JOURNAL OF PURE AND APPLIED MICROBIOLOGY, October 2009. Vol. **3**(2), p. 769-776

In vitro Studies on Antimicrobial and Antioxidant Activities of *Dillenia indica* Seed Extract

K. Himakar Reddy[1], K. Bala Nagi Reddy[1], P.V.G.K. Sharma[2] and O.V.S. Reddy[1]*

[1]Department of Biochemistry, Sri Venkateswara University, Tirupati - 517 502, India.
[2]Department of Biotechnology, Sri Venkateswara Institute of Medical Sciences, Tirupati, India.

(Received: 10 July 2009; accepted: 16 August 2009)

Hexane extract (oil) of *Dillenia indica* seed powder was evaluated for antimicrobial and antioxidant activities. It exhibited broad spectrum of antimicrobial activity. The MIC values for different bacterial and fungal strains varied from 1.0 to 2.0 mg/ml concentration and DIZ from 12 to 28 mm of the extract. Screening for secondary metabolites of the extract revealed the presence of phenols, triterpenoids, carbohydrates and fatty acids. To evaluate antioxidant activity, TLC-DPPH, DPPH, H_2O_2, ABTS radical, SOD and reducing power activities method were carried out. The distinct antimicrobial activity was observed against prominent pathogens indicates that this oil may be useful in treatment of microbial infections. The hexane extract of *D. indica* shows moderate antioxidant activity when compared with controls.

Key words: Antimicrobial, Antioxidant, *Dillenia indica*, Secondary metabolites.

In recent years, there has been increasing search for new antimicrobial compounds owing to the lack of efficacy, side effects and or resistance associated with some of the existence drugs. Plants have served as a source of new pharmaceutical products and inexpensive starting materials for the synthesis of some known drugs. Natural products and their derivatives represent more than 50% of the drugs in clinical use in the world (Cowan, 1999; Sofowora, 1984). Naturally occurring plant biologically active products are used as an alternative strategy to prevent the spread of diseases (Elkovich, 1998). A large number of oils and their constituents have been investigated for antimicrobial properties against bacteria and fungi (El-Shazly *et al.,* 2002; Ioannou *et al.,* 2007). The oils and extracts of many plants have become popular in recent years and attempts to characterize their bioactive principle have recently gained momentum in pharmaceutical applications (Moleyar and Narasimham, 1986; Cavaleiro *et al.,* 2006). Contrary to synthetic drugs, antimicrobials of plant origin are not associated with many side effects and have an enormous therapeutic potential to heal many infectious diseases (Iwu *et al*., 1999). Different chemo-types of the same species may grow in the same place and produce different oils

* To whom all correspondence should be addressed.
Tel.: +91 877 -2289495
E-mail: ovsreddy@yahoo.com

with different activity (Kalemba and Kunicka, 2003).

The term antioxidant refers to the activity of numerous vitamins, minerals and other phyto chemicals to protect against the damage caused by reactive oxygen species (ROS) (Khlifi *et al.,* 2006). Free radicals are implicated in several degenerative diseases such as atherosclerosis, diabetes, arthritis and cancer (Halliwell *et al.,* 1997). The disturbances in redox homeostasis occurring when antioxidant defenses are inadequate can damage lipids, proteins, carbohydrates and DNA. Synthetic antioxidants BHA (Butylated Hydroxyl Anisole) and BHT (Butylated Hydroxy Toluene) are carcinogenic in nature. On the basis of above facts, natural antioxidants would be promising alternative for synthetic antioxidants (Brown and Rice-Evans, 1998; Liu *et al*., 1997). The development of alternative natural antioxidants such as those found in plants origin is of worthy consideration for our health industry and hold promising commercial potential.

Dillenia indica plant belongs to family *Dilleniaceae* and it is found in tropical forests. It is commonly called as *Dillenia*. The fruit ripens in the month of November to December (Abdille *et al.,* 2005). Green calyx part is eaten in various forms of pickles. Locally it is called as *Uvya* or *Kalinga*. The main objectives of the present study are (i) *in vitro* antimicrobial activity are (ii) *in vitro* antioxidant activity of the hexane extracted oil.

MATERIAL AND METHODS

Sample preparation

Seeds of *Dillenia indica* were procured from Gautam Global, Dehradun (India) and the seeds were authenticated by the Herbarium keeper, Department of Botany, S.V.University, Tirupati. Seeds were shade dried, powdered and subjected to solvent extraction.

Solvent extraction

100 g of seed powder of *Dillenia indica* was taken separately and soxhlated for 3-4 h with n-hexane and the hexane extract was distilled off to remove the solvent. The yield of the extract was 10.6% (v/w). The extract has been found to be soluble in acetone.

Test microorganisms

The bacterial and fungal strains used in the present study were procured from IMTECH, Chandigarh.

Antimicrobial activity

Antimicrobial activity of plant extract was tested using Disc-diffusion method according to NCCLS (2000) Bauer *et al*., (1966). The bacterial strains were maintained on Nutrient Agar and fungal strains were maintained on Potato Dextrose Agar (PDA) medium (Hi-Media). A loopful of culture from the slant were inoculated into the Nutrient broth and Potato Dextrose broth and incubated at 37°C for 24 h in case of bacteria and for fungi at 28°C for 48-72 h and 0.1 ml of this culture was evenly spread on the plates containing medium respectively. Sterile discs of Whatman No.1 filter paper of about 6 mm diameter were impregnated on the surface of the media. Hexane extracts of 0.5 mg and 1 mg/ml were prepared using acetone and were applied on the discs and incubated. The results were recorded by measuring the diameter of the inhibition zone (DIZ) around the discs.

Minimum Inhibitory Concentration (MIC)

Minimum Inhibitory Concentration was determined for the extracts that showed total growth inhibition using the protocol described below. The minimum concentration at which there was no visually detectable growth was taken as MIC. Different concentrations from 0.05 to 2 mg/ml in steps of 0.1 mg/ml were prepared separately. 0.1 ml of inoculum was added to each test tube and incubated at 28°C for 48-72 h. The lowest concentration (highest dilution) of the extract that produced no visible signs of fungal growth (no turbidity) was regarded as MIC.

Qualitative analysis

The qualitative analysis for screening of secondary metabolites were carried out using the following methods of Gibbs, (1974); Dey and Harborne, (1989); Evans, (1989); Harborne, (1998) and Akinyemi *et al*., (2005).

Determination of total phenolic content

Total soluble phenolics in the extracts were determined with Folin-Ciocalteau reagent according to the method of Slinkard and Singleton (1977) using gallic acid as a standard phenolic compound. 1.0 ml of extract solution containing 1 g extract in a volumetric flask was diluted with

46 ml of distilled water. 1 ml of Folin-Ciocalteau reagent was added and the content of the flask mixed thoroughly. Three minutes later 3 ml of 2% sodium carbonate was added and the mixture was allowed to stand for 2 h with intermittent shaking. The absorbance of the blue color that developed was read at 760 nm. The concentration of total phenols was expressed as mg/g of dry extract. The concentration of total phenol compounds in the extract was expressed as gallic acid equivalent.

TLC-DPPH antioxidant screening

This method is generally used for the screening of potential antioxidant activity in crude extracts. It involves the chromatographic separation of the crude plant extract, after which the developed chromatogram is sprayed with a coloured radical solution and the presence of antioxidant compounds indicated by the disappearance of radical colour. 10 µl of each extracts was loaded as a 1 cm band on the origin of TLC (Merck, Silica gel 60 F_{254} plates). Plates were developed using the hexane and ethyl acetate solvents (7:3) ratio. To detect antioxidant activity, chromatograms were sprayed with 0.2% DPPH in methanol as an indicator (Deby and Margotteaux, 1970) and until just wet and dried in fume hood. The presence of antioxidant compounds were detected as yellow spots against a purple background on TLC plates.

DPPH radical scavenging activity

The hydrogen atom or electron donation abilities of the corresponding extracts and some pure compounds were measured from the bleaching of the purple-coloured methanol solution of 2,2'-diphenly-1-picrylhydrazyl (DPPH). This spectrophotometric assay uses the stable radical DPPH as a reagent (Burits and Bucar, 2000). One thousand micro litres of various concentrations of the extracts in methanol were added to 4 ml of 0.004% methanol solution of DPPH. After a 30 min incubation period at room temperature, the absorbance was read against blank at 517 nm. Inhibition of free radical by DPPH in percent (I %) was calculated by using the following equation:

$$I\ \% = [(A\ control - A\ sample) / A\ blank] \times 100$$

Where A control is the absorbance of the control reaction (containing all reagents except the test compound), and A sample is the absorbance of the test compound. Tests were carried out in triplicate.

H_2O_2 scavenging activity

The H_2O_2 scavenging activity of plant extracts was determined according to the method of Ruch *et al.,* (1989). A solution of H_2O_2 (40 mM) was prepared in phosphate buffer (pH 7.4). Different concentrations of plant extracts in 3.4 ml phosphate buffer were added to a H_2O_2 solution (0.6 ml, 40 mM). The absorbance value of the reaction mixture was recorded at 230 nm. The % of inhibition was calculated.

Superoxide anion scavenging activity

Measurement of super oxide anion scavenging activity of the extracts was based on the method described by Liu *et al*., (1991) with slight modification. Superoxide radicals are generated in PMS-NADH systems by oxidation of NADH and assayed by the reduction of nitro blue tetrazolium (NBT). In this experiment, the super oxide radicals were generated in 3.0 ml of Tris-HCl buffer (16 mM, pH 8.0) containing 1.0 ml of NBT (50 ìM) solution, 1.0 ml NADH (78 ìM) solution and sample solution of ethanol and aqueous extracts of plants in water (200-1000 ìg/ml). The reaction started by adding 1.0 ml of phenazine methosulphate (PMS) solution (10 ìM) to the mixture. The reaction mixture was incubated at 100°C for 5 min, and the absorbance at 560 nm was measured against blank samples. L-ascorbic acid was used as a control. Decreased absorbance of the reaction mixture indicates increased super oxide anion scavenging activity. The % inhibition of super oxide anion generation was calculated using the following formula:

$$I\ \% = [(A\ control - A\ sample) / A\ blank] \times 100$$

Where A control is the absorbance of the control reaction (containing all reagents except the test compound), and A sample is the absorbance of the test compound. Tests were carried out in triplicate.

ABTS radical scavenging activity

The antioxidant activity of the extract was determined with stable $ABTS^+$ cation radical method of Re *et al.,* (1999). ABTS (2 mM) was prepared by dissolving in 50 ml of phosphate

buffered saline (pH 7.4). $ABTS^+$ was produced by reacting 50 ml of stock solution with 200 µl of 70 mM potassium per sulphate ($K_2S_2O_8$) water solution. The mixture was left to stand in the dark at room temperature for 15–16 h before use. For the evaluation of antioxidant activity, the $ABTS^+$ solution was diluted with PBS to obtain the absorbency of 0.80 ± 0.030 at 734 nm. Different concentrations of the extract were prepared and mixed with 3 ml of ABTS solution. The absorbance was read at room temperature after 10 min at 734 nm. PBS solution was used as a blank sample. The % of inhibition was calculated.

Reducing power

The reducing power was determined according to the Oyaizu (1986) method. Different concentrations of hexane extract of *D. indica* was prepared in methanol and mixed with phosphate buffer (2.5 ml, 0.2M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (1%, 2.5 ml). The mixture was incubated at 50°C for 20 min and 2.5 ml of trichloroaceticacid (10%) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.1%, 0.5 ml). The absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. Ascorbic acid was used as a standard.

RESULTS AND DISCUSSION

Medicinal plants possess a variety of compounds of known therapeutic properties (Chopra *et al.,* 1992; Harborne and Baxter, 1995; Ahmad and Beg, 2001). Hence, much attention has been paid to plant derived antifungal compounds based on the knowledge that plants have their own defense system. The therapeutic use of such plant products is an alternative strategy to prevent the spread of diseases (Elkovich, 1998). Hexane extract of *D. indica* was evaluated for antimicrobial activity which showed a broad spectrum of activity against bacteria and fungi used in the present study. In case of bacteria, at 1 mg/ml concentration, *P. aeruginosa* exhibited 19 mm followed by *S. aureus* (16 mm) followed by *B. subtilis* (12 mm). The hexane extract of *Dillenia indica* did not inhibit the growth of *S. typhimurium* and *E. coli*. The control of *S. aureus*

Table 1. Antimicrobial activity of hexane extracts of *D. indica* seed

Organisms	Control	DIZ		MIC
		0.5	1.0	
	Bacteria			
B. subtilis	25	-	12	2.0
E. coli	26	-	-	nt
S. aureus	22	12	16	1.4
S. typhimurium	25	-	ft	nt
P. aeruginosa	25	ft	19	1.8
	Fungi			
A. niger	22	21	28	1.0
A. fumigatus	-	21	25	1.2
C. tropicalis	15	-	ft	nt
C. neoformans	12	-	-	nt

Control- Gentamycin (Bacteria) and Ketcanazole (Fungi) 10µg/disc; ft – Faint Traces; nt – Not Tested.; DIZ and MIC were expressed in mg/ml concentration

Table 2. Screening tests for the presence of secondary metabolites in hexane extract of *D. indica* seed

Secondary metabolites	Qualitative test	Hexane extract of *D. indica* seed
Alkaloids	Iodine test	-
	Dragendorff's test	-
	Wagners test	-
Flavonoids	Pews test	-
	Shinda test	-
	NaOH test	-
Carbohydrates	Benedict's test	+
	Fehlings test	+
	Molisch test	+
Phenols	Ellagic acid test	+
	Phenols test	+
Saponins	Foam test	-
Oils	Saponification	+
	Spot test	+
Tannins	Gelatin test	-
Triterpenoids	Salkowski test	+
	Tschugajiu test	+

Table 3. Reducing power activity of *D. indica* seed

Concentration	OD at 700 nm			
(µg/ml)	25	50	250	500
Ascorbic acid	0.011	0.018	0.022	0.048
D. indica	0.09	0.012	0.020	0.045

increased markedly with the increase in concentrations. At the concentration of 250 µg/ml, the % of inhibition of SOD activity was 71 % in *D. indica* (Fig. 3).

Hydrogen peroxide is a weak oxidizing agent and can inactivate a few enzymes directly, usually by oxidation of essential thiol (-SH) groups. Hydrogen peroxide can cross cell membranes rapidly, once inside the cell, H_2O_2 can probably react with $Fe^{2+,}$ and possibly Cu^{2+} ions to form hydroxyl radical and this may be the origin of many of its toxic effects (Halliwell and Gutteridge, 1993). It is therefore biologically advantageous for cells to control the amount of hydrogen peroxide that is allowed to accumulate. At the concentration of 250 µg/ml the % of inhibition of H_2O_2 scavenging activity was found to be 52% (Fig.4).

ABTS is also a relatively stable free radical. The ABTS chemistry which, involves the direct generation of ABTS radical mono cation with no involvement of any intermediary radical is a decolorization assay. Here, the radical cation is formed prior to addition of the antioxidant test system, rather than the generation of the radical taking place continually in the presence of antioxidant. This method, used for the screening of antioxidant activity, is applicable to both lipophilic and hydrophilic antioxidants (Long *et al.,* 2000). The free radicals were scavenged by concentration dependant manner. Maximum ABTS scavenging activity was observed at 250 µg/ml (71%) and minimum activity was found to be at10 µg/ml (51%) for the extract (Fig. 5).

Increased absorbance with the increased concentrations of the reaction mixture indicated the increased reducing power (Table 3). The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity (Meir *et al.,* 1995).

CONCLUSIONS

The hexane extract of *Dillenia indica* seeds showed good antimicrobial and antioxidant activity. The present study becomes important due to occurrence of multi drug resistant strains of *S. aureus* and *P. aeruginosa*. In addition to the above the extract may be useful in treating the fungal infections caused by *A. niger* and *A. fumigates.* Further studies are under progress in our laboratory to identify the active principle involved.

ACKNOWLEDGEMENTS

We thank Dr. S.C. Basappa, former Deputy Director and Scientist, Central Food Technological Research Institute (CFTRI), Mysore (India), for his suggestions, encouragement and critical comments on the manuscript. We thank Ashvin Gautham, Dehradun (India), for providing the seeds of *Dillenia indica.* Finally we thank the CSIR, DBT and UGC, New Delhi for the financial assistance.

REFERENCES

1. Cowan, M.M., Plant products as antimicrobial agents. *Clin Microbiol Rev,* 1999; **12**: 564-582.
2. Sofowora, A., Medicinal plants and traditional medicine in Africa. John Wiley publishers, New York (2nd ed) Chichester, 1984: 66-72.
3. Elkovich, S.D., Terpenoids from the genus *Artemisia* as potential pesticides. In Cutler, HG (ed): Natural products and their potential role in agriculture: ACS Symposium Series; 380 *Ame Chem. Soc*, Washington DC 1988: 250-261.
4. El-Shazly, A., Dorai, G., and Wink, M., Composition and antimicrobial activity of Essential oil and hexane–ether extract of *Tanacetum santolinoides* (DC) Feinbr. and Fertig. *Z.Naturforsch*, 2002; **57 c**: 620-623.
5. Ioannou, E., Poiata, A., Hancianu, M., and Tzakou, O., Chemical composition and *in vitro* antimicrobial activity of the essential oils of flower heads and leaves of *Santolina rosmarinifolia* L. from Romania. *Nat Prod Res*, 2007; **21(1)**:18-23.
6. Cavaleiro, C., Pinto, E., Gonçalves, M.J., and Salgueiro, L., Antifungal activity of Juniperus Essential oils against dermatophyte, *Aspergillus* and *Candida* strains. *J. Appl, Microbiol,* 2006; **100(6)**:1333-8.
7. Moleyar, V., and Narasimham, P., Antifungal activity of some Essential oil components. *Food Microbiol*, 1986; **3**: 331-336.
8. Iwu, M.W., Duncan, A.R., and Okunji, C.O., New antimicrobials of plant origin. In: J. Janick (ed.), Perspectives on new crops and new uses. ASHS Press, Alexandria, VA.1999:457-462.
9. Kalemba, D., and Kunicka, A., Antibacterial and antifungal properties of Essential oils. *Curr Med Chem*. 2003; **10(10)**:813-829.

10. Khlifi, S., El Hachimi, Y., Khalil, A., Es-Safi, N., Belahyan, A., Tellal, R., and El Abbouyi, A., *In vitro* antioxidant properties of *Salvia verbenaca* L. hydromethanolic extract. *Indian J. Pharmacol*, 2006; **38(4)**: 276-280.
11. Halliwell, B., Antioxidants and human disease: a general introduction. *Nutr Rev,* 1997; **55**: 44-52.
12. Brown, J.E, and Rice-Evans, C.A., Luteolin-rich artichoke extract protects low-density lipoprotein from oxidation *in vitro*. *Free Radical Research,* 1998; **29**:247-255.
13. Liu, F., Ooi, V.E.C., and Chang, S.T., Free radical scavenging activity of mushroom polysaccharide extracts. *Life Science,* 1997; **60**: 763-771.
14. Abdille, Md.H., Singh, R.P., Jayaprakasha, G.K., and Jena, B.S., Antioxidant activity of the extracts from *Dillenia indica* fruits. *Food Chemistry*, 2005; **90**: 891-896.
15. National Committee for Clinical Laboratory Standards (NCCLS)., Approved standard M7-A5. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically 5th edn.2000: Wayne, PA.
16. Bauer, A.W., Kirby, W.M., Sherris, J.C., and Turck, M., Antibiotic susceptibility testing by a standardized single disc method. *Am J Clin Pathol,* 1966; **45 (4):** 493-496.
17. Burits, M., and Bucar, F., Antioxidant activity of *Nigella sativa* Essential oil. *Phytother Res*, 2000*;* **14**: 323–328.
18. Deby, C., and Margotteaux, G., Relationship between essential fatty acids and tissue antioxidant levels in mice. *C R Soc Biol Fil,* 1970; **165**: 2675-2681.
19. Ruch, R.J., Cheng, S.J., and Klaunig, J.E., Prevention of cytotoxicity and inhibition of intracellular communication by antioxidant catechins isolated from Chinese green tea. *Carcinogenesis*, 1989*;* **10**: 1003-1008.
20. Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., and Rice-Evans, C., Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Rad Biol Med*, 1999; **26**: 1231-1237.
21. Oyaizu, M., Studies on product of browning reaction prepared from glucose amine. *Jap J Nutr*, 1986; **44**: 307-315.
22. Liu, G., Zhu, G., and Huang, P., Antiinflammatory, analgesic and sedative effects of *Leontice kiangnanensis*. *Zhong guo zhong Yao Za Zhi*, 1991; **161**: 50-65.
23. Gibbs, R.D., *Chemotaxonomy on flowering plants*. Mc Gill Queens University Press, Montreal and London, 1974: 633-634.
24. Dey, P.M., and Harborne, J.B., Plant phenolics. In: *Methods in Plant Biochemistry*. Vol 1, Academic Press, London, 1989:180-250.
25. Evans, W.C., In: Trease and Evans, *Pharmacognosy*, 13th ed., Bailliere Tindall. London, 1989: 829-830.
26. Harborne, J.B., *Phytochemical Methods, A guide to modern techniques of plant analysis*, 3rd edn. Chapman and Hall, London, 1998: 302-312.
27. Akinyemi, K.O., Oladapo, O., Okwara, C.E., Ibe, C.C., and Fasure, K.A., Screening of crude extracts of six medicinal plants used in South-West Nigerian unorthodox medicine for anti-methicillin resistant *Staphylococcus aureus* activity. *BMC Complement Altern Med*, 2005; **5**: 6-13.
28. Slinkard, K., and Singleton, V.L., Total phenol analysis: automation and comparison with manual methods. *Am J Enol Vitic*, **1977; 28**: 49-55.
29. Chopra, R.N., Nayer, S.L., and Chopra. I.C., Glossary of Indian Medicinal plants, 3rd edn.1992; *Council of Scientific and Industrial Research*, New Delhi, India.
30. Ahmad, I., and Beg, A.Z., Antimicrobial and phytochemical studies of 45 Indian medicinal plants against multi-drug resistant human pathogens. *J Ethnopharmacol, 2001*; **74**: 113-123.
31. Harborne, S.B., and Baxter, H., Phytochemical Dictionary. A handbook of bio-active compounds from plants. Taylor and Francis, London, 1995.
32. Sanchez-Moreno, C., Larrauri, J.A., and Saura-calixto, F.A., A procedure to measure the antiradical efficiency of polyphenols. *J Sci Food Agric*, 1998; **76**: 270-276.
33. Yen, G.C., and Duh, P.D., Scavenging effect of methanolic extracts of peanut hulls on free-radical and active oxygen species. *J Agric Food Chem*, 1994; **42**: 629-632.
34. Halliwell, B., and Gutteridge, J.M.C., *Free Radicals in Biology and Medicine*, Oxford, Clarendon, 1993: 419.
35. Long, L.H., Kwee, D.C., and Halliwell, B., The antioxidant activities of seasonings used in Asian cooking. Powerful antioxidant activity of dark soy sauce revealed using the ABTS assay. *Free Rad Res*, 2000; **32(2)**: 181-186.
36. Meir, S., Kanner, J., Akiri, B., and Hadas, S.P., Determination and involvement of aqueous reducing compounds in oxidative defense systems of various senescing leaves. *J Agric Food Chem*, 1995; **43**: 1813-181.