Available online at www.ijpcr.com
International Journal of Pharmaceutical and Clinical Research 2010; 2(3): 101-105

Research Article ISSN 0975 1556

2, 3-Dihydroxybenzoic Acid: An Effective Antifungal Agent Isolated from *Flacourtia inermis* Fruit

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ABSTRACT
In the present study, the thrust was given to the isolation, purification and characterization of the active principle from fruit of *Flacourtia inermis* Roxb for developing a simple, but novel, antifungal agent of natural origin. The active principle was separated and purified by column chromatography and the molecular structure of the active compound was elucidated by spectroscopic analysis. The antifungal compound was identified as, 2, 3-dihydroxybenzoic acid, a simple phenolic compound. Antifungal efficiency of 2, 3-dihydroxybenzoic acid was tested against human opportunistic pathogens by agar well diffusion method. Results showed that 2, 3-dihydroxybenzoic acid was effective for inhibiting the growth of tested fungi at a concentration range of 15-60 mg/ml. Among the fungi, *Aspergillus fumigatus* showed highest susceptibility followed by *Aspergillus flavus*, *Aspergillus niger* and *Chrysosporium* species. From the results of the present studies, it is evident that 2, 3-dihydroxybenzoic acid isolated from the fruit extract of *Flacourtia inermis* possesses powerful antifungal activity against human opportunistic fungal pathogens and it can be developed as a prototype for large scale preparation of new antifungal drug.

Keywords: *Flacourtia inermis*, Loika, Louvi, 2, 3-dihydroxybenzoic acid, opportunistic pathogen, antifungal, *aspergillus.*

INTRODUCTION

Antibiotic resistance by fungal pathogens and less availability of efficient antifungal drugs are the growing challenges in antifungal therapy. To overcome these problems, increased attentions have paid in isolating antifungal compounds from natural sources including plants. The last few decades have witnessed for the isolation and characterization of biologically active natural compounds as a natural remedy against cancer, viral, fungal, bacterial and other life threatening diseases and infections. [1-2] World Health Organization's report reveals that the plant based natural compounds are the effective agents for developing potential drugs against various diseases and infections. [3-5] Traditional systems of medicines are widely used with high degree of efficacy in different parts of the world. India, a country with highest population is still depending on plant derived medicines for curing various diseases. [6-7] Abundant occurrence in the local habitat, minimum side effects and availability at affordable prices are the major reasons for increased demand for plant-derived medicines. [8-9] In traditional medicine, crude extract of whole plant or their parts are used for treating microbial and other infections. [10] In the present era, efforts have given for purification of biologically active compounds from natural sources. Detecting the molecular structure of the compound is highly helpful for understanding the nature of the compound, expected mode of action and also for developing a prototype for large scale preparation of drugs to meet the needs of millions of people all around the world.

Reports say that mycosis or fungal diseases are the prime cause for increased morbidity and mortality in many developing countries. [11-12] Fungal spores once established on the skin are very difficult to eliminate permanently and this shows the medicinal significance of fungi. The increased ability of fungal pathogens to resist against antibiotics creates new challenges in the modern antifungal therapy. [13] In this context, isolation of plant derived antifungal compounds for developing new drug is worthy.

The present study deals with the identification of the fruit of *Flacourtia inermis* Roxb for its antifungal activity against human opportunistic fungi, isolation and purification of the active principles, characterization of the antifungal agent and studies on its antifungal activity against various fungal strains. *Flacourtia inermis* is a tree of *Flacourtiaceae* family and is very common in the rural areas of Kerala State, India. In Kerala, its fruits are commonly known as Louvi, Loika, or

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Lavalolikka. Its medicinal properties are still uncertain. However, the antifungal and antibacterial properties of the crude extracts of its fruits were reported. [14-15] In the present investigation, the active principle was isolated and characterized from the fruit extracts of *Flacourtia inermis* and it was purified, characterized and studies were done to establish its antifungal efficiency against human opportunistic pathogens.

MATERIALS AND METHODS

Soxhlet extraction followed by chromatography was employed for the isolation and purification of the antifungal compound form the fruit extract of *Flacourtia inermis*. Fresh fruits were collected from rural areas of Kerala State, India and washed thoroughly, cut into small pieces, dried in hot air oven at 60°C and were powdered in a mixer grinder. 250 g of the dried powder was serially extracted with 500 ml each of petroleum ether, chloroform and acetone using a Soxhlet extractor. When these crude extracts were tested for antifungal activity by agar well diffusion method against opportunistic pathogenic fungi, the acetone extract was found to be more active; therefore, this fraction was used for further studies. The acetone fraction was concentrated on a vacuum rotary evaporator and the extract was subjected to chromatographic analyses.

Thin Layer Chromatography (TLC)

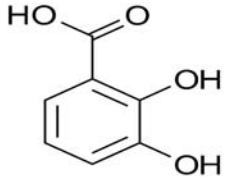
From TLC study, a mixture of chloroform-methanol in the ratio of 85:15 was identified as the eluent for column separation.

Column chromatography

Using activated silica gel of 60-120 mesh size, the acetone extract was eluted out with chloroform-methanol in the ratio 85:15. The different fractions were concentrated by evaporation and antifungal activities were tested. The active antifungal fraction was identified and again subjected to stepwise gradient elution using chloroform- methanol mixture for removing the impurities. The antifungal fraction eluted with chloroform-methanol in the ratio of 90:10 was found to be pure and subjected to spectroscopic analysis.

Spectroscopic analysis of the antibacterial compound of *Flacourtia inermis* fruit

Mass of the compound obtained in GCMS was 154.1 (Fig. 1). CHN analysis showed 54% C, 41.5% O and 3.8% H in the compound. UV-visible spectrum showed peaks at 294 nm and 269 nm. Infrared (IR) spectral analysis showed peaks at 3204 cm^{-1}, 1675 cm^{-1}, 1598 cm^{-1}, 1296 cm^{-1} and 1126 cm^{-1} (Fig. 2). In ^{1}H NMR spectrum, peaks were obtained at δ 9.518, two doublets at δ 7.519 and δ 6.880, double doublets at δ 7.449 and δ 7.469, and a peak at δ 3.45 (Fig. 3). ^{13}C-NMR spectrum showed signals at 167.07, 149.97, 144.73, 122.70, 122.17, 116.56 and 114.77 ppm (Fig. 4). Based on the data obtained from CHN analysis, GC-MS, IR, UV, ^{1}H NMR and ^{13}C NMR, the isolated antifungal compound of *Flacourtia inermis* fruit was identified as **2, 3-dihydroxybenzoic acid (2, 3 -DHB),** a phenolic compound with a molecular formula of $\mathbf{C_7H_6O_4}$.



2, 3-dihydroxybenzoic acid

For determining the antifungal efficiency, different concentrations of 2, 3-DHB was tested against human opportunistic pathogenic fungi.

Fungal strains

Antifungal activity of the 2, 3-DHB was tested against opportunistic pathogenic fungi such as *Chrysosporium* sp, *Aspergillus fumigatus*, *Aspergillus flavus* and *Aspergillus niger*. These strains were collected from Tropical Institute of Ecological Sciences, Kottayam-Kerala. They were sub-cultured on Sabouraud's Dextrose Agar (SDA) medium, incubated at 25°C to 28°C for 2 to 10 days, and were maintained at 4°C in a refrigerator.

Antifungal test

Antifungal activity of the 2, 3-DHB was tested by Agar Well Diffusion Method. [16-17] After adding the fungal spores into the SDA medium aseptically, it was poured into petri plates and allowed to set. Into a well of 6 mm, 1 ml each of various concentrations, 60mg/ml, 30mg/ml, 15mg/ml, 8mg/ml and 4mg/ml of 2, 3-DHB dissolved in warm distilled water was added. For control test, 1 ml of sterile distilled water, without compound, was added. Test and control plates were incubated 25°C to 29°C for 2-10 days. After the incubation period, activity was determined by measuring the diameter of zone of inhibition in millimeters. Tests were repeated three times and the mean value was calculated (mean fractions were avoided) and recorded.

RESULTS

Results are given in the Table 1. The control experiment did not show any activity where as all the tested fungi were sensitive towards the sample. Among the strains, *Aspergillus fumigatus* showed highest susceptibility and *Chrysosporium* species showed least susceptibility against all the tested concentrations of the compound. *Aspergillus fumigatus* (Fig. 5) was active even at a concentration of 8 mg/ml where as *Aspergillus flavus* and *Aspergillus niger* were active at 15 mg/ml. Activity was found to be increased along with increasing the concentration of the 2, 3-DHB. *Chrysosporium* species showed effective inhibition at a concentration of 30 mg/ml. However, a concentration of 60mg/ml is ideal for controlling the growth of *Chrysosporium*.

Table 1: Antifungal activity of 2, 3-dihydroxybenzoic acid of *Flacourtia inermis* fruit against opportunistic pathogenic fungi

S. No.	Tested strains	Zone of inhibition in mm				
		60 mg	30 mg	15 mg	8 mg	4 mg
1.	*Aspergillus fumigatus*	37	30	25	16	0
2.	*Aspergillus flavus*	31	24	18	0	0
3.	*Aspergillus niger*	33	22	19	0	0
4.	*Chrysosporium species*	27	15	0	0	0

DISCUSSION

Like bacteria, fungi have developed drug resistance. Injudicious, indiscriminate and over uses of antibiotics are the major factors causing drug resistance. [18] Most of the early day's non-pathogenic saprophytic fungi have now transformed as opportunistic and invasive human pathogen and their infection is more severe in immuno-incompetent patients. [19-20] Among the invasive pathogens, Aspergillus species such as *Aspergillus fumigatus*, *Aspergillus flavus* and *Aspergillus niger* stand the first place. Nasal to bronchial membranes of the respiratory tract are the major sites for their attack. HIV infected patients and those with over use of drugs are found to more susceptible to aspergillus infections. [21] 'Aflatoxin', a secondary metabolite of *Aspergillus*

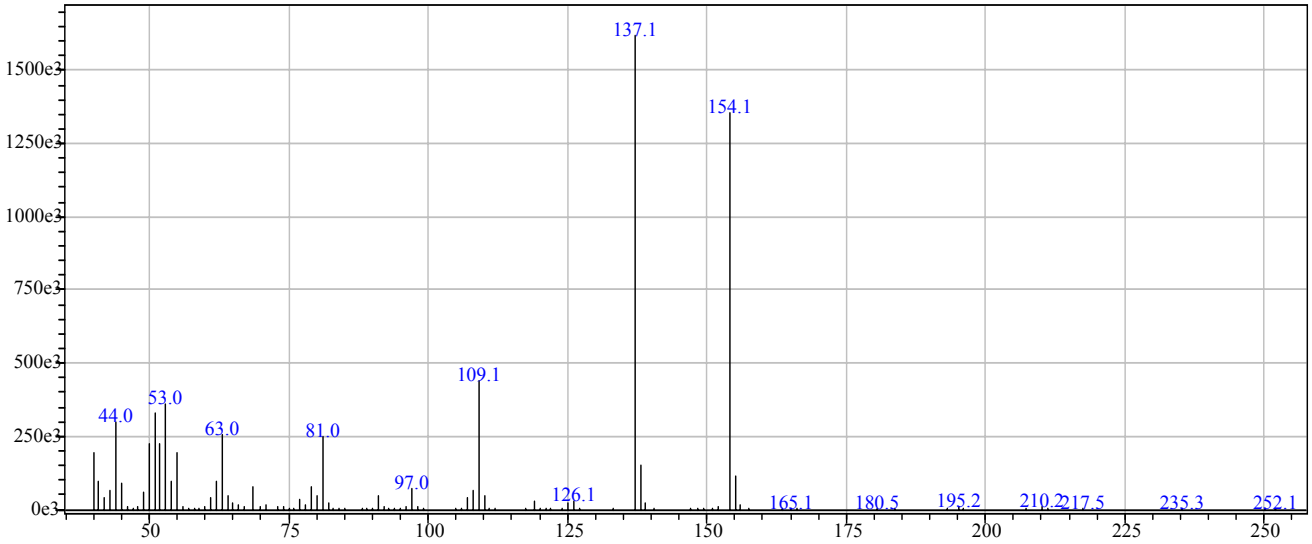


Fig. 1: Mass spectrum of 2, 3-DHB

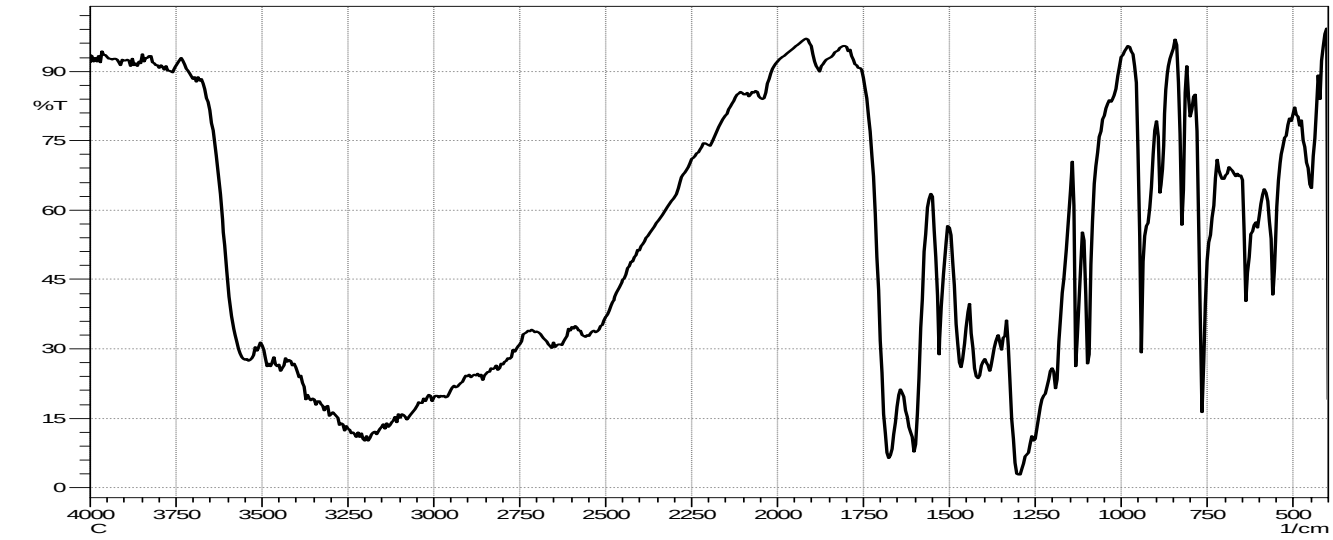


Fig. 2: IR spectrum of 2, 3-DHB

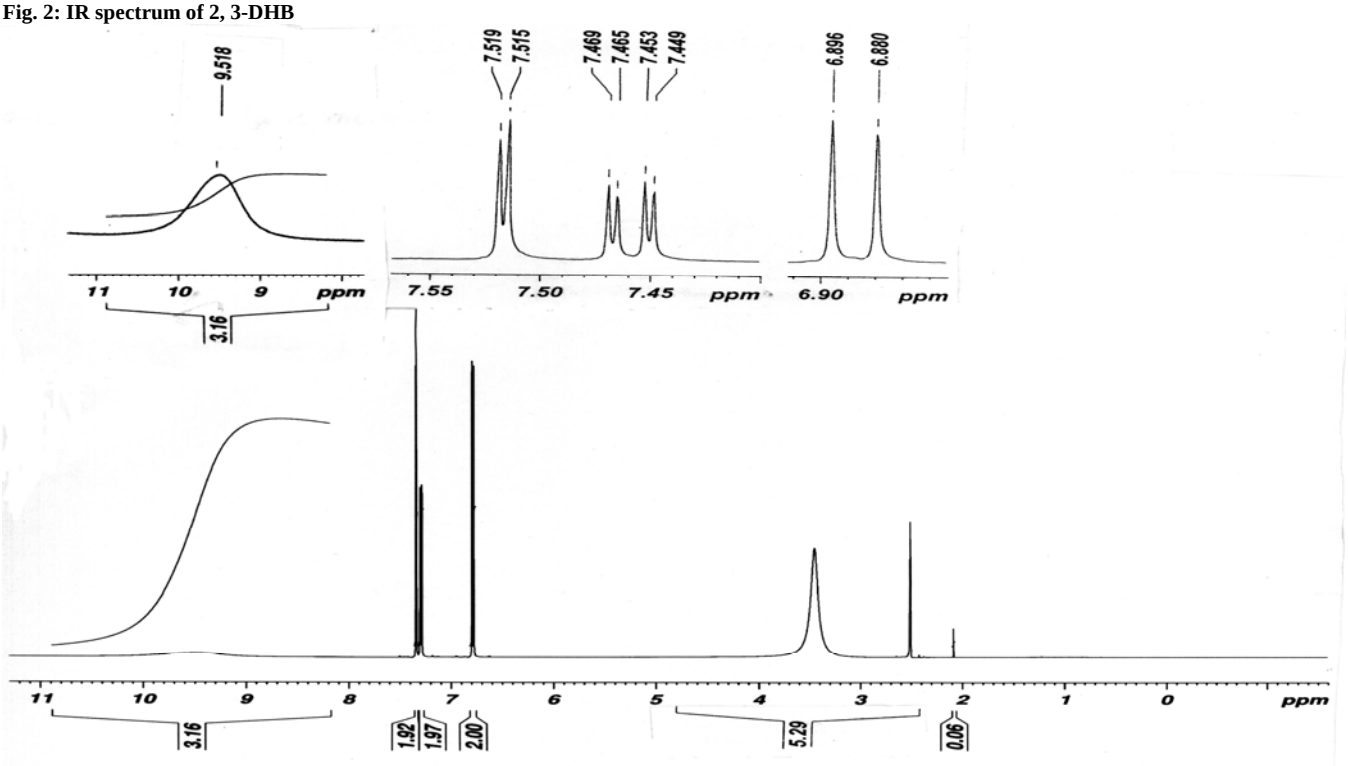


Fig. 3: ^{1}H NMR of 2, 3-DHB

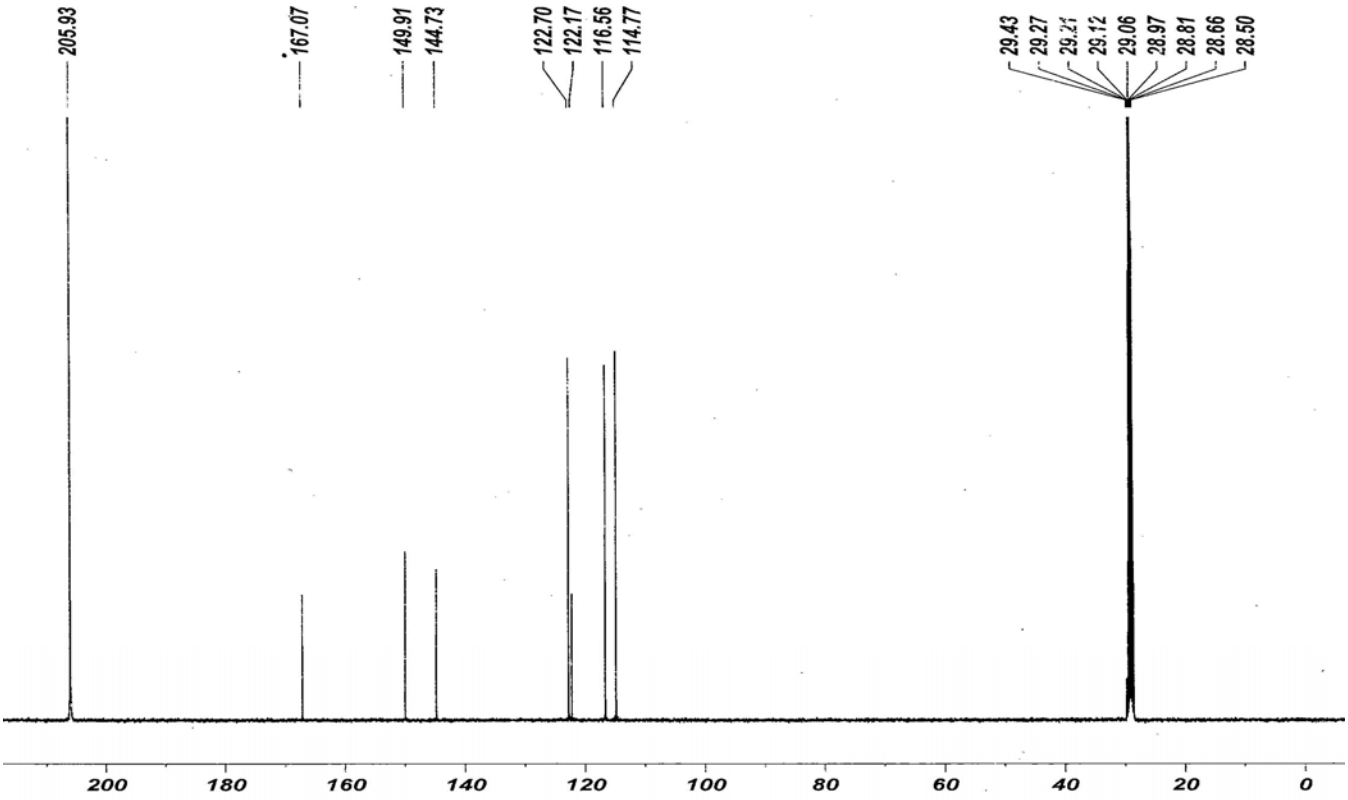


Fig. 4: 13C NMR of 2, 3-DHB

Fig. 5: *Aspergillus fumigatus*:-30mg/ml sample

fumigatus and *Aspergillus flavus* are dangerous human carcinogens. [22] *Chrysosporium* is also developed as an opportunistic skin and respiratory tract pathogen. [23]

The tested pathogens were effectively inhibited by the isolated 2, 3-DHB of the *Flacourtia inermis*. The antifungal activity of the crude extract of *Flacourtia inermis* fruit has already been reported but the active fraction was uncertain. [14] In the present study, the active fraction was identified as 2, 3-DHB. The antifungal activity of 2, 3-DHB is limited in the literature. The present studies suggest that the 2, 3- DHB can be used as a powerful antifungal agent against *aspergillus* and *chrysosporium* species.

For centuries, the people of Kerala have been used the fruits of *Flacourtia inermis* in fresh form or cooked. No toxicity or side effects of this fruit have reported so far. Therefore, 2, 3-DHB of *Flacourtia inermis* is expected to be a non-toxic compound to human cells. Simple compounds are easy to metabolize and eliminate by the body cells. Hence, 2, 3-DHB will be a valuable compound in modern medicine.

ACKNOWLEDGMENTS

Authors are thankful to Dr. N. Sasidharan, Scientist, Kerala Forest Research Institute, Peechi, Trissur and Dr. Jomy Augustine, Head of the Department of Botany, St.Thomas College, Palai, for their valuable help given for identifying the taxonomic position of the fruit of *Flacourtia inermis*. Authors are also grateful to Directors and Technicians of Tropical Institute of Ecological Sciences, Kottayam-Kerala for helping antifungal studies.

REFERENCES

1. Bankole MA, Shittu LAJ, Ahmed TA, Bankole MN, Shittu RK, Kpela T **et al**. Synergistic antimicrobial activities of phytoestrogens in crude extracts of two sesame species against some common pathogenic microorganisms. Afr J Trad CAM 2007; 4(4): 427 – 433.
2. Koshy P, Sri Nurestri AM, Wirakarnain S, Sim KS, Saravana K, Hong SL **et al**. Antimicrobial activity of some medicinal plants from Malaysia. Am J Applied Sci 2009; 6(8): 1613-1617.
3. Upadhyaya A P, Chandrikasingh S. Screening for the antimicrobial potentials of some of the plant extracts and phytochemical on the test organisms. International Research Journal 2009; 1(6): 118-119.
4. Sandipan C, Syed AA. Studies on the antibacterial activity of *Tagetes erecta* Linn leaves. Arch Pharm Sci and Res 2010; 2(2): 360-361.
5. Vinod NV, Haridas M, Sadasivan C. Isolation of 1, 4-naphthalenedione, an antibacterial principle from the leaves of *Holoptelea integrifolia* and its activity against β- lactam resistant *Staphylococcus aureus*. Indian J Biochem Biophys 2010; 47: 53-55.
6. Pirzada AJ, Shaikh W, Ghani KU, Laghari KA. Study of antifungal activity and some basic elements of medicinal plant C*ressa cretica* Linn against fungi causing skin diseases. Sindh Univ Res Jour (Sci Ser) 2009; 41(2): 15-20.

7. Harish Kumar K, Hullatti KK, Sharanappa P, Paras S. Comparative antimicrobial activity and TLC bioautographic analysis of root and aerial parts of A*ndrographis serpyllifolia*. International Journal of Pharmacy and Pharmaceutical Sciences 2010; 2(1): 52-54.
8. Nair R, Chanda S. Activity of some medicinal plants against certain pathogenic bacterial strains. Indian J Pharmacol 2006; 38(2):142-144.
9. Seema JP, Nithin V, Pradeep S. Screening for antimicrobial activity of weeds. The Internet Journal of Microbiology 2007; 4(1):1-8.
10. Mahesh B, Satish S. Antimicrobial activity of some important medicinal plant against plant and human pathogens. World J Agric Sci 2008; 4(S): 839-843.
11. Wang A, Zhang Y, He L, Shen Z, Liao W, Han M **et al**. Clinical study on the efficacy and safety of intravenous Itraconazole infusion for the treatment of invasive fungal infection in China. Jpn J Infect Dis 2006; 59: 370-376.
12. Schelenz S, Barnes RA, Kibbler CC, Jones BL, Denning DW. Standards of care for patients with invasive fungal infections within the United Kingdom: A national audit. Journal of Infection 2009; 58: 145-153.
13. Sunita B, Mahendra R. Antifungal activity of essential oils from Indian medicinal plants against human pathogenic *Aspergillus fumigatus* and *A. niger*. World J Med Sci 2008; 3(2): 81-88.
14. George S, Benny PJ. Antifungal activity of acetonic extract of *Flacourtia inermis* fruit against human opportunistic pathogens. Journal of Global Pharma Technology 2010; 2(6): 28-34.
15. Shibumon G, Benny PJ. Antibacterial potency of fruit extracts of *Flacourtia inermis* against multidrug resistant strains and comparison of its activity with that of standard antibiotics. Int J Pharm Sci Bio 2010; 1(2):96-99.
16. Varaprasad B, Prasanth KK, Chandrasekhar NK , Somasekhar P. Antifungal activity of selected plant extracts against phytopathogenic fungi *Aspergillus niger* F 2723. Indian Journal of Science and Technology 2009; 2(4):87-90.
17. Zarei AM, Gharib Nasery MK. Antifungal activity of shallot, *Allium ascalonicum* Linn. (Liliaceae), *in vitro*. J Med Plant Res 2009; 3(5): 450-453.
18. Sharma B, Kumar P. Extraction and pharmacological evaluation of some extracts of *Tridax procumbens* and *Capparis deciduas*. International Journal of Applied Research in Natural Products 2009; 1(4): 5-12.
19. Navarro Garcia VM, Gonzalez A, Fuentes M, Aviles M, Rios M Y, Zepeda G **et al**. Antifungal activities of nine traditional Mexican medicinal plants. Journal of Ethnopharmacology 2003; 87: 85–88.
20. Francisco MG, Adriana DG, Marcelo FM, Ana MR, Paul GML, Marcela R **et al**. New small-size peptides possessing antifungal activity. Bioorg Med Chem 2010; 18:158–167.
21. Kontoyiannis DP, Mantadakisy E, Samonisz G. Systemic mycoses in the immunocompromised host: an update in antifungal therapy. Journal of Hospital Infection 2003; 53: 243-258.
22. Goncalez E, Felicio JD, Pinto MM. Biflavonoids inhibit the production of aflatoxin by *Aspergillus flavus*. Braz J Med Biol Res 2001; 34(11): 1453-1456.
23. Abdel-Razik M, Zaki SM. Experimental pathogenicity and molecular characterization of an environmental isolate of *Chrysosporium zonatum* Al-Musallam and Tan (Family: Onygonaceae, Order: Onygonales). Int J Agri Biol 2008; 10(3): 273-277.