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Chemical Analysis and Anti-MRSA Activity of Mat Extract from *Aspergillus terreus* VIT 2013

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The growth parameters required for the growth and production of anti-MRSA metabolites of soil fungus *Aspergillus terreus* VIT 2013 was accomplished by partial optimization. Mass production of metabolites was carried out on malt extract broth (MEB) and metabolites were extracted by solvent extraction method. The mat extracts of *Aspergillus terreus* VIT 2013 showed high anti-MRSA activity. Further, purification of anti-MRSA fraction of mat extract, and analysis by GCMS revealed high amounts of pentamethyl ethanol followed by undecyl ester of dichloroacetic acid, mono (2-ethylhexyl) ester and 1, 2-benzenedicarboxylic acid. Its Minimum Inhibitory Concentration (MIC) was found to be 40μg against 10 isolates of MRSA. The MIC of standard vancomycin was assessed as 1.6 μg for comparison. The extract was active against other MTCC isolates of gram positive and gram negative bacteria but no activity was seen against fungi. The 28S rDNA sequence results of *Aspergillus terreus* VIT 2013 was found to be 99% similar to *Aspergillus terreus*. GC-MS analysis of the ethyl acetate extract of the mat of *Aspergillus terreus* VIT-2013 suggests the presence of a compound which could be labelled as a broad spectrum antibiotic, active against MRSA and also several other pathogens.

Key words: *Aspergillus terreus* VIT-2013, Secondary metabolites, Anti-MRSA activity, 28S rDNA sequencing.

In 1940s, Sir Alexander Fleming isolated the first life saving drug penicillin, during World War II but two years after of its discovery, penicillin resistant *S. aureus* emerged. Similar studies have been reported for contemporary drugs from several parts of the world[1]. During and after the few years of "Golden Age of Antibiotic" many isolates were reported to be drug resistance with worldwide distribution and community settings. This created a horrible situation among infectious disease caused by these isolates. Different approaches have been made to conquer the problem of drug resistance by the isolation of bioactive fungal metabolites, using antibacterial bacteriocin and bacteriophages[2-4]. Among fungal metabolites, 226 secondary metabolites have been isolated from *A. fumigatus* and 145 from *A niger*[5,6]. Fungi synthesize a variety of primary and secondary metabolites which include vitamins like riboflavin, anticancer drugs and drugs related to human health and also antibacterial metabolites[5,7,8]. Erinacine A and Erinacine B are two such anti-MRSA compounds, isolated from fruiting bodies and mycelium of *Hericium erinaceus*[9]. *Aspergillus flavipes* has been reported for the synthesis 1, 3-dihydroisobenzofuran and FR198248, active against MRSA[10]. Since soil fungi are huge source for secondary metabolites active against drug resistance bacteria present work aims at study of metabolites of *Aspergillus terreus* VIT 2012 with antiMRSA activity.

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MATERIALS AND METHODS

All experiments were conducted in triplicate and diameter of the zone of inhibition was expressed excluding the dia of well

Soil was collected from Vellore ($12^0$542 N $79^0$82 E), Tamil Nadu during the month of August. Physical, chemical and biological properties of the soil sample were analyzed in Sri A.M.M Mugurappa Chettiar Research Centre (MCRC), Taramani, Chennai by following an alternative analytical indigenous technology developed by them and IIT (M). *Aspergillus terreus* VIT 2013 was isolated from soil and identified by three point inoculation method using Malt extract Agar, Yeast extract sucrose Agar and Sabouraud Dextrose Agar. It was confirmed by SEM analysis and 28S rDNA sequencing. It was then grown in Sabouraud Dextrose Broth (SDB), Malt extract broth (MEB), Czapek dox Yeast Extract Broth (CYB), Yeast Extract Sucrose Broth (YESB) and a specially Designed Broth (DMB). The last medium was based on the soil analysis report. 100 microliter of each broth was assessed for Anti MRSA activity using ATCC 43300 culture of MRSA.

Optimum temperature and pH for anti MRSA activity, and also for biomass production of *Aspergillus terreus* VIT 2013, was determined on malt extract broth and its bulk production was carried out by inoculating 10^6 conidia/ml of production medium at pH 8.0 and the medium was incubated at 30°C for 20 days. Subsequently, the mat and the broth were separated and the respective metabolites were extracted with different solvents. Both set of extracts were concentrated in rotary evaporator and 1mg/ml of each were used to check the anti-MRSA activity. Since maximum activity was found in the mat extract of ethyl acetate**,** its activity at 4mg/ml was tested against several MTCC cultures of pathogenic bacteria and fungi along with 10 clinical isolates of MRSA, following CLSI guidelines. Subsequently the MIC was determined for 10 clinical isolates of MRSA. Chemical tests for all crude extracts were carried out by following standard protocols[11]. The crude metabolites from mat, extracted by ethyl acetate were separated into four fractions using petroleum ether (1:4) for which the anti-MRSA activities were checked. The one fraction which showed activity was analysed by GC-MS and the spectrum was matched with National Institute Standard and Technology (NIST) library.

RESULTS

Aspergillus sp was isolated from soil and its presence was confirmed by observing its colony morphology on three fungal media, Scanning Electron Microscope (SEM) image (Fig. 1) and the blastn results of 28S rDNA sequence (Fig. 2), wherein a match of 99% similarity was observed with *Aspergillus terreus.*

Fig. 3 shows the results of anti MRSA activity of the medium, collected during the growth of this organism from 0^{th} day to 20^{th} day.

Among the five fungal media, including the one designed using the soil report, the highest anti-MRSA activity was found in MEB followed by CYB and SDB**.** Though effort had been made to

Table 1. Physical, chemical and biological properties of soil sample

Soil parameters	Results	Recommended level of metals in soil by MCRC
pH	7.12	-
Temperature (^OC)	31	-
Organic carbon (%)	0.41	0.75-1.5
Humus[a]	140.82	18 -31
Nitrogen[a]	98.72	113-182
Phosphorous[a]	13.95	18-36
Potassium[a]	140.65	60-138
Calcium[b]	516.9	>300
Magnesium[b]	195.88	10-15
Sodium[b]	131.3	-
Iron[b]	6.37	6-8
Manganese[b]	3.66	1.2-2.5
Copper[b]	1.59	0.3-1
Zinc[b]	1.14	0.5-1
Sulfate[b]	21.54	10-15
Protease[c]	135.6	NA
Cellulase[c]	0.763	NA
Invertase[c]	0.93	NA
Alk.Phosphatase[c]	87.5	NA
Bacteria[d]	13	NA
Actinomycetes[e]	0.5	NA
Rhizobium[e]	400	NA
Fungi[e]	56	NA

Note: [a] kg/acre, [b]mg/kg, [c] µgs/Tyr/g/hr, [d]10^9cfu/gm, [e]10^6cfu/gm

Table 2. Antimicrobial activity of crude mat extract of *Aspergillus terreus* VIT-2013

Microorganism	MTCC no	Zone of inhibition (mm)
Micrococcus luteus	4300	14±0.12
Escherichia coli	443	10 ± 0.5
Pseudomonas aeruginosa	8076	Nil
Klebsiella pneumonae	7407	Nil
Staphylococcus aureus	3160	9 ± 0.3
Bacillus subtilis	441	11 ± 0.2
Salmonella typhi	3231	Nil
Enterobacter aerogenes	111	Nil
Proteus mirbilis	9493	Nil
Saccharomyces cerevisiae	170	Nil
Candida albicana	1637	Nil
Aspergillus niger	282	Nil
Fusarium oxysporum	284	Nil
Penicillium chrysogenum	160	Nil

create soil stress conditions in laboratory by designing growth medium using soil analyses report, organism was failed to show any activity in designed medium**.** Partial optimization of physical parameters indicated that the optimum temperature for anti-MRSA activity and biomass production of fungus was 30°C and 40°C respectively. The pH of 8.0 ±0.2 was found to be optimum for anti-MRSA activity and production of biomass of the fungus. These results were compared with soil analysis report in which temperature and pH of the soil were found to be 31°C and pH 7.12 (Fig. 4).

Soil analysis revealed that, it was rich in humus and also in magnesium, copper, manganese, sulfate and zinc in comparison with recommended levels of metals in soil by MCRC. The concentration of nitrogen, phosphorous and organic carbon was found to be low. Calcium and iron were present in optimum levels. The stress due to a high metal

Table 3. Chemical analysis of mat and broth extracts of *Aspergillus terreus* VIT-2013

Chemical tests	Crude extracts					
	Pet ether	n-Butanol	Chloroform		Ethyl acetate	
	Broth	Mat	Mat	Broth	Mat	Broth
Alkaloids	-	+	+	+	+	-
Carbohydrates	+	+	+	+	+	+
Glycosides	+	-	-	+	-	+
Saponins	+	+	-	-	-	+
Proteins	+	+	+	+	+	+
Sterols	-	+	+	-	+	-
Fats and oils	+	+	+	+	+	-
Phenols	+	-	-	-	-	-
Flavonoids	+	+	+	-	+	+

content and pH conditions may be one of the several factors for the stimulation of metabolite production. Among soil enzymes, protease was predominantly present followed by alkaline phosphatase and other enzymes (Table 1).

Mass production and extraction of secondary metabolites of *Aspergillus terreus* VIT-2013 showed a high yield of 1.754 grams in ethyl acetate extract of mat. This was followed by 980 mg of mat and 430 mg of broth extract of n-butanol. Petroleum ether and chloroform yielded lesser amounts of crude extracts in contrast to others. In general, the yields of mat extracts were higher than those of broth. Ethyl acetate extract from mat showed highest anti-MRSA activity followed by the n-butanol extract (Fig. 5). No activity was noticed in crude extracts carried out with highly polar solvents like methanol, acetonitrile and water.

The mat extract of ethyl acetate was showed strong MRSA activity and its MIC was found to be 40µg against ten clinical isolates of MRSA in contrast to that of vancomycin which was found to be 1.6µg. MIC values of crude extracts can be further reduced by purification of the antibacterial metabolites. The extract was also active against other Gram positive, Gram negative MTCC strains but no activity was seen against fungal pathogens (Table 2).

Chemical tests, performed on all extracts (both mat and broth), and the results are shown in Table 3. Further, separation of ethyl acetate extract of mat by precipitation using petroleum ether, resulted in four fractions, out of which one fraction was found to be active against MRSA. GCMS analysis of this fraction indicated three possible compounds *i.e.*, pentamethyl ethanol, undecyl ester of dichloroacetic acid and mono (2-ethylhexyl) ester of 1, 2-benzenedicarboxylic acid.

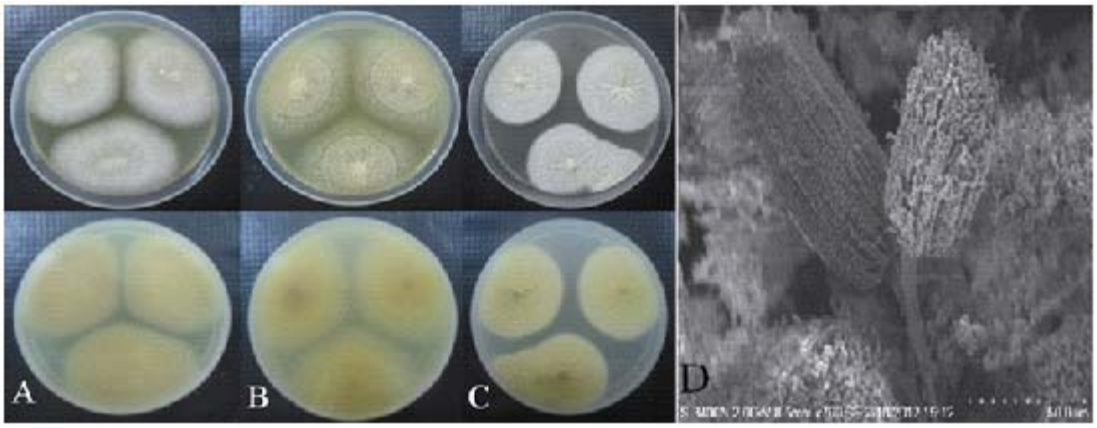

Fig. 1. Identification of *Aspergillus terreus* VIT-2013. A. MEA B. YESA C. SDA D. SEM

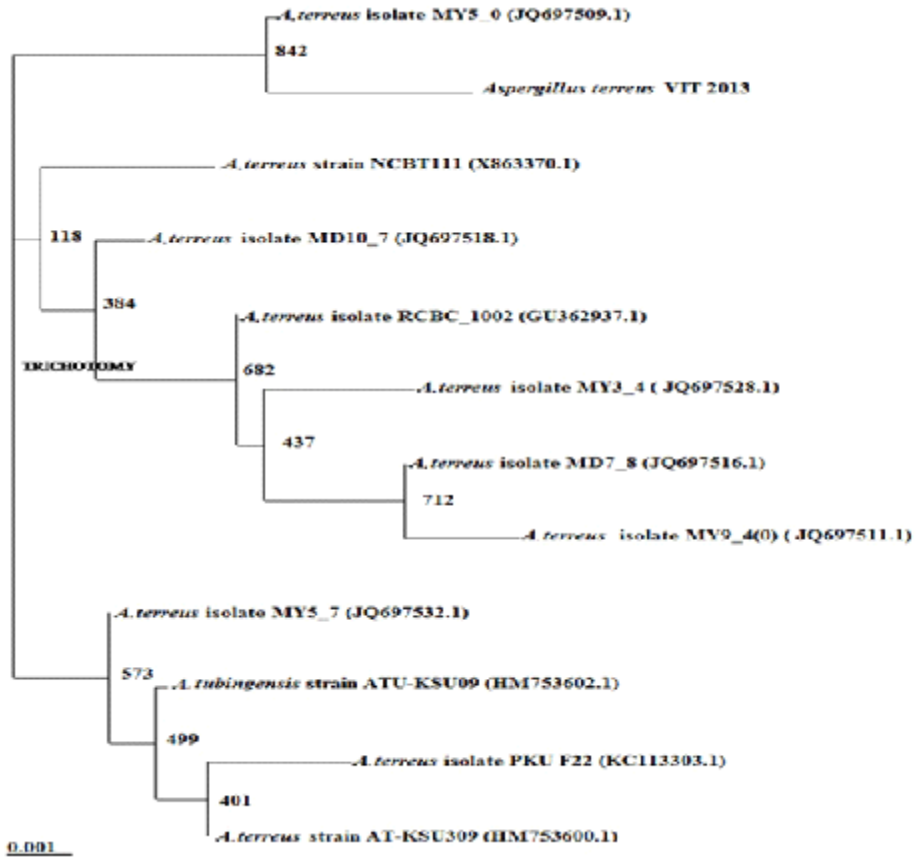

Fig. 2. Phylogenetic tree constructed using partially sequenced 28S rDNA gene of *Aspergillus terreus* VIT 2013

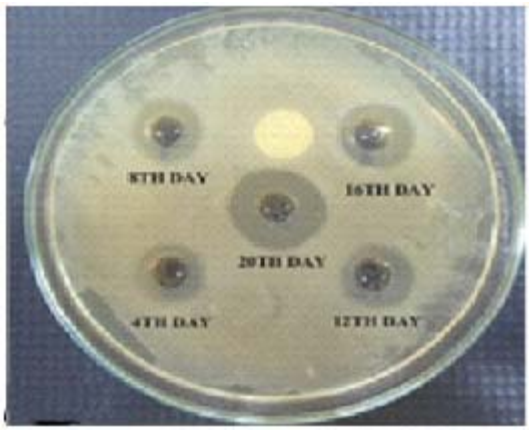

Fig. 3. Anti-MRSA activity of growth medium of *Aspergillus terreus* VIT-2013

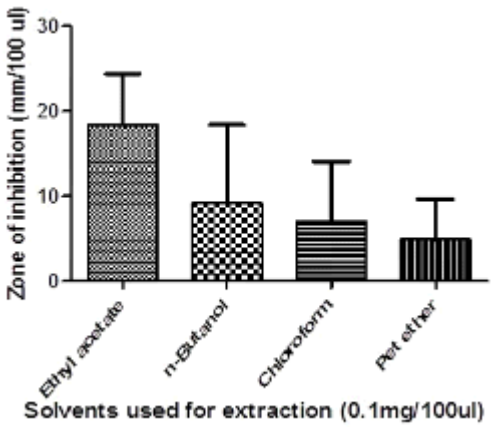

Fig. 4. Partial optimization of medium for production of anti-MRSA metabolites and biomass

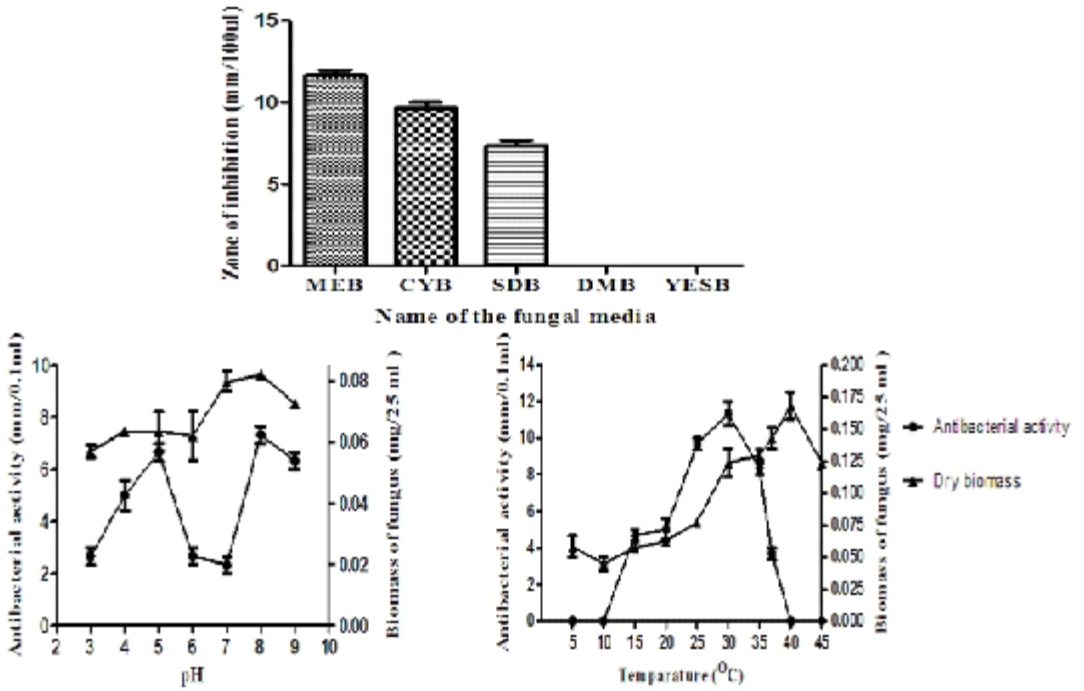

Fig. 5. Anti-MRSA activity of crude extracts of mat and broth

DISCUSSION

Soil fungus *Aspergillus terreus* VIT 2013 was matched 99% similarity with *Aspergillus terreus* identified based on 28S rDNA gene sequence. Its Anti-MRSA activity was found to be highest in malt extract broth but no activity was noticed in designed medium. Since the pH of designed medium and temperature it was incubated for the growth of the fungus were almost correlated with that of soil pH and temperature, these parameters may not be the responsible factor for reduced activity in designed medium. The improper concentration of metals and other ingredients in designed medium would have inhibited the synthesis of anti-MRSA metabolites. Further it

implies that optimization of each components of the designed medium at different concentration is essential for stimulated production of anti-MRSA metabolites. Chemical analysis revealed that, alkaloids were not found in all extracts though Asterrelenin and 1,6 alpha-hydroxy-5 N-acetylardeemin an inhibitor of acetylcholinesterase along with seven other alkaloids were reported by the same species[12,13]. Interestingly, sterols were found in all mat extracts which were absent in all broth extracts. Similarly, glycosides were found in all broth extracts but not in all mat extracts. This signifies that the anti-MRSA metabolites in mat extract may be sterols, fats/oils and flavonoids as the color of the mat extract was jelly red. Lavistatin is one of the cardiac glycoside synthesized by *A.terreus* used for lowering cholesterol biosynthesis by means of inhibiting the HMG-CoA reductase[14]. Fungal lipids plays very important role in synthesis of biomass. The low amount of lipids were noticed along with increase in fungal dry biomass and among the fungal sterols, egrosterols were found to be predominant (64-94%)[15]. Finally, Analysis of one of the anti-MRSA fraction of crude ethyl acetate extract of mat by GCMS showed three components in which the highest amount was that of pentamethyl ethanol. For best of our knowledge, this compound, identified by GCMS have not been reported previously for their anti-MRSA activity from any organism including, *Aspergillus terreus*. But the derivatives of tetra and pentamethyl like quercetin 3,7,3',4'-tetramethylether and quercetin 3,5,7,3',4'-pentamethylether, are known to possess vasorelaxing effect in male impotence[16]. However, Mono[2-ethylhexyl]ester of 1,2-benzenedi carboxylic acid, has been reported for its antimicrobial, antioxidant and anti-inflammatory activities[17]. This study establishes a strong platform for further purification of novel anti MRSA components from mat and broth extracts of *Aseprgillus terreus* VIT 2013.

CONCLUSION

Soil fungi serve as huge resource for anti-MRSA metabolites. The MIC and activity of mat extract of *Aspergillus terreus*VIT 2013 in current study indicates the potential and broad spectrum antimicrobial(s) against various Gram positive and Gram negative bacteria. In addition, none of the metabolites revealed in GCMS have been reported in the literatures for their anti-MRSA activity. There is an immense scope for utilization of novel metabolites produced by The *Aspergillus terreus* VIT 2013 in the pharmaceutical vicinity as antimicrobials drugs.

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REFERENCES

1. Appelbaum, P.C. MRSA—the tip of the iceberg. *Clin. Microbiol. Infect.*, 2006; **12**(2): 3-10.
2. Ogata, M., Ueda, JY., Hoshi, M., Hashimoto, J., Nakashima, T., Anzai, K., et al. A novel indole-diterpenoid, JBIR-03 with anti-MRSA activity from *Dichotomomyces cejpii* var. *cejpii* NBRC 103559. *J. Antibiot* (Tokyo)., 2007; **60**(10): 645-648.
3. Aunpad, R., Na-Bangchang, K. Pumilicin 4, a novel bacteriocin with anti-MRSA and anti-VRE activity produced by newly isolated bacteria *Bacillus pumilus* strain WAPB4. *Curr. Microbiol.*, 2007; **55**(4): 308-313.
4. O'Flaherty, S., Ross, RP., Meaney, W., Fitzgerald, GF., Elbreki, MF., Coffey, A., Potential of the Polyvalent Anti-*Staphylococcus* Bacteriophage K for Control of Antibiotic-Resistant *Staphylococci* from Hospitals. *Appl. Environ. Microbiol.*, 2005; **71**(4): 1836-1842.
5. Frisvad, JC., Rank, C., Nielsen, K.F., Larsen, T.O., Metabolomics of *Aspergillus fumigates*. *Med. Mycol.*, 2009; **47**(1): S53-S71.
6. Nielsen, K.F., Mogensen, J.M., Johansen, M., Larsen, T.O., Frisvad, J.C. Review of secondary metabolites and mycotoxins from the *Aspergillus niger* group. Anal. Bioanal. Chem., 2009; 395(5):1225-1242.
7. Balde, E.S., Andolfi, A., Bruyère, C., Cimmino, A., Lamoral-Theys, D., Vurro, M. *et al*. Investigations of fungal secondary metabolites with potential anticancer activity. *J. Nat. Prod.*, 2010; **73**(5): 969–971.
8. Bills, G.F., Platas, G., Gams, W. Conspecificity of the cerulenin and helvolic acid producing ''*Cephalosporium caerulens*'', and the hypocreaelan fungus *Sarocladium oryzae*. Mycological. Res., 2004; **108**(Pt 11):1291-1300.

9. Kawagishi, H. Anti-MRSA Compounds from *Hericium erinaceus* (Bull.: Fr.) Pers. Int. J. Med. Mushrooms., 2005; **7**: 350.
10. Know, Y.J., Zheng, C.J., Kim, W.G. Isolation and identification of FR198248, a hydroxylated 1, 3-Dihydroisobenzofuran from *Aspergillus flavipes* as an inhibitor of peptide deformylase. Biosci. Biotechnol. Biochem., 2010; 74(2): 390-393.
11. Harborne, J.B. Method of plant analysis *Phytochemical Methods*, London: Chapman and Hall, 1973; 1-32.
12. Ge, H.M., Peng, H., Guo, Z.K., Cui, J.T., Song, Y.C., Tan, R.X. Bioactive alkaloids from the plant endophytic fungus *Aspergillus terreus*. *Planta. Med*., 2010; **76**(8): 822-824.
13. Li, G.Y., Li, B.G., Yang, T., Yin, J.H., Qi, H.Y., Liu, G.Y *et al*. Sesterterpenoids, Terretonins A"D, and an Alkaloid, Asterrelenin, from *Aspergillus terreus*. *J. Nat. Prod.*, 2005; **68**(8): 1243–1246.
14. Topliss, J.G., Clark, A.M., Ernst, E., Hufford, C.D., Johnston, G.A.R., Rimoldi, J.M. *et al*. Natural and synthetic substances related to human health (IUPAC Technical Report). Pure. Appl. Chem., 2002; **74**(10): 1957–1985.
15. Nemec, T., Jernejc, K., Cimerman, A. Sterols and fatty acids of different *Aspergillus* species. FEMS. Microbiol. Let., 1997; 149: 201-205.
16. Hnatyszyn, O., Moscatelli, V., Rondina, R., Costa, M., Arranz, C., Balaszczuk, *et al*. Flavonoids from *Achyrocline satureioides* with relaxant effects on the smooth muscle of Guinea pig *corpus cavernosum*. Phytomedicine., 2004; **11**(4): 366–369.
17. Ezhilan, B.P., Neelamegam, R. GC-MS analysis of phytocomponents in the ethanol extract of *Polygonum chinense* L. *Pharmacognosy. Res.*, 2012; **4**(1): 11–14.