Pak. J. Weed Sci. Res., 23(2): 143-154, 2017

SCREENING OF FUNGAL EXTRACTS FOR WEEDICIDAL ACTIVITY AGAINST *Lemna minor* (DUCK WEED)

Zafar Iqbal[1], Sana Irshad Khan[2], Mudassar Iqbal[1], Muhammad Numan[1], Saiqa Jabeen[1], Shabeer Jan[1], Muhammad Amin[1], Muhammad Luqman[1] and Sartaj Alam[3]

ABSTRACT

Mycellial extracts of ethyl acetate of four fungal species i.e. Rhizopus stolonifer, Trichoderma harzianum, Penicillium sp. EU0013, and Aspergillus niger were screened for toxicity against Lemna minor (duck weed). The agar plate culture of all the strains were prepared and inoculated into static flask cultures using potato dextrose broth (PDB) media. The crude extracts were tested against the weed Lemna minor as an indication of the potential of the extract possessing phytotoxic activity and prospect of developing future candidate(s) as herbicides. Different concentrations of the crude 10, 50, 100 and 200 ppm were prepared in ethyl acetate as test solutions in comparison with negative (blank) and positive (Atrazine) reference plates. Total 30 Lemna minor plants were raised on E-medium and test solutions were applied in triplicate to determine percent mortality. The results showed that the ethyl acetate extracts of Penicillium sp. EU0013 and Trichoderma harzianum exhibited very weak or no toxicity towards the weed (0-10% mortality). Organic extract from A. niger was the most potent with > 60% mortality followed by R. stolonifer with 36% mortality at 200 ppm. It is opined that the fungal extracts showing significant phytotoxic activity may contain useful natural products and could be utilized in weed management. HPLC analysis of the defatted organic extracts showed many peaks signifying biosynthesis of phytotoxic metabolites by A. niger and R. stolonifer. However, retention time (Rt) and UV (λm) spectra of these unidentified compounds provide little information related to the elucidation of structure of the peaks. Detailed investigation of these compounds might need advanced spectroscopic studies such as NMR and HRMS analysis.

[1]Dept. of Agricultural Chemistry, Faculty of Nutrition Sciences, The University of Agriculture Peshawar, Pakistan
[2]Soil and Water Testing Laboratory, Directorate of Soil Plant Nutrition, ARI Tarnab-Peshawar, Pakistan
[3]Dept. of Plant Pathology, Faculty of Crop Protection Sciences, The University of Agriculture Peshawar, Pakistan
*Corresponding author's email: zafar.iqbal@aup.edu.pk

Key words: Fungi, *Lemna minor*, mycelia extract, Plant growth inhibition.

Citation: Iqbal, Z., S.I. Khan, M. Iqbal, M. Numan, S. Jabeen, S. Jan, M. Amin, M. Luqman and S. Alam. 2017. Screening of fungal extracts for weedicidal activity against *Lemna minor* (Duck weed). Pak. J. Weed Sci. Res. 22(2): 143-154.

INTRODUCTION

Weeds are among the harshest agricultural and environmental pest causing severe losses to productivity and economy. In agriculture different classes of synthetic herbicides are used for weeds control to minimize the damage caused by them. However use of synthetic chemicals in agriculture has provoked serious issues to environment and human health (Evidente *et al.,* 1998).

Investigation of phytotoxic compounds from various biological sources offers useful clues in searching new candidates of natural herbicides with useful properties that could be more safe and specific than the synthetic weedicids (Gonçalves *et al.,* 2009). Natural products used as herbicides usually considered less toxic, biodegradable and environmental friendly for plants and other organisms and are needed to replace synthetic chemicals.

Fungi are considered as important biosynthetic factory for their bioactive metabolites production and exhibiting interesting bioactivities like antibacterial, cytotoxic and weedicidal properties (Saxena and Pandey, 2001).

Screening of fungi for potent secondary, metabolites against weeds has been one of the most interested areas in weed management research. Literature indicated many reports that fungal metabolites are toxic to certain weeds. Some fungal metabolites are toxic against both monocotyledonous and dicotyledonous weeds, e.g. cornexistin from *Paecilomyces variotii* (Pearce, 1997) and prehelminthosporal from *Helminthosporium* sp. (Pena-Rodriguez *et al.,* 1988). Similarly maculosin produced by *A. alternata* is host-specific to spotted knapweed (Steirle *et al.,* 1988). These natural compounds have been playing in developing environment friendly herbicides. Zhang *et al.,* (2008 a, b) reported potent phytotoxic activity of Ethyl acetate extract obtained from the culture filtrate of the fungal strain FH01 against the growth of *Echinochloa crusgalli*.

In this study crude extract of four fungi were tested for growth inhibitory activity against *Lemna minor* (duckweed). *Lemna minor* is a small plant having three fronds. It is native to stagnant water and is found to be highly sensitive so can be effectively used for screening phytotoxicity level, when tested against different doses of chemicals.

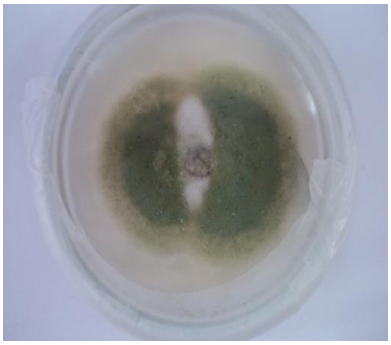
The main objective of this project is to reveal prospective fungal species which could have a promising future for their use in weed control.

MATERIALS AND METHODS

Collection of fungal species

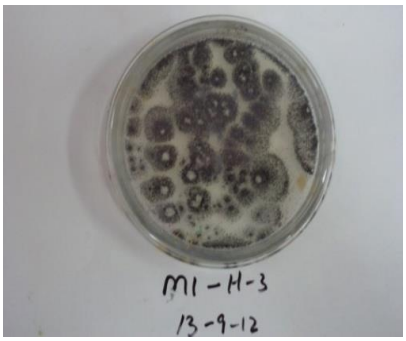
Four fungal species *Rhizopus stolonifer*, *Trichoderma harzianum, Penicillium* sp. EU0013 and *Aspergillus niger* were collected from the Dept. of Plant Pathology, The University of Agriculture, Peshawar as live cultures. The strains were maintained as frozen culture in 20% glycerol solution.

Trichoderma harzianum



Penicillium sp EU0013

Rhizopus stolonifer



Aspergillus niger

Cultivation of the Fungal Cultures and Growth Conditions

Agar plate cultures and storage of the selected fungal species

Potatoes dextrose agar (PDA) medium was subjected to autoclave sterilization at 121 °C for 15 min. Agar medium was poured into pre-autoclaved petri dishes. All petri dishes were inculcated from the frozen glycerol culture of *Rhizopus stolonifer*, *Trichoderma*

harzianum, Penicillium EU0013, and *Aspergillus niger* and incubated at 25 °C to grow for 5-7 days.

Seed cultures of the fungi

In order to obtain secondary metabolites the pure fungal cultures were grown on pre-sterilized Potatoes dextrose broth (PDB) medium (1L) at 25 °C for 3 days. After that a pre-inoculum was prepared by pouring into 250 mL flasks containing 100 mL PDB and incubated at 25 °C on a rotary shaker at 200 rpm for 10-15 days. pH of the production medium (PDB) was maintain to 7.5 with dil. Sodium hydrooxide solution before sterilization. Then the mycelium of each fungus was separately subjected to solvent extraction.

Preparation of crude extract

For the extraction of crude extract procedure of Yin *et al.* (2010) was followed. Fungal cultures were filtered using vacuum and cells were washed with distilled water. The cells were homogenized and extracted with EtOAc for 24 hrs at room temperature. The extracts were filtered and the filtrate was dried over anhydrous $MgSO_4$ and dried using rotary evaporator. The crude extract 300-500 mg L^{-1} was obtained as dark brown oil. The crude extract solution (50 mg mL^{-1} in EtOAc) was first centrifuged to remove solids and then spotted for thin layer chromatography (TLC) analysis.

Phytotoxic Bioassay

Lemna minor plants were used as subject to test the phytotoxicity of crude extract according to the procedure described by Atta-ur-Rahman, 1991. *Lemna minor* plants were obtained from the Dept. of botany, University of Peshawar. Stock solution (2000 ppm) was prepared and different amounts from this solution were shifted to plastic cups (250 mL). After solvent evaporation, test solutions of 10 ppm, 50 ppm, 100 ppm and 200 ppm of crude extract in E-medium were prepared. Then *Lemna minor* plants (30) were transferred to each cup containing solutions of different concentrations. E-medium was added to these cups on daily on daily basis when their amount was reduced because of evaporation. Experiments were performed in triplicate and compared with standard Atrazine and negative control. After 7 days, % mortality of *Lemna minor* was calculated.

High Performance Liquid Chromatography (HPLC) Analysis

Crude extract solution (10 mg mL^{-1}) was prepared in acetonitrile (HPLC grade) and 10 uL of solution was injected to HPLC phenomenex Luna $5uC_{18}$ (II) (250 × 4.6 mm) reversed phase column after the removal of solids. phenomenex Luna $5uC_{18}$ (II) (250 × 4.6 mm) reversed phase column. A solvent system of ACN (pump B) and water (pump A) with 0.05% formic acid each was used. The sample was run for 15 min gradient program (0.00 min 10% B, 10.00 min

90% B, 12.00 min 90% B, 13.00 min 10% B and 15.00 min 10% B) at 1 mL min^{-1} flow rate.

Statistical analysis

The recorded data was analyzed using CR design. Each data value is the replicate of three observations.

RESULTS AND DISCUSSION

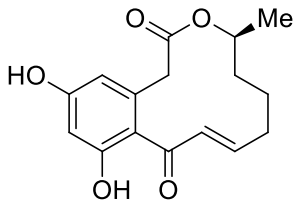
Phytotoxic Bioassay

This project was designed to screen the phytotoxicity of organic extracts of the selected fungal species: *Rhizopus stolonifer*; *Trichoderma harzianum*; *Penicillium* sp. EU0013 and *Aspergillus niger* against the weed *Lemna minor* (duck weed). Various concentration of the crude extracts (i.e. 0 ppm, 50 ppm, 100 ppm, 200 ppm) in E-medium was prepared. Negative (blank) and positive control (Atrazine) were also run as parallel for reference. Total of 30 *Lemna minor* plants were taken and tested against each concentration. Results (Figure-1) showed that among the extracts from four fungal species, *R. stolonifer* extract showed strongest toxicity and growth inhibition activity against the weed (> 60% mortality) followed by *A. niger* (55% mortality) at 200 ppm.

All the fungal extracts showed varying degree of phytotoxicity. Negative control and standard treatments displayed 0% and 100 % mortality respectively against the tested weed. The extracts from *Trichoderma harzianum* exhibited very low toxicity and percent mortality (2-10%) in all concentrations tested.

It is observed that with increase in concentration of the fungal extracts from 10 to 200 ppm, mortality of the plants increase as shown in Figure-2. However the *Trichoderma* sp. exhibited higher rate of mortality at 100 ppm rather than 200 ppm. Both the extracts from the *Rhizopus* sp. and *Aspergillus niger* possess strong lethality and > 50% *Leman minor* plants were found dead when treated with the extract of the both fungi at 200 ppm concentration.

Several fungal species such as *Penicillium* sp. (Kobayashi *et al*., 1988), *A. macrospora* (Roberson *et al*., 1985), *A. cinerariae* (Arai *et al.,* 1989), *Alternaria zinniae* (Vurro *et al.,* 1998), *Cochliobolus spicifer* (Ghisalberti and Rowland, 1993), *Nectria galligena* (Gutierrez *et al.,* 2005) and *Aspergillus* sp. (Kusano *et al*., 2003) are reported to produce natural phytotoxic metabolites. Compound α,β dehydrocurvularin 1, isolated and identified that showed strong phytotoxic activities against important crops including tomato rice lettuce) and millet and weak phytotoxicity toward the weed *Xanthium occidentale* (Munro *et al*., 1967; Hyeon *et al.,* 1976; Vurro *et al.,* 1998; Kusano *et al.,* 2003; Gutierrez *et al.,* 2005).



α,β dehydrocurvularin 1

HPLC Analysis

HPLC chromatogram of four fungal species i.e. *Rhizopus stolonifer*, *Trichoderma harzianum, Penicillium* sp. EU0013 and *Aspergillus niger* showed many peaks at different retention times as shown in Fig. 3. Whiles UV chromatogram of these peaks is presented in Fig. 4. The RP-HPLC chromatogram of *Rhizopus stolonifer* organic extract showed many peaks at different retention time. Peak 1 has absorbance maxima at 200 nm eluted in 5.23 min. *Rhizopus stolonifer* peak 2 has absorbance maxima at 223 and 275 eluted in 6.20 min, peak 3 has absorbance maxima 227 and 276 eluted at 7.70 min. Peak 4 has Rt 8.18 and UV maxima 224 and 278 nm and peak 5 has UV absorbance at 227 and 276 and Rt 8.93. Peak 6 eluted at 9.46 min and UV is 223 and 278 nm. Peak 7 having UV 200 and 230 nm and Rt is 12.05 min. Applying Fishier Woodward rules on the UV spectrum of these peaks, it was suggested that UV maxima (λm) at 223 and 275 nm might indicates the presence of a cis 1,3 pentadien and a cisoid having alkyl group or ring residues with exocyclic double bond in peak 2 compound. Except peak 2 no sufficient information has been found related to the functionality of all other peaks.

Aspergillus niger showed two main peaks and many peaks of trace metabolites. Peak 1 with UV maxima of 200, 224 and 306 nm eluted at 6.37 min of *Aspergillus niger* showed that it is an aromatic compound containing with carboxylic acid and –C (O) CH3 or –C (O) O- groups. Peak 2 of *Aspergillus niger* eluted at 7.36 min and having UV maxima of 203 and 304 nm. UV and Rt information of Peak 2 represented that it might be aniline residue of peak 1.

Similarly RP-HPLC chromatogram of *Trichoderma harzianum* extract showed eight (8) peaks. However we select only last three peaks (peak # 1, 2 and 3) eluted at retention time of 11.56, 12.08 and 12.60 minutes respectively, for analysis. Retention time of the three selected peaks of *Trichoderma harzianum* indicated them as non polar. A UV spectrum of peak 1 showed λm at 269 and 280 nm while peak 2 showed λm at 200 and 231 nm and peak 3 at 200 nm and 228 nm. Applying Woodward rules on these peaks UV spectrum, it was suggested that Peak 1 (λm at 269 and 280 nm) might contains an -OR group and phenolic group. Literature indicates UV absorption at

280 nm for many phenolic structures (Hoteling, 1933; Darton, 1988), however this spectra did not provide any information through which particular phenolic compound structure can be suggested. Similarly peak 2 might represent a compound having arene group and pentacyclic group or phenyl functionality in its structure. Peak 3 indicates a conjugated aromatic compound with a 1-Buta, 3-yne side chain ($CH2{=}{=}CHC{\equiv}{\equiv}CH$) (Paton, 2011). It might be a degradation product of peak 2 eluted at 11.56 min. Absorbtion of UV at 200 nm and 228 nm of peak 3 might be due to conjugation outside benzene ring which leads to absorption other than 205 nm. This shift in wavelength might be because of the movement of electrons from bonding orbital to antibonding orbitals.

Penicillium sp. EU0013 extract showed three prominent peaks and various small peaks. Small peaks might be trace metabolites. Peak 1eluted at Rt of 1.16. Peak 2 elute at 1.36 and peak 3 at 12.56 having UV absorbance at 200 and 259, 211 and 266, 205 and 222 nm. *Penicillium* sp. EU0013, Peak 1 Rt and UV maxima represents that it is 1,3- cyclohexadiene. UV information related to Peak 2 of *Penicillium* sp. EU0013 also indicate it a cyclohexadiene with an alkoxy or –OH group. Literature showed that majority of complex organic compounds (similar to Benzene) does have absorption maxima in the range of 150-350 nm (Paton, 2011). Retention time and UV maxima of peak 3 indicates the presence of organic acid or ester (-COOR) Br functionality or conjugated carbonyl compound containing α-alkyl groups or phenyl, β-alkyl groups or phenyl residues and an endocyclic double bond in 7-membered ring.

In the past many compounds of agrochemical and biomedical importance have been derived from fungi. For example *Aspergillus niger* has outstanding capability to convert most of the existing carbon (95%) to organic acids. Evidence suggested that these organic acids of *A. niger* helps in the degradation of the plant cell (Andersen *et al.,* 2009). Similarly *Penicillium rubrum* had been found rich of herbicidal compounds like rubralactone (polyketoid), rubralides A (3R-5-formyl-4,6-dihyroxy-3-methoxyphthlide), B (5-formyl-3,4,6-trihydroxyphthlide), C (3R-4,6-dihydoroxy-3-methoxy-5-methylphthlide) and rubramin (2,4-diformyl-3,5-dihydroxybenzoic acid and 2–formyl–3,5-dihyrxy-methyl benzoic acid (Kimura *et al*., 2007). This represents that fungi are rich source of agrochemicals.

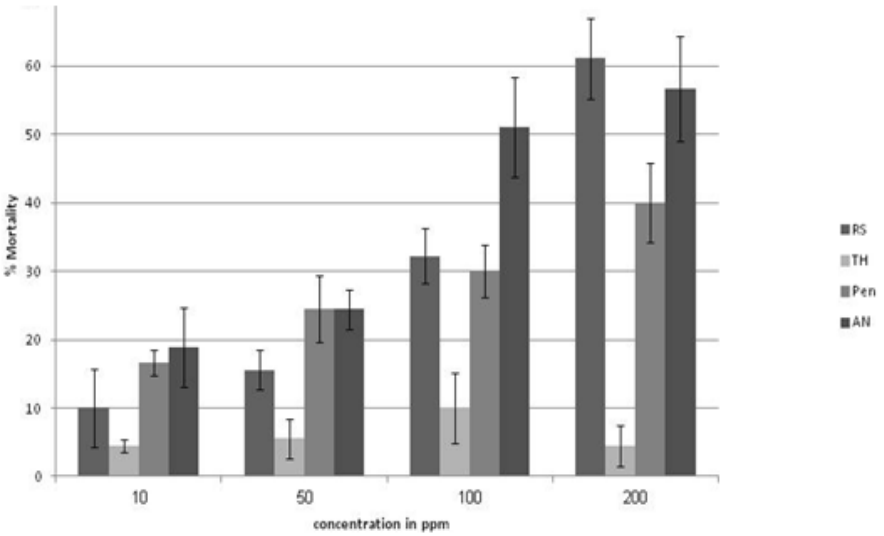


Figure 1. Percent mortality of the ethyl acetate extracts from four fungal species against *Lemna minor* (RS= *R. stolonifer*, TH = *Trichoderma harzianum*, Pen = *Penicillium* sp. EU0013 and AN = *A. niger*)

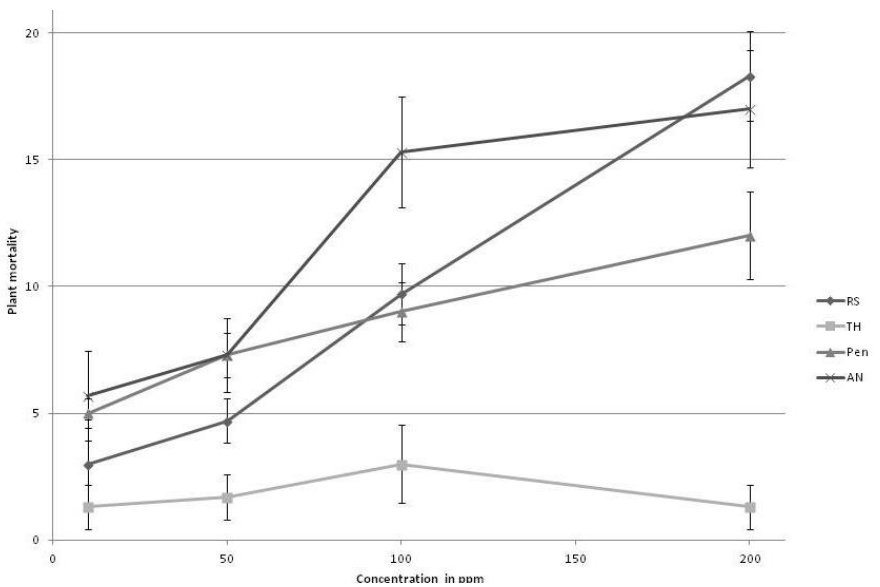


Figure 2. Number of *Lemna minor* plants dead out of total 30, against different concentration from the extract of selected fungal species. (RS= *R. stolonifer*, TH = *Trichoderma harzianum*, Pen = *Penicillium* sp. EU0013 and AN = *A. niger*)

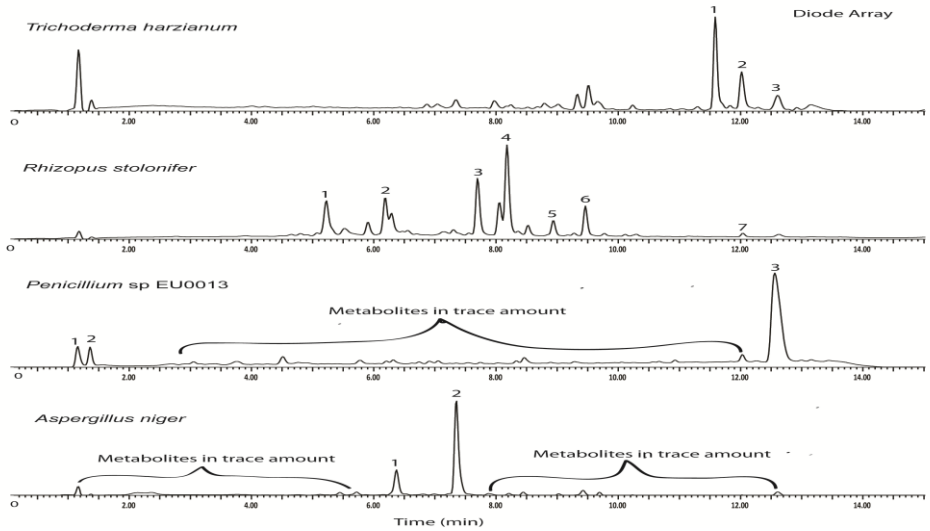


Figure 3. HPLC chromatogram of selected fungal species organic extracts

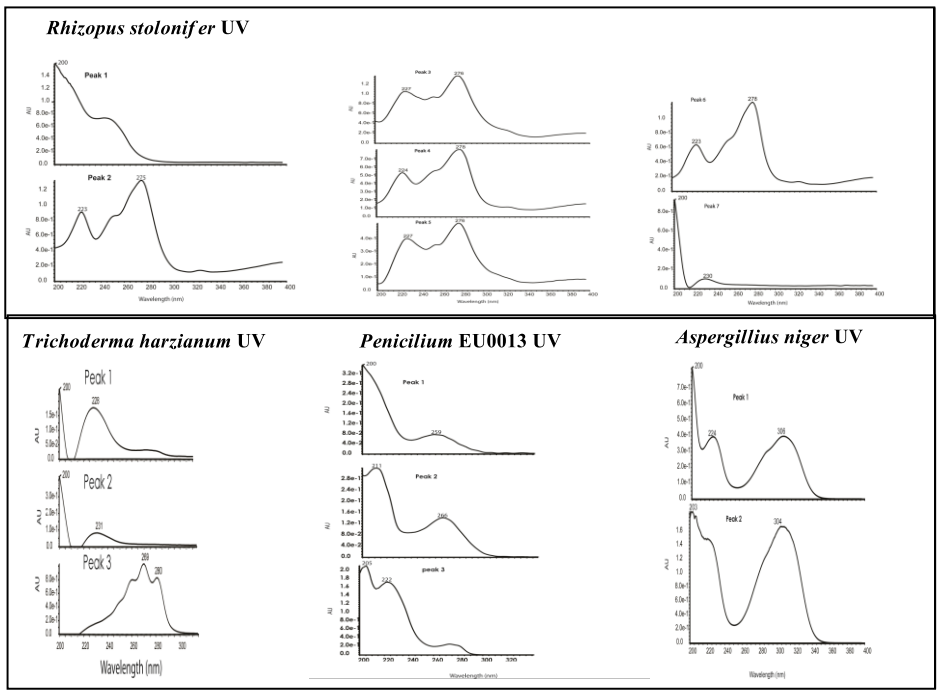


Figure 4. UV spectrum of peaks selected from HPLC chromatogram

CONCLUSION

Organic extracts from *A. niger* and *Rhizopus* sp. was revealed to possess strong growth inhibitory activity against *Lemna minor* and should be further investigated for weed control program. UV absorbance and retention time (Rt) provided little corroborate information related to the structure of compounds Therefore further investigation of these compounds might need advance studies such as LCMS and NMR analysis, as well for development of natural products based future herbicidal leads.

ACKNOWLEDGEMENTS

This work is part of the project entitled "Bioactive natural projects from fungal species of Pakistan'. The authors acknowledge the Higher Education Commission (HEC), Pakistan, Pakistan Science Foundation and Directorate of Science and Technology (DoST), Govt. of Khyber Pakhtunkhwa, for provision of funding for this project.

REFERENCES CITED

Andersen, M.R., L. Lehmann and J. Nielsen. 2009. Systemic analysis of the response of *Aspergillus niger* to ambient pH. Gen. Biol. 10(5): 1-13.

Arai, K., B.J. Rawlings, Y. Yoshizawa, and L.C. Vederas. 1989. Biosynthesis of antibiotic A26771B by *Penicillium turbatum* and dehydrocurvularin by *Alternaria cinerariae*: comparison of stereochemistry of polyketide and fatty acid enoyl thiol ester reductase. J. Amer. Chem. Soc. 111: 3391–3399.

Atta-ur-rahman. 1991. Studies in Natural Products Chemistry Bench Top Bioassays or Discovery of Bioactive Natural Product an update. Elsevier Science Publishers B.V.Netherland.9.

Darton, R. A. 1988. Rotation in factor analysis, The Statistician, 29, 167-194.

Evidente, A., R. Capasso, A. Cutignano, O. Taglialatela-Scafati, M. Vurro, M.C. Zonno, and A. Motta. 1998. Ascaulitoxin, a phytotoxic bis-amino acid N-glucoside from *Ascochyta caulina*. Phytochem. 48:1131–1137.

Ghisalberti E.L., and C.Y. Rowland. 1993. 6-Chlorodehydrocurvularin, a new metabolite from Cochliobolus spicifer. J. Nat. Prod.. 56: 2175–2177.

Gonçalves, S., M. Ferraz, and A. Romano. 2009. Phytotoxic properties of *Drosophyllum lusitanicum* leaf extracts and its main compound plumbagin. Sci. Hortic- Amsterdam. 122.:96–101.

Gutierrez M., C. Theoduloz, J. Rodriguez, M. Lolas, and G. Schmeda-Hirschmann. 2005. Bioactive metabolites from the fungus

Nectria galligena, the main apple canker agent in Chile. J. Agric. Food Chem. 53: 7701–7708.

Hoteling, H. 1933. Analysis of a complex of statistical variables into principal components. J. Educ. Psych. 24: 417-441.

Hyeon S., A. Ozaki, A. Suzuki, and S. Tamura. 1976. Isolation of a,b-dehydrocurvularin and b-hydrocurvularin from Alternaria tomato as sporulation-supressing factors. J. Agric. Biol. Chem. 40: 1663–1664.

Kimura, Y., T. Yoshinari, H. Koshino, S. Foujioka, K. Okada and A. Shimada. 2007. Rubralactone (polyketoid), rubralides A, B, C and rubramin produced by *Penicillium rubrum* .Biosci. Biotechol. Biochem.71(8): 1896-1901.

Kobayashi A., T. Hino, and S. Yata. 1988. Unique spindle poisons curvularin and its derivatives isolated from *Penicillium* species. Agric. Biol. Chem. 5:3119–3123.

Kusano M., K. Nakagami, S. Fujioka, T. Kawano, A. Shimada, and Y. Kimura. 2003. b,c-dehydrocurvularin and related compounds as nematicides of Pratylenchus penetrans from the fungus *Aspergillus* sp. J. Biotech. Biochem. 6: 1413–1416.

Munro H.D., O.C. Musgrave, and R.Templeton. 1967. Curvularin. Part V. The compound C16H18O5, a,b-dehydrocurvularin. J. Chem. Soc. (C). 947–948.

Paton, R. 2011. Organic Spectroscopy.. Michaelmers. 1-26. http://paton.chem.ox.ac.uk

Pearce, C. 1997. Biologically active fungal metabolites. Adv. Appl. Microbiol. 44: 1-68.

Pena-Rodriguez, L. M., N. A. Armingeon, and W. S. Chilton. 1988. Toxins from weed pathogens, I. Phytotoxins from a *Bipolaris* pathogen of Johnson grass. J. Nat. Prod. 51: 821-828.

Roberson D.J., G. Strobel, and R.N. Strange. 1985. The identification of a major phytotoxic component from *Alternaria macrospora* as alpha-beta-dehydrocurvularin. J. Nat.l Prod. 48: 139–141.

Saxena, S., and A. K. Pandey. 2001. Microbial metabolites as eco-friendly agrochemicals for the next millennium. Appl. Microbiol. Biotechnol. 55: 395-40.

Stierle, A. C., J. H. Cardellina, II, and G. A. Strobel. 1988. Maculosin, a host-specific phytotoxin for spotted knapweed from *Alternaria alternata*. Proc. Natl. Acad. Sci. U S A. 85: 8008–8011.

Vurro M., A. Evidente, A. Andolfi, M.C. Zonno, F. Giordano, and A. Motta. 1998. Brefeldin A and a,b-dehydrocurvularin, two phytotoxins from *Alternaria zinniae*, a biocontrol agent of *Xanthium occidentale*. Pla. Sci. 138: 67–79.

Yin, G., W. Wang, S. Sha, L. Liu, and X. Yu. 2010. Inhibition and control effects of the ethyl acetate extract of *Trichoderma*

harzianum fermented broth against *Botrytis cinerea*. Afri. J. Micr.Bio. Res. 4(15): 1647-1653.

Zhang, Y.L., H.M. Ge, F. Li, Y.C. Song, and R.X. Tan. 2008b. New phytotoxic metabolites from *Pestalotiopsis* sp. HC02, a fungus residing in *Chondracris rosee* gut. Chem. Biodivers. 5:2402–2407.

Zhang, Y.L., H.M. Ge, W. Zhao, H. Dong, Q. Xu, S.H. Li, J. Li, J. Zhang, Y.C. Song, and R.X. Tan 2008a. Unprecedented immunosuppressive polyketides from *Daldinia eschscholzii*, a mantis-associated fungus. Angew. Chem. Int. Edit. 47:5823–5826.