.84±0.05	17.32±0.001	23.31±0.01	26.39±0.001
		TBA	10.73±0.001	14.52±0.05	19.47±0.43	24.33±0.002	29.49±0.01
		DPPH	8.23±0.12	15.06±0.01	21.12±0.43	24.25±0.005	29.63±0.021
6	Ascorbic acid	RP	0.093± 0.01	0.279±0.05	0.837±0.001	1.286±0.05	1.893±0.005
		FTC	38.98±0.05	54.73±0.01	65.91±0.002	70.16±0.001	78.92±0.05
		TBA	32.94±0.001	47.98±0.05	56.23±0.01	63.87±0.001	74.43±0.05
		DPPH	40.12±0.001	46.15±0.05	55.38±0.002	64.52±0.001	73.63±0.05

Values are mean ± SEM of 3 replicates

Table 3: IC_{50} value from FTC, TBA and DPPH scavenging activity of *Crateva magna*

Sl.No	Activity	Extracts	IC $_{50}$
1	FTC	BZ	191.314
		DE	89.678
		CH	316.716
		EA	61.167
		MT	78.942
		Ascorbic acid	0.025
2	TBA	BZ	156.568
		DE	133.249
		CH	203.334
		EA	50.843
		MT	78.442
		Ascorbic acid	0.047
3	DPPH	BZ	228.679
		DE	63.670
		CH	85.617
		EA	58.968
		MT	174.724
		Ascorbic acid	4.514

Values are mean ± SEM of 3 replicates

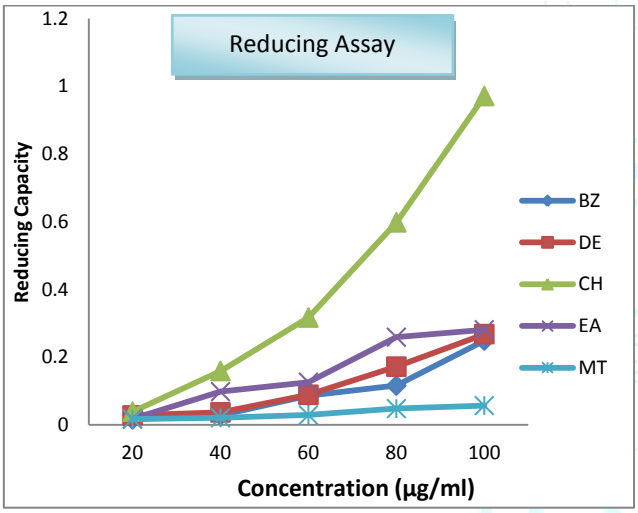

Fig 1: Reducing Power Assay of different extracts of *Crateva magna.*

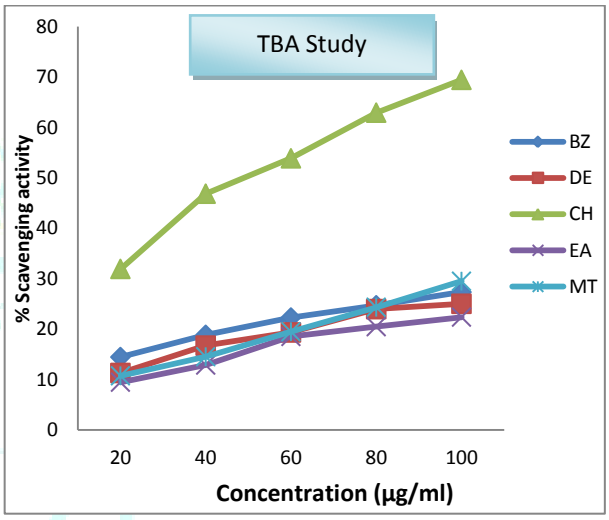

Figure 3: TBA scavenging activity of different extracts of *Crateva magna.*

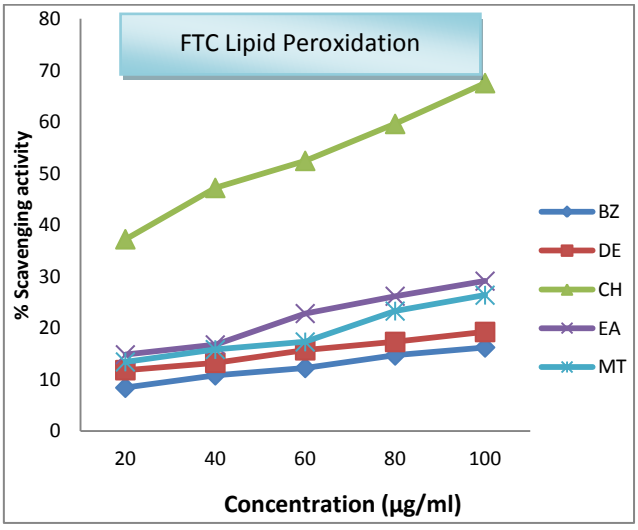

Figure 2: FTC lipid peroxidation study of different extracts of *Crateva magna.*

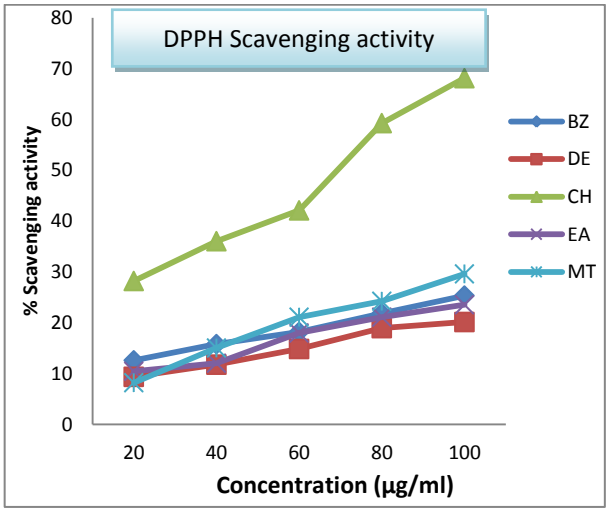

Figure 4: DPPH scavenging activity of different extracts of *Crateva magna*.

Antimicrobial assay and Minimum inhibitory concentration (MIC± SD of three replicates) at 600nm OD of Chloroform extract of *Crateva magna* bark

The results of the antimicrobial assay (Table 9.1) shows that out of 54 bacterial strains, the growth of 33 strains were inhibited by the extract at concentrations within the range of 128 – 512 µg/ml, 14 strains were inhibited at a concentration of 1000µg/ml, while the remaining 07 strains were inhibited at concentration >2000 µg/ml, which was the highest concentration for extract. The MIC study shows that 5 out of 18 Gram-positive strains were sensitive between 128 and 256 µg/ml (Zone of inhibition 12-16 mm); whereas 15 out of 36 Gram-negative strains were sensitive at a concentration range between 128-256 µg/ml of the chloroform extract (Zone of inhibition 08-12 mm). So we can conclude that, the Chloroform extract of *Crateva magna* has shown antimicrobial activity against both Gram-positive and Gram –negative isolates.

The results show good antimicrobial potential for the chloroform extract of *Crateva*, when compared with the standard drug lincomycin.

Table 4: *In vitro* antimicrobial assay and Minimum inhibitory concentration (MIC± SD of three replicates) at 600nm OD of chloroform extract of *Crateva magna* bark.

Pathogens	Number of strain	MIC of Chloroform extracts (µg/ml)					Zone of inhibition (mm)	MIC of lincomycin (µg/ml)						
		128	256	512	1000	>2000		0.25	0.5	8	64	128	256	>1000
S. aureus	07	01	01	02	03	-	++	02	-	01	02	-	02	-
K. pneumoniae	06	03	01	01	-	01	+	-	01	-	01	01	01	02
E. coli	06	01	02	01	02	-	++	-	01	01	01	-	01	02
Salmonella. sp	09	-	03	02	02	02	++	-01	01	01	02	01	02	01
V. cholerae	07	02	-	02	03	-	+	-	01	01	02	02	-	01
B. subtilis	06	-	01	02	02	01	++	01	01	01	-	02	-	01
S. faecalis	05	-	02	01	01	01	++	-	01	01	-	01	01	01
P. aeruginosa	08	-	03	02	01	02	+	01	01	01	02	-	02	01
Total	54	07	13	13	14	07		05	07	07	10	07	09	09

Chloroform extract of *Crateva magna*; +: ≤ 10mm; ++: ≥12mm; the inoculum size used was 10^5 cfu per spot for all the organisms except *S.aureus*, where the inoculum size per spot was 10^6 cfu. The result represents the mean value of triplicate tests.

CONCLUSION

The Study has shown the potential of chloroform extract of *Crateva magna* bark as an antioxidant and antimicrobial agent. The bark of the plant can be explored further for isolation of bioactive compound and exploration of the isolated compound further towards preparation of pharmaceutical formulation from the herbal isolated which are safer in terms of their long use in traditional medicine. The ailing human race presently needs safer alternatives for healthy living, which was the prime reason for undertaking this piece of work.

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