ISSN NO. 2320-5407

Journal homepage: http://www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

CORDIA DICHOTOMA CRUDE EXTRACTS: POTENT SOURCE OF NATURAL ANTIBACTERIAL AND ANTIOXIDANT AGENTS

***Konka. Rajashekar, Murali Krishna Thupurani, Siddhartha Marupati, Thota Shiva Prasad, Kiran reddy Kanubaddi**

Department of Biochemistry, Biotechnology and Chemistry, Chaitanya Postgraduate College (Autonomous), Kishanpura, Hanamkonda, Kakatiya University, Waranagal

Manuscript Info

Manuscript History:

Received: 10 December 2013
Final Accepted: 20 January 2014
Published Online: February 2014

Key words:
antibacterial, antioxidant, *Cordia dichotoma*, methycilin resistant,

****Corresponding Author***

Konka. Rajashekar

Abstract

The present investigation was carried out for the evaluation of antibacterial and antioxidant activities of *Cordia dichotoma* leaves, stem, fruit peel and fruit pulp extracts. Among them fruit pulp extract was found to have significant antibacterial and antioxidant properties. Antibacterial activity was more effective against gram negative organisms than aganist gram positive organisms. The current studies also revealed that methycilin resistant *Staphylococcus aureus*, was susceptible to fruit pulp extracts. Fruit pulp exhibited good antioxidant activities. Antioxidant activities of the extracts were correlated with its scavenging property of free radicals.

Introduction

The herbal products usually symbolize safety in contrast to the synthetic drugs that are harmful to human beings and environment (Chin et al., 2006). Majority of the medicinal plants especially of Indian origin have been in continuous use for the treatment of different types of aliments. However, there are still several plants which have not been evaluated for their use as traditional medicine. Extraction and isolation of therapeutically valuable natural compounds is necessary to scientifically validate the newly found medicinal plants.

Cordia dichotoma Foster (*C.obliqua*) of Cordiaceae (*Borginaceae)* is a small to moderate-sized deciduous tree with a short trunk and spreading crown. The stem bark is greyish brown, smooth or longitudinally wrinkled. Flowers are short-stalked, bisexual, and white in color which open only at night. The fruit is yellow or pinkish-yellow, shining and globose which turns black on ripening while the pulp gets viscid. It is mostly distributed around tropical and subtropical regions of India especially in the dry deciduous forests of Rajasthan and Western Ghats in Myanmar.

Cordia dichotoma commonly called Sebastian plum is an important plant as its leaf extract has been used in wound healing, anthelmintic activity and other pharmaceutical purposes (Kuppast *et al.,* 2006). Fruit extracts of *Cordia dichotoma* has been reported antihelmintic activity on adult Australian warms (*Eudrilus giniae*) **(**Maisale *et al*., 2010). Literature survey also reveals the *In vivo* anti-inflammatory activities (Sharma *et al*., 2010**)**.

In view of these findings, the present investigation was carried out for the evaluation of antibacterial and antioxidant activities of *Cardia dicotoma* leaves, stem, fruit and fruit pulp.

2.0 MATERIALS AND METHODS

2.1 COLLECTION OF PLANT MATERIAL

The stem, leaves and fruits of *Cordia dichotoma* were collected during rainy season from the village Pembarthi, District Warangal, Andhra Pradesh, INDIA. Authenticity of the plant has been confirmed by Prof. Thirupatiah, Taxonomist, Plant Systematic laboratory, Department of Biotechnology, Chaitanya Postgraduate College

(Autonomous), Kishanpura, Hanamakonda, Warangal, A.P, INDIA. The parts of the plants were registered and stored in the departmental herbarium and allotted specimen number. The voucher number is 115.

2.2 PREPARATION OF EXTRACTS

Leaves were washed with water, dried under shade, homogenized to coarse powder. Whereas, fruits were further processed to separate peel and pulp. Pulp was shade dried and homogenized to coarse powder (100 grams) was used for extraction with methanol and subjected for dryness under reduced pressure by rotavapor at 40-50 ^{0}C for 3 h. Leaf, stem, fruit peel, fruit pulp extracts were marked as CDL, CDS, CDFPL, CDFP for experimental convenience.

2.3 CHEMICALS

Thiobarbituric acid, DPPH**,** 2, 2'-azino-bis (3-ethylben - zthiazoline- 6-sulphonic acid, were purachased from Hi-media laboratories, Mumbai, India. All other chemicals used were of analytical grade.

2.4 REAGENTS PREPARATION

Preparation of Nash Reagent: 75.0 g of ammonium acetate, 3 ml of glacial acetic acid and 2 ml of acetyl acetone were mixed and distilled water was added to total volume of 1 L.

Preparation of Griess Reagent: 1% Sulphanilamide, 2% Phosphoric acid and 0.1% N-1-napthylethylenediamine di hydrochloride in distilled H_2O.

Preparation of Ferrous EDTA: 0.13% ferrous ammonium sulfate and 0.26% EDTA in distilled H_2O.

2.5 QUALITATIVE ANALYSIS OF PHYTOCHEMICALS

The extracts were subjected for the preliminary phytochemical analysis using standard methods described by Harbone (1984) and Trease and Evans (1989).

2.6 DETERMINATION OF TOTAL PHENOL CONTENT

The amount of total phenolics in extract was determined with Folin–Ciocalteu reagent by with slight modifications (Siglelton and Rossi 1965)· Separately, 1 ml of aliquots various alcoholic extracts of CDL, CDS, CDFPL and CDFP of different concentrations (50,100 and 150 µg/ml) and standard solution of tannic acid (10 µg/ml) were added separately in to a 100 ml volumetric flask, that contained about 60 ml distilled water followed by the addition of 5 ml of Folin–Ciocalteu reagent. The content was mixed thoroughly and kept constant for about 10 min. To this was added 15 ml Na_2CO_3 (20 %) and made up to 100 ml using distilled water. The mixture was allowed to stand for 2 h with intermittent shaking. The absorbance was measured at 760 nm using a UV-visible spectrophotometer.

2.7 DETERMINATION OF FLAVONOID ASSAY

Flavonoid content was measured by aluminum chloride colorimetric assay with slight modification (Kartashova and Sudos, 1997).1 ml of each CDL, CDS, CDFPL and CDFP alcoholic extracts with different concentrations (50,100 and 150 µg/ml) and standard solution of catechin (10 µg/ml) were added separately to a 100ml volumetric flask containing 4 ml of distilled water. To the above mixture 0.3 ml of 5% $NaNO_2$ was added, followed by the addition of 0.3 ml of 10% $AlCl_3$ after 5 min. After incubation period of 6 min 2 ml of 1 M NaOH was added and the total volume was made up to 10 ml with distilled water. The solution was mixed well and the absorbance was measured against a prepared reagent blank at 510 nm.

2.8 ANTIBACTERIAL ACTIVITY

The bacterial species selected were Methycilin *resistant Staphylococcus aureus*, *Bacillus subtillis*, *Bacillus cereus*, *Pseudomonas aeurginosa*, *Escherichia coli*, *Proteus vulgaris*. All these strains were obtained from department of Biochemistry Chaithanya Postgraduate College (autonomous), affiliated to Kakatiya University, Warangal.

2.8.1 PREPARATION OF BACTERIAL SUSPENSION

The bacterial strains were inoculated into sterilized nutritive broth and incubated at 35 ± 2°C for 24 h. The turbidity of the resulting suspensions are diluted with same nutritive broth to obtain a transmittance of 25% at 580 nm, this percentage was calculated spectrophotometrically using Bausch & Lomb spectrophotometer comparable to McFarland turbidity standard. This level of turbidity is equivalent to approximately 3.0 × 108 CFU/ml (a stock standard from which a working standard was drawn with concentration of 1 × 108 CFU/ml).

The antibacterial activity of these extracts was carried out according to the method described by Raman *et al*., (2009) with slight modifications. Each selective medium was inoculated with the test organism suspended in nutritive broth. Once the agar was solidified, it was punched with a six millimeters-diameter wells and filled with 25

µL of the plant extracts of CDL, CDS, CDFPL and CDFP at various concentrations and corresponding wells with positive and negative control. The concentrations of the methanolic extracts employed were 50, 100 and 150 µg/ml simultaneously, Azythromycin (10 µg/ml) was used as positive control. The test was carried out in triplicate. The plates were incubated at 35 ± 2°C for 24 h. The inhibition-zone diameter was measured in mm.

2.9 ANTIOXIDANT ACTIVITIES

2.9.1 LIPID PEROXIDATION ASSAY

In vitro lipid peroxidation was determined by the described method (Satoh 1978; Bouchet *et al*., 1998). Briefly, 1 ml of rat liver microsomal fraction was added to 1.0 ml of 150 mM Tris-HCl buffer (pH 7.4) containing various concentrations of CDL, CDS, CDFPL, CDFP and 0.2 ml FeCl3 (1 mM), 0.2 ml ascorbic acid (0.5 mM) to induce lipid peroxidation. The mixtures were incubated at 37°C for 30 minutes. At the end of the incubation, 0.5 ml of glacial acetic acid and 0.5 ml of 0.33% TBA were added to each mixture. The mixtures were kept in a water bath at 97°C for 45 min; for cooling and extraction of pink chromogen with 2 ml of butanol. The absorbance of the organic layer was measured at 535 nm, and the thiobarbituric acid reactive substances produced were estimated using the MDA standard curve. BHT or BHA was used as a control substance.

2.9.2 DPPH (1, 1-DIPHENYL-2-PICRYLHYDRAZYL) RADICAL SCAVENGING ACTIVITY

The free radical scavenging activity of *Cordia dichotoma* extracts was measured by 1,1-diphenyl-2-picryl-hydrazil (DPPH) method described by Blois with slight modifications (Blois, 1958). 0.1 mM solution of DPPH in methanol was prepared and 1ml of this solution was added to 3 ml of various concentrations of CDL, CDS, CDFPL and CDFP extracts and the reference compound (10 µg/ml). After 30 min, absorbance was measured at 517 nm. Ascorbic acid was used as the reference material. All the tests were performed in triplicate and the graph was plotted with the mean values. The percentage of inhibition was calculated by comparing the absorbance values of the control and test samples.

% DPPH radical scavenging = [(Absorbance of control – Absorbance of test sample)/ (Absorbance of control)] × 100

2.9.3 ABTS^{*+} (2, 2'-AZINO-BIS (3-ETHYLBEN - ZTHIAZOLINE- 6-SULPHONIC ACID) RADICAL CATION DECOLOURISATION ASSAY

ABTS^{*+} (54.8 mg) was dissolved in 50ml of distilled water to 2mM concentration and potassium persulphate (17 mM, 0.3 ml) was added. The reaction mixture was left to stand at room temperature overnight in dark before use. To 0.2 ml of various concentrations of the CDL, CDS, CDFPL and CDFP extracts and reference standards, 1.0 ml of distilled DMSO and 0.16 ml of ABTS^{*+} solution was added to make a final volume of 1.36 ml. Absorbance was measured spectrophotometrically, after 20 min at 734 nm **(**Pellegrini *et al*., 1999).

3.0 STATISTICAL ANALYSIS

The data from the experiments of antioxidant activities are represented as mean ± S.E.M (n=3). Student's t-test was used for statistical analysis (SAS software 9.0). Values were considered statistically significant when $P<0.5$.

3.1 RESULT AND DISCUSSION

3.2 PHYTOCHEMICAL ANALYSIS OF THE PLANT EXTRACTS

Methanol extracts of *Cordia dichotoma* extracts resulted in the presence of various types of chemicals. Fruit and fruit pulp extracts were given positive results for all phyto chemicals tested. The results were shown in table 1.

3.3 DETERMINATION OF TOTAL PHENOL CONTENT

Total phenolic content of CDL, CDS, CDFPL and CDFP alcoholic extracts revealed that all the plant extracts possessed concentration dependent increase in the amount of phenols. The percentage yield of phenolic content was found to be 52, 70, 78 and 42, 54, 66 and 52, 60, 76, and 60, 79, 88 at 50, 150, 250 µg/ml of plant extracts respectively. The highest yield was noticed with stem bark and leaf extracts were comparable with reference standard tannic acid 58.9, 74.5, 88.9 at 10 µg/ml (Figure 1).

3.4 DETERMINATION OF TOTAL FLAVONOID CONTENT

The percentage yield of total flavonoid content is found to be 39, 52, 69 and 58, 69, 75 and 45, 53, 62 and 55, 78, 84 at 50, 150, 250 µg/ml of plant extract respectively. The highest yield was noticed with stem bark and leaf extracts and are comparable with reference standard catechin 60.8, 73.4, 86.9 at 10 µg/ml respectively (Figure 2).

Table 1: Pytochemical analysis of *Cordia dichotoma* extracts

Phyto chemicals	Leaves	Stem	Fruit peel	Fruit pulp
Glycosides	+	+	+	+
Coumarins	+	+	+	+
L. anthocyanins	-	-	-	-
Steroids	+	+	+	+
Saponins	+	+	+	+
Anthocyanins	-	-	-	-
Phototanins	-	-	-	-

Table 2: Antibacterial activity of *Cordia dichotoma* extracts at various concentrations compared with standard azythromycin at 10µg/ml

Strains	**Leaves**			**Stem**			**Fruit Peel**			**Fruit pulp**			**Standard**
Gram negative	**50***	**100***	**150***	**50***	**100***	**150***	**50***	**100***	**150***	**50***	**100***	**150***	
E. coli	5	7	9	-	-	-	5	9	13	7	13	16	21
P. aeurginosa	4	8	11	4	6	8	4	7	11	6	10	13	19
MRSA	4	6	10	3	5	6	2	5	9	8	14	19	24
Gram positive													
B. subtilis	4	7	11	3	6	11	6	9	12	7	12	14	20
B. cereus	5	7	8	4	7	10	4	8	13	5	9	12	18
P. vulgaris	3	5	8	3	5	6	5	8	10	4	7	9	15

MRSA-Methycilin resistant staphylococcus aureus,
*Concentration of plant extract in µg/ml

Table 3: Antioxidant activity of *Cordia dichotoma* extracts tested at various concentrations

	Leaves			**Stem**			**Fruit Peel**			**Fruit pulp**		
	50*	**100***	**150***	**50***	**100***	**150***	**50***	**100***	**150***	**50***	**100***	**150***
DPPH	2.5 ± 1.4	2.0 ± 1.1	2.0 ± 1.2*	3.0 ± 17	3.5 ± 2.0	4.1 ± 2.4*	6.0 ± 3.4	6.5 ± 3.7	8.0 ± 4.6*	4.0 ± 2.3*	8.5 ± 4.9	10.5 ± 6.0*
LPO	2.5 ± 1.4	5.5 ± 3.2	5.5 ± 3.1*	3.0 ± 1.7	5.5 ± 3.1	7.5 ± 4.3	3.0 ± 1.7	6.5 ± 3.7	5.5 ± 3.1*	3.0 ± 1.7*	2.5 ± 1.4	3.0 ± 1.7*
ABTS	3.5 ± 2.0	2.5 ± 1.4	6.5 ± 3.7*	4.0 ± 2.3	5.5 ± 3.1	3.5 ± 2.0	4.0 ± 2.3	3.0 ± 1.7	4.1 ± 2.4*	4.0 ± 2.3*	3.0 ± 1.7	4.5 ± 2.6*

Values are represented as mean±S.E.M
Significance is considered at *P<0.05

Figure 1: Determination of Total Phenol content at various concentrations of *Cordia dichotoma* extract

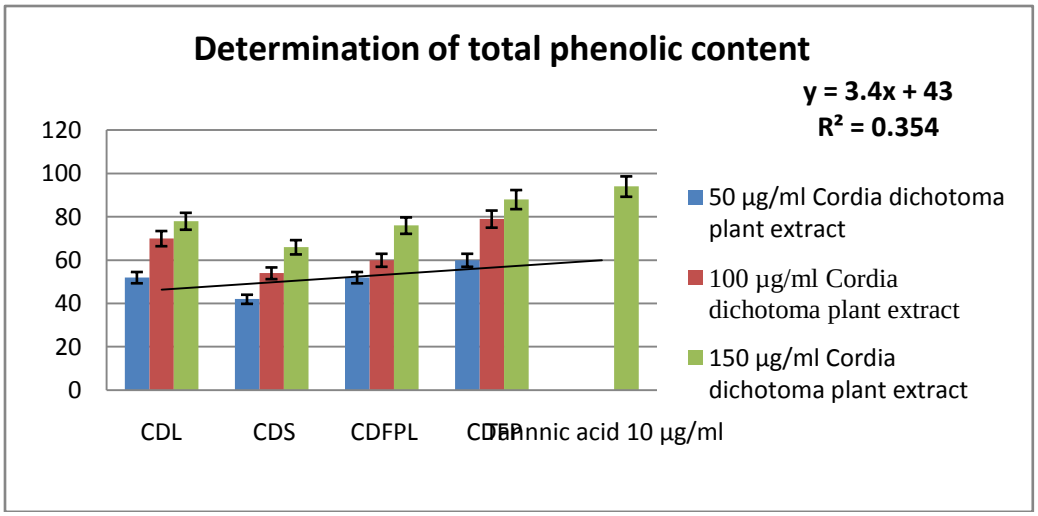


Figure 2: Determination of Total Flavanoid content at various concentrations of *Cordia dichotoma* extract

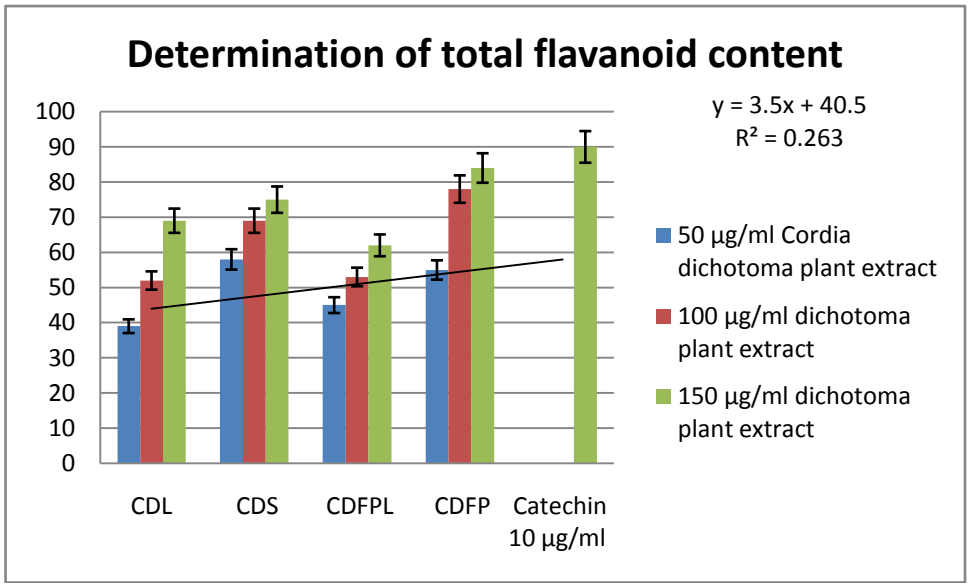
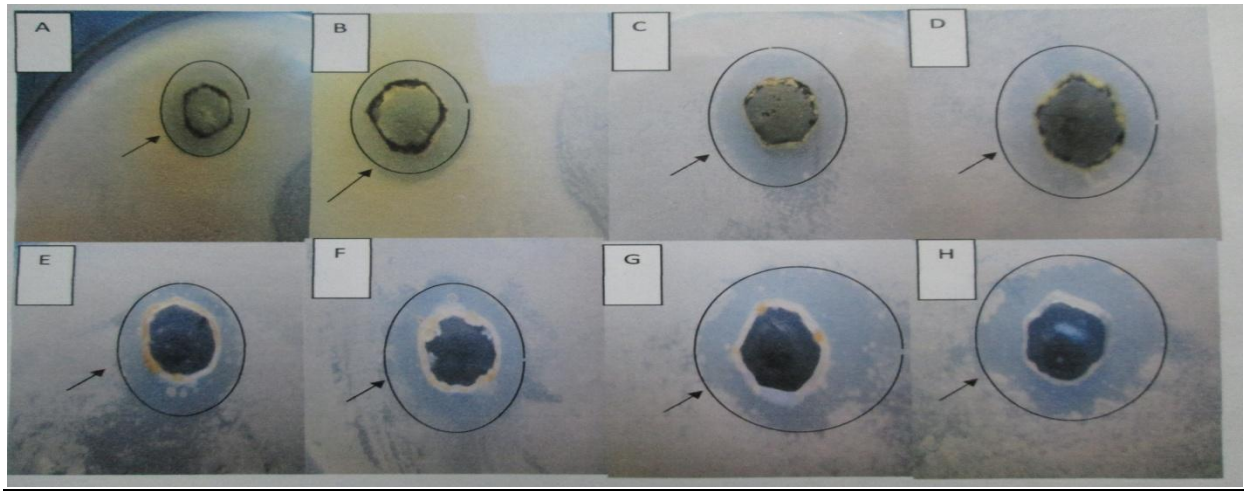
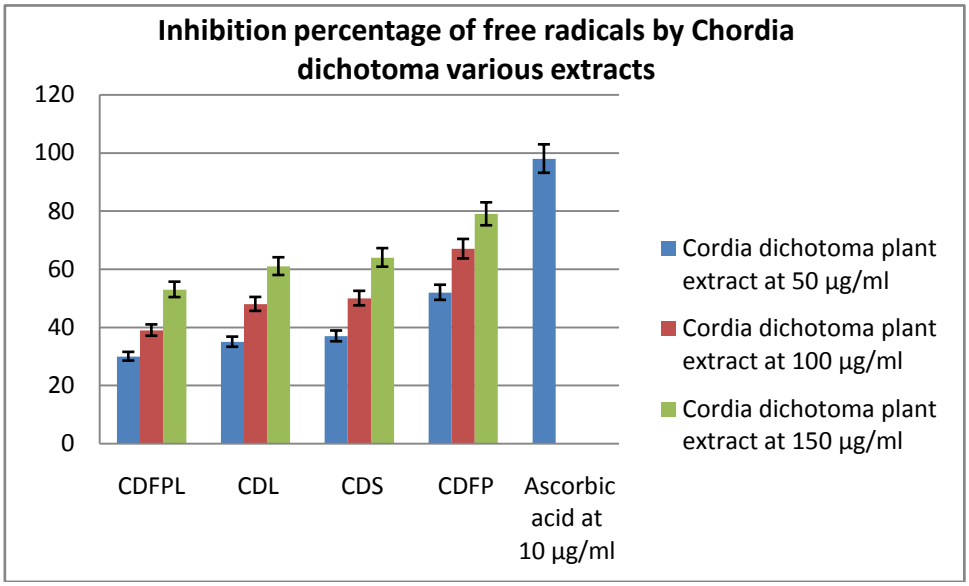


Figure 3: Zone of inhibition produced against various concentrations of *Cordia dichotoma* extract



A-B Antibacterial activity of stem at 150 µg/ml, C-D Antibacterial activity of leaves at 150 µg/ml , E-F Antibacterial activity of fruit peel at 150 µg/ml, G-H Antibacterial activity of fruit pulp on Methycillin reisistant *Staphylococcus aureus* at 150 µg/ml

Figure 4: Inhibition percentage of free radicals by *Cordia dichotoma* various extracts compared with reference standard ascorbic acid



3.5 ANTIBACTERIAL ACTIVITY

The *Cordia dichotoma* extracts of leaves, stem, fruits and fruit pulp showed good antibacterial activities. (Table 2). Fruit pulp exhibited significant activity against MRSA (*Methycilin Resistant Staphylo coccus aureu*s) by producing inhibition zones 8, 14, 19 at 50, 100 and 150 µg/ml respectively. *E.coli* and *Pseudomonas aurgenosia* were also more susceptible towards fruit pulp extract and exhibited 7, 13, 16 and 6, 10, 13 inhibition zones at 50, 100 and 150 µg/ml respectively. Among the gram positive strains, *Bacillus subtilis* and *Bacillus cereus* were more susceptible and produced 7, 12, 14 and 5, 9, 12 inhibitory zones towards fruit pulp extract at 50 100 and 150 µg/ml respectively. The highest inhibition zone 13 was produced by fruit extract against *E. coli* and *Bacillus cereus*. Results of antibacterial activity of the extracts were compared with known standards.(Table 2).

3.6 LIPID PEROXIDATION ASSAY

Methanol extracts of *Cordia dichotoma* leaves, stem, fruit peel and fruit pulp showed concentration-dependent lipid peroxidation inhibition. Among, various extracts methanol extract of fruit pulp noticed significant inhibition. Next to the fruit pulp, extract of fruit peel showed good inhibition percentage (Table 3). The inhibition percentage 52, 67, 79 % was noticed at 50, 100, 150 µg/ml respectively. The results were comparaed with 98 % standard ascorbic acid at 10 µg/ml (Figure 4).

3.7 DPPH RADICAL SCAVENGING ACTIVITY

DPPH radical scavenging activity of the various extracts, resulted good inhibition percentage of DPPH free radical. Fruit pulp was showed highest scavenging activity when compared with that from leaves, stem and fruit peel (Table 3). The percentage of free radical scavenging activity of fruit pulp at 50, 100, 150 µg/ml noticed are 63, 81, 95 % respectively. Fruit pulp extracts showed inhibition activity at 150 µg/ml is comparable to value obtained with standard ascorbic acid (Figure 4).

3.8 ABTS^{*+} RADICAL CATION DECOLOURISATION ASSAY

All the extracts showed concentration dependent scavenging activity. (Table 3, Figure). The percentage of inhibition noticed with fruit pulp was at 50, 100, 150 µg/ml are 48, 63, 84 % respectively (Figure 4).

Investigations on the primary phytochemical screening of *Cordia dichotoma* leaves, stem, fruit peel, fruit pulp extracts revealed the presence of saponins, steroids, tannins, glycosides, alkaloids and flavonoids. Among these compounds, tannins can be ttribute to antibacterial activity by inhibition of protein synthesis via, binding to proline-rich protein (Shimada, 2006). Among the various parts, fruit pulp extracts were more significant against all tested microbial strains. *Methycilin Resistant Staphylococcus aureus* (MRSA) was more susceptible towards fruit pulp extracts when compared with others. The current studies noticed that gram negative strains are more susceptible than gram positive strains. This might be because of the variations in the composition of cell wall of both types of strains (Guo *et al*., 1998). Apart from fruit pulp, fruit peel extracts also revealed notable antibacterial activity on all the tested bacterial strains. Stem extracts showed least antibacterial activity aganist microbial species tested. *E. coli* showed resistance against stem extracts at all concentrations tested. The antibacterial activity of *Cordia dichotoma* extracts were

fruit pulp>fruit peel>leaves>stem.

Cordia dichotoma methanol extracts at all tested concentrations proved as significant inhibitors of free radicals generated *in vitro*. Among the free radicals tested, DPPH was found successfully scavenged by all the extracts, which exhibited direct inhibition of lipid peroxidation. However, there was variability towards the potentiality of scavenging free radicals ions. Probably, this might be due to electron potential differences within free radical species (Pasha *et al*., 2000). Ascorbic acid in combination with Fe induced lipid peroxidation can be studied as non-enzymatic process by which the total antioxidant potentialities of the extracts were determined. The studies also revealed that extracts possessed both metal chelation and free radical scavenging properties, thus the total lipid peroxidation activity generated by the extracts could be interpreted as integrated effect of these two processes. Fruit pulp extracts are found with high potent action which was equivalent to the reference standard ascorbic acid. The number and position of OH groups of the phytochemicals present in various extracts builds a direct correlation with antioxidant activities. Phytochemicals with 3-OH group exhibited greater activity than the compound 5-OH group (Sakir *et al*., 2003).

DPPH forms stable diamagnetic molecule by accepting an electron or hydrogen atom and reactivity with reducing agent and consequently the electron becomes paired off for the formation of corresponding hydrazine. Therefore number of electron consumption was estimated by the color of solution that was lost as measured spectrometrically at 517 nm (Mukherjee, 1989).

Cordia dichotoma extracts possess ability to donate electrons and resulted significant antioxidant potentialities. ABTS*+ is a suitable method for the determination of antioxidant potentials. Its decolorization potential is more suitable to lipophilic and hydrophilic antioxidants such as plasma antioxidants, hydroxycinnamates, flavonoids, and carotenoids. Comparison to TEAC assay, ABTS*+ radical monocation does not require any intermediary radical. A green color is observed whose intensity was determined when ABTS*+ is formed complex with transition metal ion (Deng *et al* ., 2009). A good reduction reaction kinetics was observed with the extracts of *Cordia dichotoma*. All the extracts recorded strongest ability of donating electron or hydrogen to ABTS*+ radical cation /metal complex tested. However, both metals have chance to undergo redox reactions that may lead to the reduction in $Cu2^{+}$ to Cu+ and/or Fe3+ to Fe 2^{+} and makes the electron interception. The ABTS*+ assay is well documented and perfectly executed method that has ability to detect the potentiality of antioxidant to donate hydrogen to free radical in the reaction system (Huang *et al.*,2005; Sun and Tanumihardjo, 2007).

4.0 CONCLUSION

The extracts of *Choridia dichotoma* possessed antibacterial and antioxidant activities. Therefore, the extracts of this plant can be used for the treatment of bacterial infections. This report gives a preliminary scientific validation of the plant which has been used as traditional medicine for the treatment of various human aliments.

ACKNOWLEDGEMENTS

We duly thank to Dr. Ch.V Purshothamm Reddy, Chairmen of Chaitanya colleges for providing the required funds for the undertaking and successful accomplishment of this research work.

REFERENCES

1. **Chin, Y.W., Balunas, M.J., Chai, H.B., Kinghorn, A.D. (2006).** Drug discovery from natural sources. AAPS Pharma Science. 8:239-53.
2. **Kuppast, I J, Vasudeva Nayak, P. (2006).** Wound healing activity of *Cordia dichotoma* Forst. F. Fruits. Natural products Radiance. 5: 99-102.
3. **Maisale, A.B., Atlimarad, S.L., Harada gatti, D.S., Karigar, A. (2010).** Antihelnintic activity of fruit pulp of Cordia dichotoma. International jouranal of Ayureveda and Pharmacy, 1: 597-600.
4. **Sharma, U.S., Sharma, U.K., Sutar, N., Singh, A., Shukla, D.K. (2010).** Anti-inflammatory activity of *Cordia dichotoma forst f*. seeds extracts. International Journal of Pharmaceuticals Analysis. 2: 1-4.
5. **Trease, G.E., Evans, W.C. (1989).** A text book of Pharmacognosy. Balliere Tinall Ltd, Lond. 53.
6. **Harbone, J.B. (1984).** Phtochemical methods: A guide to modern techniques of plant analysis. 2nd edition. Chapman and Hall, London.
7. **Siglelton, V.L., Rossi, J.A. (1965).** Colorimetric of total phenolic substances. American journal enology and viticulture. 16: 144-158.
8. **Kartashova, G.S., Sudos, L.V. (1997).** Quantitative determination of flavanoids in the above ground part of agrimony plants. Pharmaceutical Chemistry Journal. 31: 488-491.
9. **Raman, B.V., Sai Ramkishore, A., Uma Maheshwari, M., Radhakrishnan T.M. (2009).** Antibacterial activities of some folk medicinal plants of Eastern Ghats. J. Pure and Applied Microbiology. 3: 187-194.
10. **Satoh, K. (1978).** Serum lipid peroxide in cerebrovascular disorders determined by a new colorimetric method. Clinica Chimica Acta.90: 37-43.
11. **Bouchet, Nathalie., Laurence, Barrier., Bernard, Fauconneau. (1998).** Radical scavenging activity and antioxidant properties of tannins from Guiera senegalensis (Combretaceae). Phytotherapy Research. 12: 159–162.
12. **Blois, M.S. (1958).** Antioxidant determinations by the use of a stable free radical. Nature. 181: 1199-1200.
13. **Pellegrini, RReN., Proteggente, A., Pannala, A., Yang, M., Rice-Evans, C. (1999).** Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radical Biology and Medicine. 26: 1231-1237.
14. **Shimada T. (2006).** Salivary proteins as a defense against dietary tannins. J. Chem. Ecol. 32: 1149-1163.
15. **Guo, L., Lim, K.B., Poduje, C.M., Daniel, M., Gunn, J.S., Hackett, M., Miller S.I. (1998).** Lipid Aacylation and bacterial resistance against vertebrate antimicrobial peptides. Cell. 95:189-198.
16. **Pasha, F.A., Cho, S.J., Beg, Y., Tripathi, Y.B. (2000).** Quantum chemical QSAR study of flavones and their radical-scavenging activity. Medicinal Chemistry Research. 16: 408-417.

17. **Sakir, E., Figen, E., Nevin, K. (2003).** Theoretical investigation of quercetin and its radicalisomers. Journal of Molecular Structure: Theochem. 631:141-146.
18. **Mukherjee, K.L. (1989).** Medical laboratory technology, 1st ed, Sood, New delhi: Tata McGraw Hill Publishing Company limited.
19. **Deng, W., Tan ,Y., Fang, Z., Xie, Q., Li, Y., Liang, X., Yao, S. (2009).** ABTS-multiwalled carbon nanotubes nanocomposite/Bi film electrode for sensitive determination of Cd and Pb by differential pulse stripping voltammetry," Electroanalysis**,** 21: 2477–2485.
20. **Huang, D.J., Ou, B.X., Prior, R.L. (2005).** The chemistry behind antioxidant capacity assays, J Agricultural Food Chemistry, 53(6): 1841–1856, (2005).
21. Sun, T., Tanumihardjo, S.A. (2007). An integrated approach to evaluate food antioxidant capacity. J. Food Science. 72: 159–165.