JOURNAL OF PURE AND APPLIED MICROBIOLOGY, October 2009. Vol. **3**(2), p. 777-784

Anti-candidal Activity of *Vitex negundo* L.: An Ethnomedicinal Plant

S.K. Panda, B.C. Behera and S.K. Dutta*

PG Department of Zoology, North Orissa University, Baripada - 757 003, India,

(Received: 14 April 2009; accepted: 07 June 2009)

The anti-candidal activity and phytochemicals of the leaf and bark of *Vitex negundo* L. (Verbenaceae) was evaluated against four species of *Candida* viz. *C. albicans, C. krusei, C. parapsilosis* and *C. tropicalis.* Both polar and non-polar extracts viz. petroleum ether, ethyl acetate, ethanol, methanol and aqueous were prepared and studied for anti-candidal activity using agar cup and broth dilution methods. Although all five extracts showed substantial inhibitory action against all tested *Candida* species, yet maximum activity was observed in ethanol extract of bark. Minimum Inhibitory Concentration (MIC) values for most of the extracts ranged from 0.156 to 2.5 mg/ml; while the least Minimum Fungicidal Concentration (MFC) value was observed at 2.5 mg/ml. Phytochemical analysis exhibited the presence of carbohydrates, tannin and phenolic compound, glycoside, saponin, steroid & sterols and flavonoid in different extracts. These results, exhibit the anti-candidal activity of *Vitex negundo* L. extracts would have potentials as an herbal treatment of candidiasis. However, the active components responsible for antifungal activity need to be evaluated.

Key words: Similipal Biosphere Reserve, candidiasis, *Vitex negundo,* Phytochemical, MIC, Medicinal plant.

Due to increasing development of drug resistance in human pathogens as well as the appearance of the undesirable effects of certain antimicrobial agents, there is a need to search for new agents. Candidiasis is an acute or chronic, superficial or deep infection with a very wide clinical spectrum occurs mostly in patients who are predisposed to an overgrowth of their own yeast flora. Oropharyngeal candidiasis occurs in patients with diabetes mellitus, those receiving antibacterial antibiotics and those infected with HIV (Kwon-Chung, 1992). Oral candidiasis is usually treated by topical antifungal agents, which include nystatin, miconazole, fluconazole, itraconazole and amphotericin B (Goff *et al.,* 1995; Nolte *et al.,* 1997). However, the management of *Candida* infections faces a number of problems including; limited number of effective antifungal agents (Mehta *et al.,* 2002); toxicity of the available antifungal agents (Mehta *et al.,* 2002); resistance of *Candida* to commonly used antifungals (Perea *et al.,* 2001); relapse of *Candida* infections (Debruyne, 1997); and the high cost of antifungal agents (Mehta *et al.,* 2002). When relapses occur, the infections tend to be increasingly refractory to treatment.

The difficulties associated with the management of *Candida* infections necessitate the discovery of new antifungal agents, in order to

* To whom all correspondence should be addressed.
Tel.: + 91-9437143598; Fax: +91-679-2253908
E-mail: sk_dutta@yahoo.com

widen the spectrum of activity against *Candida* and combat strains expressing resistance to the available antifungal agents. Either nystatin suspension or the clotrimazole douches is the drug of choice in candidiasis for nonimmunosuppressed adults. Patients with advanced HIV infection or other immunosuppressed disorders may not respond to clotrimazole and may require systemic therapy with ketoconazole. *Candida albicans* is reported to be the commonest species of *Candida* causing infection in AIDS and vulvovaginal candidiasis, other species like *Candida tropicalis, Candida glabrata, Candida parapsilosis,* and *Candida krusei* are also reported (Kumar *et al.,* 1996; Chander, 2002). However, episodes due to non-albicans species of *Candida* appear to be increasing everyday.

Vitex negundo L. (Verbenaceae) commonly known as nirgundi chiefly occurring throughout India, is widely distributed in Similipal Biosphere Reserve, Orissa. Some studies have also been done on antimicrobial activity of *Vitex negundo* along with some other Indian medicinal plants (Ahmed *et al*. 1998; Kumar *et al*. 2006; Panda *et al*. 2009; Parekh and Chanda, 2008; Rusia and Srivastava 1998; Sathiamoorthy *et al*. 2007; Valasraj *et al*. 1997). These works give little information on anti-candidal property of this plant. Hence in the present experiment an attempt has been made to evaluate the anti-candidal activity of different extract (petroleum ether, chloroform, ethanol, methanol and aqueous) against four human pathogenic yeast and the biological activities of the extracts in terms of MIC and MFC were also determined. Besides, phytochemical screening of the extracts were also carried out with view assess the presence of different phytochemicals in different extracts. Selection of the medicinal plant for the present study was based on its ethnomedicinal usages and preliminary screening of antimicrobial activity made by the authors (Thatoi *et al.,* 2008; Panda *et al.,* 2009). The tribes of Similipal used seven teaspoons of juice extracted from leaves of *Vitex negundo* and barks of *Strychnos nux-vomica* are applied like ointment on the affected part. Leaves are used as insect repellent in storing the food grains. Bark paste is externally applied to cure scabies, itches and allied skin infections Young leaves are heated with mustard oil and applied in ear to cure earache. Poisonous snake change their resting place keeping the peduncle on their hole.

MATERIALS AND METHODS

Study area

The Similipal massif lies between 21°-28' and 22°-08' North latitude and 86°-04'and 86°-37' East longitude in the Mayurbhanj district of Orissa covering 5,569 Km^2 of forest land, a unique habitat of mixed tropical forest which harbor varied flora and fauna (Fig. 1). The ecosystem is enriched with variety of medicinal plants. The total number of species comprising the flora of the hills is 990, representing 145 families of vascular plants, with occurrence of more than 500 medicinal plants (Saxena and Brahmam 1989; Pandey and Rout, 2002).

Plant material

Bark and leaves of *Vitex negundo* were collected in the month of Dec, 2007 from Similipal Biosphere Reserve, Mayurbhanj, Orissa. The collected bark and leaves along with complete herbarium of the plant of *Vitex negundo* was sent for identification and finally was authenticated by Department of Botany, North Orissa University, Baripada. The shed dried healthy leaves and bark were powdered separately using mechanical grinder and then were passed through sieve so that uniform powder size is maintained.

Preparation of extract

Sequential extraction was carried out with the same powder using solvents of increasing polarity. About 250 g of dry bark and leaf powder were sequentially extracted using petroleum ether, chloroform, ethanol, methanol and aqueous solution in Soxhlet apparatus. After about forty siphons of each solvent extraction step, the materials were concentrated by evaporation.

Microorganisms and growth media

Four species of *Candida* viz. *C. albicans* (Resistant to Ak, B, Ce, Nf); *C. krusei* (Resistant to Ap, B, Cc, It, Kt, Ns); *C. parapsilosis* (Resistant to Ak, B, Ce, Nf); *C. tropicalis* (Resistant to Ak, B, It, Ce, Nf) are used as the test organisms and are obtained from S C B Medical College, Cuttack, India. These organisms were cultured on Sabouraud dextrose agar at 30 ºC for 24 h and the stock culture was maintained at 4 ºC and sub-cultured as needed.

Phytochemical analysis

Qualitative phytochemical analysis was carried out using method described by Trease and Evans (1989). Each extract was screened for presence of alkaloid, flavonoid, carbohydrates, glycosides, saponin, tannin & phenolic compound, gum & mucilage and steroid & sterols.

Assay for anti-candidal activity by agar cup method

The agar cup method of Barry (1980) was followed with little modification to ensure the anti-candidal activity of the extracts. Plates of Sabouraud dextrose agar media were seeded with a 100 µl of suspension of actively growing over night culture of yeast cells. Wells (6 mm diameter) were made on Sabouraud dextrose agar plate using a sterile standard cork borer. The bottoms of the wells were sealed by pouring 50-100 µl of molten Sabouraud dextrose agar into scooped out wells. A 50 µl (10 mg/ml) of extract were poured into the wells and allowed to evaporate water. The yeast seeded plates were incubated at 30 ºC for 24 h, after which the diameters of zone of inhibitions were measured. Each experiment was carried out in triplicates. The average diameter of the inhibition zone was taken for evaluating the anti-candidal activity of the extracts.

Evaluation of Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC)

A broth micro-dilution technique was adopted using 96 well micro-titer plates and tetrazolium salt, 2,3,5-Triphenyltetrazolium chloride (TTC) was carried out to determine the MIC by slight modification of Eloff (1998) for *Candida* species. In the plate, A1 to H1 was the blank and consisted of Sabouraud dextrose broth (SDB) only. A3 to H3 was having the stock solution of the test extract(s) and A4 to H4 till A9 to H9 were the wells in which the test extracts were serially diluted using Sabouraud dextrose broth. Wells A12 to D12 were control having 20µl of DMSO and E12 to H12 served as control over control. All wells were dispensed with 100µl of SDB. 20µl of the herbal extract was transferred from stock test solution to the first well i.e. from A4 to H4 containing 100µl of SDB. 20µl of the SDB containing herbal extract was then transferred to the next well to create serial dilutions. 100µl of the 0.5 McFarland adjusted activated culture in Sabouraud dextrose broth was then added to all the wells except the blank. 5µl of 0.5 % TTC was further added to all the dilutions, blank, control and control over control. The final volume of all the wells was 205µl. The Micro plate was sealed and incubated at 30 ºC at 110 rpm. Each assay was repeated twice. 10µl of the broth from each culture tube exhibiting MIC and control tubes were taken aseptically and were plated on one day old Sabouraud dextrose agar plate as point inoculums and allowed to dry for 10 min under the laminar air hood to find out MFC. These were then sealed and incubated at 30 ºC for 48 h and observed for growth of the yeast.

RESULTS AND DISCUSSION

Frequently *Candida* infection has risen dramatically since the advent of antibiotics and development of the drug resistant. Development of drug resistant pathogens demand new strategies and the native peoples ethnobotanical knowledge which has received less emphasis, is a valuable resource which should be utilized to advance health oriented objectives. Since the incidence of *Candida* strains with multiple antibiotic resistances is increasing world wide, it is of great importance to find effective treatments for infection of these pathogens. Novel, safe and effective compounds may be found through consultation with traditional healers or tribal peoples using herbal medicines. Certainly indigenous plants are reservoirs of novel antimicrobials; they would play important roles in providing us with such bioactive in future. This encouraged us to evaluate the natural resources of our country to identify an antifungal agent, particularly against *Candida albicans, Candida tropicalis*, *Candida parapsilosis* and *Candida krusei.*

The result of preliminary phytochemical screening of five extracts (petroleum ether, ethyl acetate, ethanol, methanol and aqueous) of both leaf and bark are shown in Table 1. Evaluation of phytochemicals such as alkaloid, flavonoid, carbohydrates, glycosides, saponin, gum & mucilage revealed the presence of most of constitutes in polar extracts such as ethanol,

Table 1. Phytochemical screening of different extract of *Vitex negundo* bark and leaf

Solvent	Plant Part (s)	Color	% of yield	Alkaloids	Carbohydrates	Tannin & Phenol	Glycosides	Flavonoid	Saponin	Steroid	Gums & Mucilage
Petroleum ether	Bark	Blackish	1.07	-	-	-	-	++	-	-	-
	Leaf	Greenish	1.24	-	-	-	-	++	-	++	+
Chloroform	Bark	Blackish	1.52	-	-	-	-	++	+	-	-
	Leaf	Deep greenish	9.86	-	-	-	-	++	+	++	-
Ethanol	Bark	Blackish	5.86	+	-	++	+	++	+	-	-
	Leaf	Greenish black	14.88	++	-	+++	+	++	+	+	+
Methanol	Bark	Greenish black	4.34	++	+	+++	+	++	+	-	-
	Leaf	Greenish	7.84	+++	++	++	+	++	+	-	+
Aqueous	Bark	Greenish black	4.82	++	+++	+++	-	++	-	-	-
	Leaf	Greenish black	8.64	+++	++	++	-	++	-	-	+

(+++) Present in high amount; (++) Present in medium amount; (+) Present in trace amount; (-) Absent

Table 2. Screening of anticandidal activity of *Vitex negundo* bark and leaf by agar cup method

Strain used	Petroleum ether		Chloroform		Ethanol		Methanol		Aqueous		Antibiotic	
	Bark	Leaf	Bark	Leaf	Bark	Leaf	Bark	Leaf	Bark	Leaf	Clotrim-azole	Cephol-axime
Candida albicans	11.3±0.57	10.6±0.57	11.6±0.57	9.6±0.57	15.0 ±2.6	12.3±0.57	14.0± 1.0	11.3±0.57	13.6 ±1.5	13.6±0.57	20.0±1.0	-
Candida krusei	11.6±0.57	9.6±1.52	11.3±0.57	-	15.0± 2.0	9.3±0.57	15.0 ±1.7	11.3±0.57	13.6 ±0.5	13.0±1.00	-	14.33± 0.5
Candida parapsilosis	13.6±0.57	12.0±0.00	10.3±0.57	11.0±0.00	15.6±0.57	12.3±0.57	13.0±0.00	13.0±1.00	11.3±3.21	11.3±0.57	28.6±1.1	-
Candida tropicalis	13.6±0.57	10.6±2.3	9.6±0.57	10.6±0.57	13.6±0.57	13.0±0.00	14.3 ±0.5	13.3±0.57	10.0±1.73	12.3±0.57	29.6±1.1	-

(Zone of inhibition of mean ± SD in mm); (-) No zone of inhibition; Zone of inhibition including 6 mm borer.

methanol and aqueous extracts compared to non polar extracts (petroleum ether and chloroform). However, flavonoid was found to be universally occurring in all the extracts.

The susceptibilities of the various *Candida* species to both the plant extract and standard antifungal agents were determined by the agar cup method are given in Table 2. It is noteworthy that most of the leaf extracts of *Vitex negundo*, produced no outstanding activity against *Candida* species used in the test. Also chloroform extract of bark and leaf has no significant activity against any of the tested strain. Ethanol and methanol extract of bark showed highest zone of inhibition against all tested *Candida* species. Oral candidiasis is usually treated by topical antifungal agents, which include nystatin, clotrimazole, itraconazole and amphotericin B (Goff *et al.,* 1995; Nolte *et al.,* 1997). However, these antibiotics are getting resistance to *Candida* species (Perea *et al.,* 2001). Two tested *Candida* species viz. *C. krusei, C. tropicalis* showing resistance to these common antibiotics such as nystatin, clotrimazole, fluconazole, itraconazole and amphotericin B. From agar cup method result obtained that there was no significant difference among the species, while there were marked differences between the activities of the plant extract and those of the pure anti-fungal drugs. Such significant differences are normally present when crude (unpurified) plant extracts are compared with pure drugs that are already in clinical use (Yoder, 1982). Also the agar cup method is not always dependable for accurate assessment and comparison. This is because of the high degree of interference inherent in this method, arising from drug diffusion problems (Dickert *et al.,* 1981).

A more generally accurate method of assessment is the broth-dilution technique. In this study, therefore, the broth dilution method was used in determining the activities measured as MIC. In using this method, higher degrees of inter-strain differences in susceptibility among *Candida* species were observed (Table 3). It would appear that *Candida tropicalis* is the most sensitive strain to the tested extracts. *Candida tropicalis* is one of the non-albicans *Candida* strains currently emerging in fungal infections (Powderly *et al*. 1999).The effect of the unrefined nature of the plant extract as compared to the reference drugs is once more apparent. But there is obvious evidence that the plant extract has a substantial level of anti-candidal activity. Result of MFC showed that at concentration 5.0 mg/ml, 42.5 % of the tested *Candida* strains were killed while rest 57.5 % was inhibited at same concentration. Similipal possess a rich tradition in the use of medicinal plants and an outstanding floral diversity of vascular plants, little research have been done on the context of phytochemicals leads for therapeutic use. The present study has clearly demonstrated that the medicinal knowledge held by the tribal peoples is relatively measurable in laboratory based assay.

At the preliminary stage of the study, it is yet not clear which of the phytochemical constituents is dominantly responsible for the anti-candidal activity. In order to clarify this aspect, a more detailed investigation of the activity of the extract is currently on-going. Sathiamoorthy *et al.* (2007) have reported that ethanol extract of the leaf contains a new flavone glycoside compound which was found to have significant antifungal activity against *Trichophyton mentagrophytes* and *Cryptococcus neoformans* at MIC 6.25 µg/ml using flucanazole as standard drug. But there is no evidence that the anti-candidal action resides in this compound. However, a first approximation, based on other literature reports, may be to attribute this activity to either flavonoid, or glycosides or tannin & phenol. Rauha *et al.* (2000) investigated that the presence of phenolic compounds in Finnish plant extracts and are effective in inhibiting the growth of the organisms particularly *Candida albicans*. Phenolic compounds like tannins found in plant cells are potent inhibitors of hydrolytic enzymes used by plant pathogens (Rath *et al.,* 2009). Favell *et al.* (2005) isolated steroidal glycosides named alexin from crude extract of *Yucca gloriosa* L. and investigated *in vitro* antifungal activity against a panel of human pathogenic fungi, yeasts as well as dermatophytes and filamentous species. They found that alexin had a broad spectrum of antifungal activity and found to reside entirely in the spirostanoid fraction. Hence, presence of these phytochemicals viz. tannin and phenolic compound, glycoside, saponin in ethanol extracts may be responsible for anti-candidal activity

Table 3. Minimum inhibitory concentration of *Vitex negundo* bark and leaf

Strain used	Petroleum ether mg/ml		Chloroform in mg/ml		Ethanol in mg/ml		Methanol in mg/ml		Aqueous in mg/ml		Antibiotic in mg/ml	
	Bark	Leaf	Bark	Leaf	Bark	Leaf	Bark	Leaf	Bark	Leaf	Clotrimazole	Cepholaxime
Candida albicans	1.25	2.50	1.25	2.50	0.156	0.625	0.312	1.25	1.25	2.50	0.002	-
Candida krusei	1.25	2.50	2.50	5.00	0.312	0.625	0.312	2.50	1.25	1.25	-	0.001
Candida parapilosis	1.25	2.50	2.50	2.50	0.312	0.625	0.312	1.25	1.25	1.25	0.002	-
Candida tropicalis	0.625	2.50	2.50	2.50	0.156	0.312	0.156	0.625	0.625	1.25	0.002	-

Table 4. Minimum fungicidal concentration of *Vitex negundo* bark and leaf

Strain used	Petroleum ether mg/ml		Chloroform in mg/ml		Ethanol in mg/ml		Methanol in mg/ml		Aqueous in mg/ml		Antibiotic in mg/ml	
	Bark	Leaf	Bark	Leaf	Bark	Leaf	Bark	Leaf	Bark	Leaf	Clotrimazole	Cepholaxime
Candida albicans	5.0	<5.0	<5.0	<5.0	2.50	2.50	5.0	<5.0	5.0	<5.0	0.002	-
Candida krusei	<5.0	<5.0	<5.0	<5.0	2.50	2.50	5.0	<5.0	<5.0	<5.0	-	0.001
Candida parapilosis	<5.0	<5.0	<5.0	<5.0	2.50	2.50	5.0	5.0	<5.0	<5.0	0.002	-
Candida tropicalis	<5.0	<5.0	<5.0	<5.0	2.50	2.50	5.0	5.0	5.0	<5.0	0.002	-

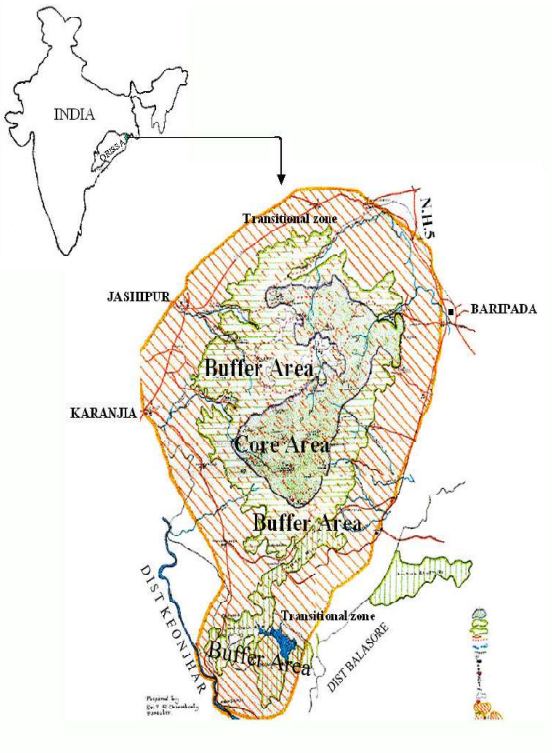

Fig. Similipal Biosphere Reserve

against all tested strain, which need further investigation. Parekh and Chanda (2008) tested antifungal activity of methanol extract of leaf of *Vitex negundo* by disc diffusion method and conclude that *C. albicans*, *C. glabrata* and *C. tropicalis* did not show any susceptibility except to *Candia albicans* ATCC2091 (10 mm zone of inhibition). However our results obtained has better inhibitory effect as compared to Parekh and Chanda (2008). Comparison of the data obtained in this study with previously published result is problematic. First, the composition of the plant extracts is known to vary according to local climatic and environmental conditions (Janssen *et al.,* 1987; Sivropoulou *et al.,* 1995). Secondly the method used to assess antibacterial activity and the choice of the test organisms also varies (Janssen *et al.,* 1987). Most frequently used methods to antibacterial activity are agar diffusion techniques and broth dilution methods. The results obtained by each of these methods may differ as many factors vary between assays (Janssen *et al.,* 1987; Hili *et al.,* 1997). *In vivo* studies may be required to confirm the values of the some of the results obtained. The experiment provides some scientific justification for the utilization of extracts from both bark and leaves to treat candidiasis. However, it is important to point out that the crude extracts such as these need to be further purified through anti-candidal activity guided fractionation to isolate and identify the compounds responsible for antibacterial activity.

ACKNOWLEDGEMENTS

The present research has been funded by the Department of Science and Technology, Government of Orissa (Grant No. 2818/ 8.6.2006). We are also grateful to the authorities of North Orissa University for providing necessary facilities to carryout this research.

REFERENCES

. Ahmad, I., Mehamood, Z., Mohammad, F. Screening of some Indian medicinal plants for their antimicrobial properties. *J. Ethnopharmacol.,* 1998; **62**: 183–193.

2. Barry, A.L. Procedure for testing antimicrobial Agents in Agar media. In: *Antibiotics in Laboratory Medicine* (Lorin V, ed). Baltimore: Williams Wilkins Co., 1980; pp 1-23.

3. Chander, J. (ed): *Textbook of Medical Mycology*, 2nd edn, New Delhi: Mehta Puublishers, 2002; 216-226.

4. Debruyne, D. Clinical pharmacokinetics of fluconazole in superficial and systemic mycoses. *Clin. Pharmacokinet.,* 1997; **33**: 52-77.

5. Dickert, H., Machka, K., Braveny, I. The uses and limitations of disc diffusion in the antibiotic sensitivity testing of bacteria. *Infection,* 1981; **9**: 18-24.

6. Eloff, J.N. A sensitive and quick microplate method to determine the minimal inhibitory concentration of the plant extracts for bacteria. *Planta Med.*, 1998; **64**: 711-713.

7. Fevell, A., Kemertelidze, E., Benidze, M., Fallague1, K., Regli, P. Antifungal Activity of Steroidal Glycosides from *Yucca gloriosa* L. *Phytother. Res.*, 2005; **19**: 158-161.

8. Goff, D.A., Koletar, S.L., Buesching, W.J., Barnishan, J., Fass, R.J. Isolation of flucanozole-resistant *Candida albicans* from HIV-negative patients never treated with azoles. *Clinc. Inf. Dis.* 1995; **20**: 77-83.

9. Hili, P., Evans, C.S., Veness, R.G. Antimicrobial action of essential oils: the effect of dimethylsulphoxide on the activity of cinnamon oil. *Lett. Appl. Microbiol.,*1997; **24**: 269-275.
10. Janssen, A.M., Scheffer, J.J.C., Baerheim-Svendsen, A. Antimicrobial activity of essential oils: a 1976-86 literature review. *Planta Med.,* 1987; **53**: 395-398.
11. Kumar, B., Rajagopalan, M., Sehgal, S. Candidal esophagitis- a marker for HIV infection. *Ind. J. Der. Venerol. Leprol.* 1996; **56**: 230-231.
12. Kumar, V.P., Chauhan, N.S., Padhi, H., Rajani, M. Search for antibacterial and antifungal agents from selected Indian medicinal plants. *J. Ethnopharmacol.,* 2006; **67**: 241-245.
13. Kwon-Chung, K.J., Bennet, J.E. (ed): *Medical Mycology: Candidiasis*, Philadelphia: Lea and Febiger; 1992; 280-336.
14. Mehta, D.K., Martin, J., Jordan, B., Macfarlane, C.R., Hashimi, F.T., Kouimtzi, M., Ryan, R.S.M., Shing, T., Wagle, S.M.S., Gallagher, G.P. (ed). *British National Formulary.* 43rd edn. London: Pharmaceutical Press; 2002; 294-298.
15. Nolte, F.S., Parkinson, T., Falconer, D.J. Isolation and charecterisation of flucanazole and amphoterecin B resistant *Candida albicans* from blood of two patients with leukemia. *Antimicrob. Agents. Chemother.,* 1997; **41**: 196-199.
16. Panda, S.K., Thatoi, H.N., Dutta, S.K. Antibacterial activity and phytochemical screening of leaf and bark extracts of *Vitex negundo* L. from Similipal Biosphere Reserve, Orissa. *J. Med. Plant. Res*., 2009; **3**(4): 294-300.
17. Pandey, A.K., Rout, S.D. Medicinal Plants of Similipal Biosphere Reserve. In: *Perspectives of Plant Biodiversity* (Das AP,), Dehra Dun: Bishen Singh Mahendra Pal Singh, 2002; 681-696.
18. Parekh, J., Chanda, S., *In vitro* antifungal activity of methanol extracts of some Indian medicinal plants against pathogenic yeast and moulds. *Afr. J. Biotechnol.,* 2008; **7**(23): 4349-4353.
19. Perea, S., Lopez-Ribot, J.L., Kirkpatrick, W.R., McAtee, R.K., Santillan, R.A., Martínez, M., Calabrese, D., Sanglard, D., Patterson, T.F. Prevalence of molecular mechanisms of resistance to azole antifungal agents in *Candida albicans* strains, displaying high-level fluconazole resistance isolated from human immunodeficiency virus-infected patients. *Antimicrob. Agents. Chemother.,* 2001; **45**: 2676-2684.
20. Powderly, W.G., Mayer, K.H., Perfect, J.R. Diagnosis and treatment of oropharingeal candidiasis in patients infected with HIV: a critical reassessment. *AIDS Res. Hum. Retroviruses,* 1999; **15**: 1405-1412.
21. Rath, S.K., Mohapatra, N., Dubey, D., Panda, S.K., Thatoi, H.N., Dutta, S.K. Antimicrobial activity of *Diospyros melanoxylon* bark from Similipal Biosphere Reserve, Orissa, *India. Afr. J. Biotechnol.,* 2009; **8**(9): 1924-1928.
22. Rauha, J.P., Remes, S., Heinonen, M., Hopia, A., Kahkonen, M., Kujala, T., Pihlaja, K., Vuorela, H., Vuorela, P. Antimicrobial effects of Finnish plant extracts containing flavonoids and other phenolic compounds. *Int. J. Food Microbiology,* 2000; **56**(1): 3-12.
23. Rusia, K., Srivastava, S.K., Antimicrobial activity of some Indian medicinal plants. *Ind. J. Pharm. Sci.,* 1998; **60**: 57-58.
24. Sathiamoorthy, B., Gupta, P., Kumar, M., Chaturvedi, A.K., Shukla, P.K., Maurya, R. New antifungal flavonoid glycoside from *Vitex negundo. Bioorganic & Medicinal Chemistry Letters*, 2007; **17**(1): 239-242.
25. Sivropoulou, A., Kokkini, S., Lanaras, T., Arsenakis, M. Antimicrobial activity of mint essential oils. *J. Agric. Food Chem.,* 1995; **43**: 2384-2388.
26. Thatoi, H.N., Panda, S.K., Rath, S.K., Dutta, S.K. Antimicrobial activity and ethnomedicinal uses of some medicinal plants from Similipal Biosphere Reserve, Orissa. *Asian J. Plant Sci.,* 2008; **7**: 260-267.
27. Trease, G.E., Evans, W.C. (ed): *Text book of Pharmocognosy*.13th edn. Oxford: Alden Press; 1989; 268-298.
28. Valsaraj, R., Pushpangadan, P., Smitt, U.W., Adersen, A., Nyman, U. Antimicrobial screening of selected medicinal plants from India. *J. Ethnopharmacol.,* 1997; **58**: 75-83.
29. Yoder, S. Biomedical and ethnomedical practices in rural Zaire: constrasts and complements. *Soc. Sci. Med.,* 1982; **16**: 1851-1857.