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Different Inhibitors of the Extracellular Pectinolytic Enzymes of *Fusarium oxysporum* and *Rhizoctonia solani*

[1,2]*Khaled M.A. Ramadan,* [3]*Afaf, Z.A. El-Meneisy,* [2]*Eslam S.A. Bendary and* [4]*Ahmed Z. Abdel Azeiz*

[1]King Faisal University, Central Laboratories, Department of Chemistry, Kingdom of Saudi Arabia
[2]Department of Biochemistry, Faculty of Agriculture, Ain Shams University, Egypt
[3]Department of Plant Pathology, Faculty of Agriculture, Ain Shams University, Egypt
[4]College of Biotechnology, Misr University for Science and Technology (MUST), Egypt

Abstract: Pectinases are one of the basic weapons for plant attack by different pathogens. Identification of pectinase inhibitors can help in plant protection against several phytopathogens. This study aimed to identify some inhibitors against *Fusarium oxysporum* and *Rhizoctonia solani* pectinases. Among the tested ten compounds, the highest inhibition percentage of *Rhizoctonia solani* pectinases were obtained from butyl-isobutyl-phthalate (64.4%) followed by nitrophenol (35.2%). The highest inhibition percentage of *Fusarium oxysporum* pectinases were obtained from thymoquinone (47.5%) followed by butyl-isobutyl-phthalate (46.1%). Phthalate derivatives are produced by roots of several plants in the root exudates that have many roles. One of these roles is as antifungal compounds. On the other hand, butyl-isobutyl-phthalate is a simple compound that can be synthesized and its structure elucidated by mass and NMR spectroscopy in the lab. So, through this study it is recommend the use of butyl-isobutyl-phthalate for phytopathogens control in the field.

Key words: *Fusarium oxysporum* · *Rhizoctonia solani* · Pectinases · Inhibitors

INTRODUCTION

The phytopathogenic fungi attack mechanisms of plants include production of several enzymes, the most important of them are the pectinases [1]. These phytopathogenic fungi cause dramatic decrease in the crops production worldwide. Several researches were conducted to find pectinases inhibitors to be used against these pathogenic fungi [2-4].

Butyl-isobutyl phthalate was identified by Rasha *et al.* [5] in the root exudates of Faba bean that enhanced the germination of *Orobanche* spp. Seeds, which are the aggressive root parasitic plant of Faba bean and many other crops. It was clearly appeared from the literature that several plants produce several phthalate derivatives that work as antimicrobial agents [6-10].

On the other hand, one of the defense systems in the plant cell against the fungal infection is the production of Leucine Rich Repeat protein (LRR) that works as pectinases inhibitor [11]. Furthermore, several phenolic compounds are considered as inhibitors for pectinases and cellulases [12-14].

The aim of the present study was to test the inhibition effect of ten different compounds, include butyl-isobutyl phthalate, leucine dimer and phenolic compounds, against the pectinases of the phytopathogenic fungi *Fusarium oxysporum* and *Rhizoctonia solani.*

MATERIALS AND METHODS

Isolation of Pathogenic Fungi: Tomato plants with typical symptoms of root rot or wilt diseases were used for pathogen isolation. The specimens were surface sterilized and transferred to water agar plates for 5 days at 3°C. a disk of each fungus was transferred to PDA plates and incubated for 4-7 days. Hyphal tip technique was used for purification. Slide cultural technique was used

Corresponding Author: Khaled M.A. Ramadan, King Faisal University, Central Laboratories, Department of Chemistry, Kingdom of Saudi Arabia.

for obtained isolates. Fungal isolates were identified according to cultural properties, morphological characters and microscopically investigation at Plant Pathology Department, Faculty of Agriculture, Ain Shams Univ., Egypt according to Both [15] for *Fusarium* and Sneh *et al*. [16] for *Rhizoctonia*.

Pathogenicity Test: Pathogenicity of isolated fungi was carried out to determine the most aggressive isolate of *R. solani* and *F. oxysporum* to use in further investigations.

Sterilized pots were filled with disinfested soil. Inocula of *R. solani* and *F. oxysporum* were prepared by growing each isolate in bottles containing sterilized barley grains and incubated at 25±2°C for 15 days. Soil infestation was achieved by mixing the inoculum of each isolate with the upper layer of the soil at the rate of 2% (w/w) potential inoculum [17]. The pots were irrigated regularly. Sterilized tomato seeds were sown in the pots and obseved for disease symptoms up to 45 days of sowing. The most aggressive isolates of *R. solani* and *F. oxysporum,* that showed more disease severity, were selected for further studies.

Chemicals and Phenolic Compounds: 2,3,5-trichlorophenol, 2,3-dimethyl phenol, 2-ethyl phenol, Nitrophenol, tannin, benzoyl chloride and 2-keto glutaric acid were purchased from Supelco, USA. Leucine derivatives, butyl phthalate and thymoquinone were synthesized at Central Laboratory, Faculty of Agriculture, Ain Shams University.

Organic Synthesis and Characterization of Butyl-Isobutyl Phthalate (BIP): Synthesis of Butyl-isobutyl-phthalate was performed according to the method described by Liu *et al*., [18]. Phthalic anhydride was added in n-butanol, in presence of 95% H_2SO_4. This mixture was refluxing over 4 h. Then, the reaction was cooled down to room temperature and cold water was added. The mixture was extracted by ethyl acetate (EtOAc). After filtration and concentration, a compound (1) was obtained as colorless and clear oil. The compound (1) was added in dry Dimethylformamide (DMF) and then 1-bromo-2-methylpropane and K_2CO_3were added. The reaction mixture was stirred at 60°C overnight. After cooling to room temperature, the mixture was diluted with water, extracted with EtOAc and the organic layer was dried over Na_2SO_4 anhydrous. The crude residue was purified by flash chromatography (10: 1 petroleum ether/ EtOAc) yielding the target compound (2) as colorless oil (Fig. 1). The structure of compound (2) was characterized using LC-MS-ESI+ spectra, ^{1}H and ^{13}C NMR.

Organic Synthesis of Thymoquinone: The thymoquinone was synthesized from thymol by the method described by Kremers *et al.* [19]. The structure was confirmed by GC/MS analysis that showed one pure compound at Rt. 12.5 min and has a MW 164. The analysis was performed using HP-5890 GC equipped with HP-5972 mass spectrometer. The HP-innowax column 30m X 0.25mm id X 0.25 µm was used. Helium was used as mobile phase with a flow rate 1ml/ min. Oven temperature start at 50°C for 2min., up to 200°C at a rate of 15°C/min. The fragmentation pattern in the mass spectrum was analyzed by Wiley 7N mass library.

Organic Synthesis of Lucine Derivatives: In basic medium, the formaldehyde can react with the alpha-amino group of the amino acids to form either dimer of the amino acid, or N-hydroxymethyl derivatives or both. One g of lucine was dissolved in 100 ml of 0.1 N NaOH and 10ml of formaldehyde solution (30%) was added and mixed well. The solution was heated under reflux at 100°C for 10 h. After cooling, the product was precipitated by drop-wise addition of four volumes of acetone, followed by centrifugation. The lucine derivatives were dried at 70°C.

HPLC/MS Analysis for Synthesized Butyl-Isobutyl Phthalate Compound: The synthesized butyl-isobutyl phthalate was analyzed by LC- MS instrument Agilent 1200 infinity II (Central lab. Faculty of agriculture - Ain Shams University) equipped with auto-injector and a single quad mass detector and fitted with a Zorbax SB- C18 (5µm, 4.6 mm x 250 mm). The crude extracts were dissolved in 60% methanol and filtered through 0.45µm pore size filter. Two µl were injected. The mobile phase was 60% methanol in water and was gradient to 100% methanol 30 min after injection. The column then washed with 100% methanol for 20 min. The flow rate was 0.8 ml/min and the column temperature was set to 30°C [20].

NMR Spectroscopic Elucidation of Synthesized Butyl-Isobutyl Phthalate Compound: The NMR spectra (^{1}H and^{13}C) were recorded on a Varian Mercury VX-300 NMR spectrometer. ^{1}H spectra were run at 300 MHz and ^{13}C spectra were run at 75.46 MHz in deuterated dimethyl sulphoxide (DMSO-d6). The analysis was performed in Department of Chemistry, Faculty of Science, Cairo University.

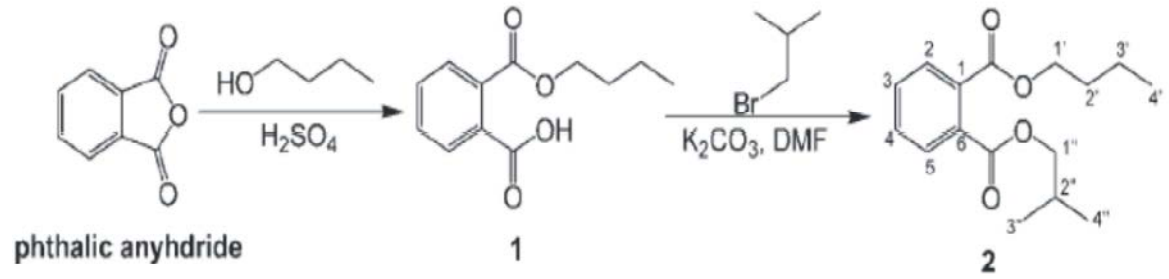


Fig. 1: Synthesis of butyl-isobutyl phthalate

Growth of Pathogenic Fungi: *Fusarium oxysporum* and *Rhizoctonia solani* isolates were grown on potato dextrose agar plates for a week. A 5mm disk from each fungus was transferred to flasks containing 50 ml of a liquid minimal medium supplemented with 0.5% of pectin. The medium consisted of (g/l): NH_4NO_3: 2, KH_2PO_4: 1, $MgSO_4$: 0.1, Yeast extract: 0.5, Malic acid: 3. pH was adjusted to 5 by NaOH 0.1N solution [21]. Inoculated flasks were then incubated at 25°C for another week and then the supernatant of each fungus culture was obtained by filtration through filter paper.

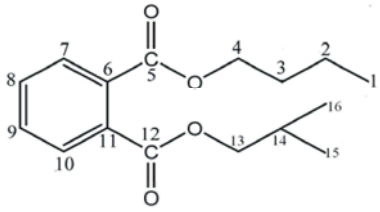
Pectinases Inhibition Test: Pectinase was determined by measuring the amount of reducing sugars released from pectin as a substrate by the method described by Miller [22].

Reagents: 1% pectin in 50mM sodium phosphate buffer, pH 7. 0.1% (w/v) galacturonic acid standard solution. Dinitrosalicylic acid solution: 0.25 g Dinitrosalicylic acid + 75 g potassium sodium tartarate + 4 g NaOH dissolved in 250 ml distilled water. a mixture of fugal supernatant (0.5 ml), pectin solution (0.5 ml) and phosphate buffer solution (100µl) or inhibitor solution 1% (100µl) were mixed in a test tube. The tubes were incubated at 37°C for one hr. Two ml of DNS solution were added for each tube and kept in a water bath at 100°C for 10 min. The absorbance was measured at 570 nm. Control tubes contain all reagents, but the DNS solution was firstly added to inhibit the enzyme activity. The standard curve was made by 250µl, 500µl, 750µl and 1000µl of the 0.1% galacturonic acid standard solution. The volume was raised in each tube to 1.1 ml by distilled water. One enzyme unite was expressed as the amount of enzyme releases one mg of galacturonic acid/ hr/ ml. Three replicates for each treatment were used.

RESULTS AND DISCUSSION

HPLC- MS analysis for synthesized butyl-isobutyl phthalate compound showed one pure compound and the mass spectrum showed the expected MW (m/z 279) and the mass fragmentation pattern of the phthalate moiety (Fig. 2). GC/MS analysis of thymoquinone showed one pure compound at retention time (Rt.) 12.5 min. The mass spectrum showed the expected MW at m/z 164 (Fig. 3).

NMR Spectroscopic Elucidation of Synthesized Butyl-isobutyl Phthalate Compound:



Chemical structure of butyl-isobutyl phthalate

Table 1: NMR (^{1}H and ^{13}C) of the synthesized butyl-isobutyl phthalate:

Carbon No.	δ_C	δ_H
1	13.4	0.88 (3H, t, j=7.4)
2	18.8	1.36 (2H, hex, j=7.4)
3	29.8	1.65 (2H, pent, j=6.6)
4	64.8	4.22 (2H, t, j=6.5)
5,12	186.9	-
6, 11	186.9	-
7-10	120.6, 128.5,131.5	7.7 (4, m)
13	71	4,02(2H, d, j=3.5)
14	29.8	1,86 (1H, non, j=4.9)
15, 16	18.6	0.91 (6H, d, j=7.3)

Inhibition of *Rhizoctonia solani* Pectinases: The highest inhibition percentage of *Rhizoctonia solani* pectinases were obtained from butyl phthalate (64.4%) followed by nitrophenol (35.2%), thymoquinone (29.5%) and benzoyl chloride (27.8%). On the other hand, three of the tested

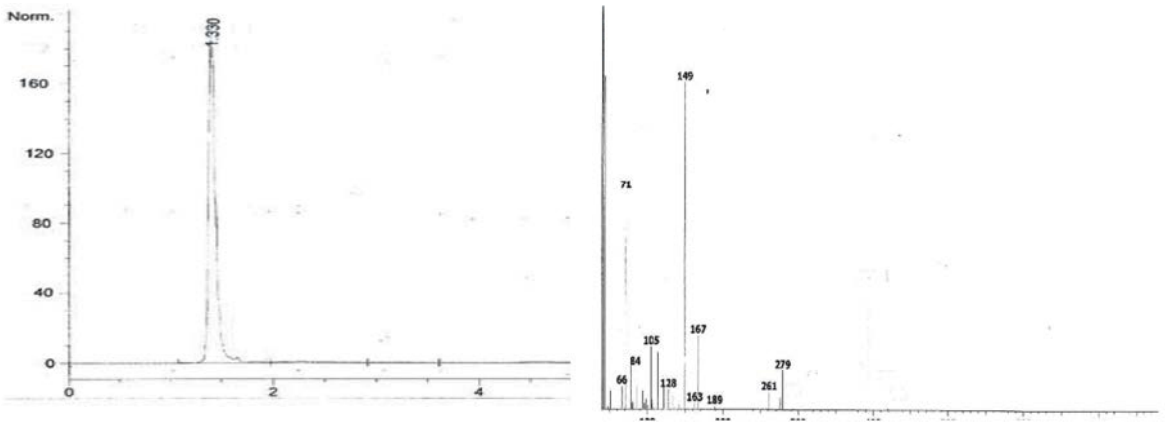


Fig. 2: HPLC-MS chromatogram and the mass spectrum of purified butyl-isobutyl phthalate

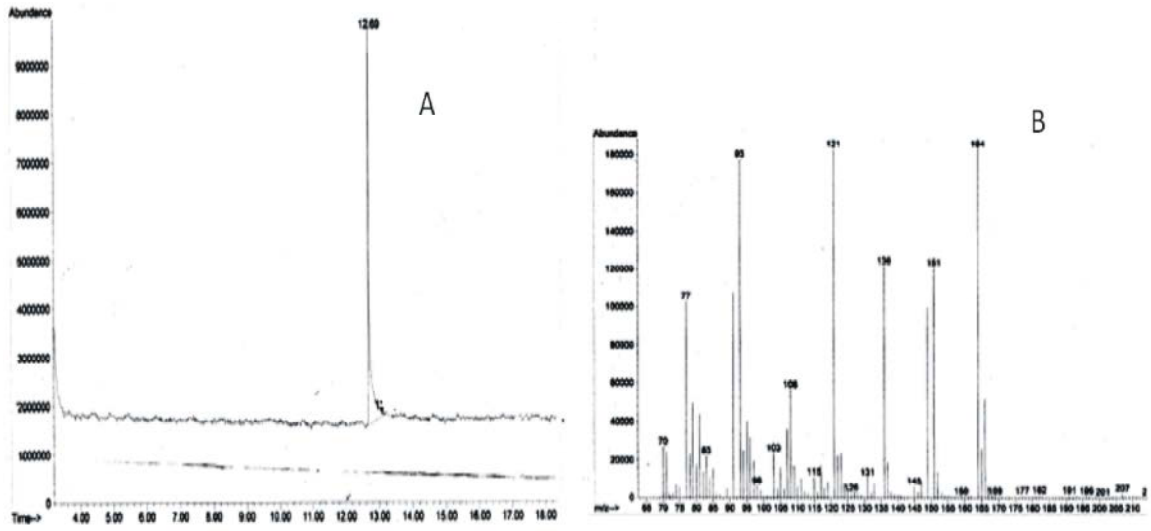


Fig. 3: (A) The GC/MS chromatogram of the thymoquinone showed one pure compound at Rt. 12.5 min. and (B) The mass spectrum of thymoquinone showed the expected MW at m/z 164.

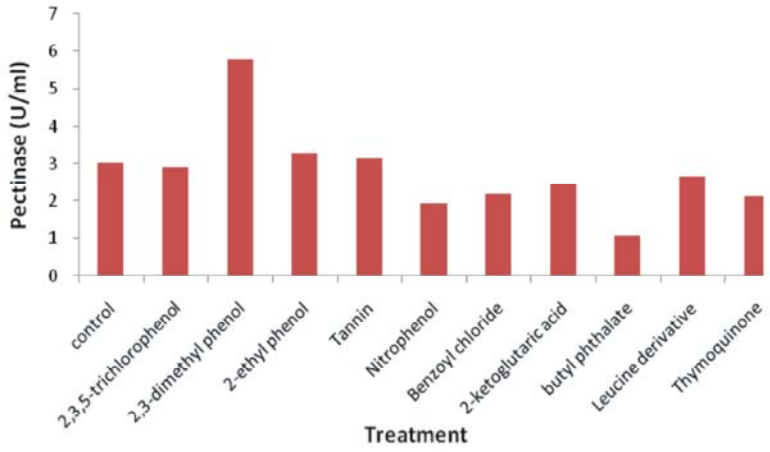


Fig. 4: The pectinases activity (U/ml) of the *Rhizoconia solani* culture as affected by different compounds

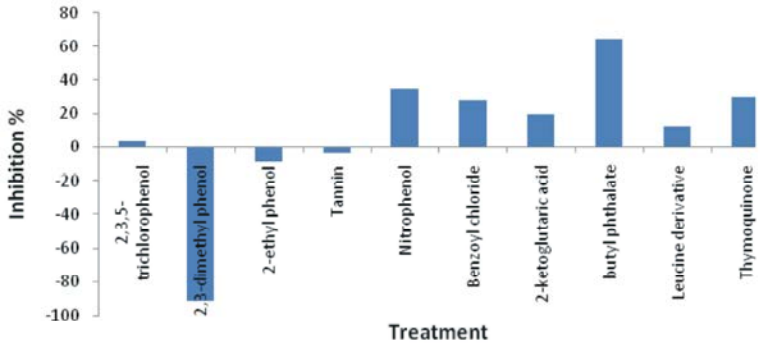


Fig. 5: Inhibition/activation percentages of *Rhizoctonia solani* pectinases by ten tested compounds

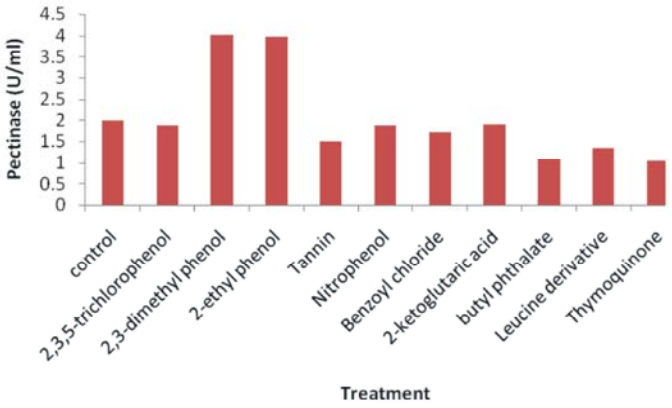


Fig. 6: The pectinases activity (U/ml) of the *Fusarium oxysporum* culture as affected by different compounds

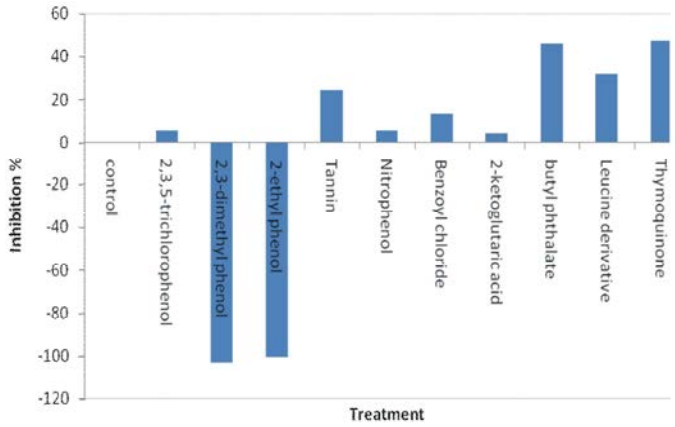


Fig. 7: Inhibition/activation percentages of *Fusarium oxysporum* pectinase by 10 tested compounds

compounds enhanced the pectinases activity. These compounds are 2,3 dimethyl phenol, 2-ethyl phenol and tannin that increased the enzyme activity by 116.6%, 10.9% and 4.7% for each compound respectively (Fig. 4,5). Ahsan *et al*. [23] reported that butyl phthalate produced by *Streptomyces* strain KX852460 considered as antifungal compound against *Rhizoctonia solani* and showed high inhibition zone in solid medium.

Inhibition of *Fusarium oxysporum* Pectinases: The highest inhibition percentage of *Fusarium oxysporum* pectinases was obtained by thymoquinone (47.5%) followed by butyl phthalate (46.1%) and leucine derivatives (31.9%). On the other hand, the enzyme activity was enhanced by the same two compounds that enhanced the *Rhizoctonia solani* pectinases, 2,3 dimethyl phenol, 2-ethyl phenol by 103.2% and 100.2% for each

compound, respectively (Fig. 6, 7). In another study by Akhtar *et al.* [24] there was 0- 100% inhibition of growth of *Fusarium solani* with 0.031- 1.0 mg thymoquinone /ml on 10th day of incubation.

Inhibition of pectinases secreted from the two tested fungi (*Fusarium oxysporum* and *Rhizoctonia solani*) by butyl-isobutyl phthalate, leucine derivative and thymoquinoine confirm the role of these compounds as pectinases inhibitors in their natural sources. Further investigations are required to test different phthalate derivatives and testing of these compounds in field study. On the other hand, improvement of pectinases activity by 2,3-dimethyl phenol and 2-ethyl phenol is required to be studied.

REFERENCES

1. Herbert, C., G. Boudart, C. Borel, C. Jacquert, M.T. Esquerre-Tugaye and B. Dumas, 2003. Regulation and Role of Pectinases in Phytopathogenic Fungi, in book: Advances in Pectin and Pectinase Research, Springer
2. Brown, A.E. and A. Nimal, 1983. A Role for Pectinase and Protease Inhibitors in Fungal Rot Development in Tomato Fruits. Journal of Phytopathology, 106(3): 239-251.
3. Muhammad, F.R., I.T. Muhammad, A. Mehwish, K. Ghulam and I. Aima, 2009. Inhibition Studies of Cellulolytic Activities Isolated from *Planococcus Citri*. The Open Enzyme Inhibition Journal, 2: 8-11.
4. Hassanien, S.E., A.Z. Abdel Azeiz, A.M. Ramadan and A.Z. EL-Meneisy, 2012. Screening for pectinase and cellulase inhibitors from the root exudates and protein contents of five plants against six phytopathogenic fungi. J. Biol. Chem. Environ. Sci., 2012, 7(3): 545- 553.
5. Mohammed, R.A. K.M. Ramadan, S.E. Hassanien, R.R. Francis and A.Z. Abdel Azeiz, 2013. Butyl- iso-butyl phthalate as an Orobanche crenata seeds germination activator secreted by roots of *Vicia Faba*. Journal of Biological Chemistry and Environmental Sciences, 8(4): 157-167.
6. Rowshanul, M.H. and M.K. Rezaul, 2009. Antimicrobial and cytotoxic activity of Di-(2-ethylhexyl) phthalate andanhydrosophoradiol-3-acetate Isolated from *Calotropis gigantean*(Linn.) Flower. Mycobiology, 37(1): 31-36.
7. Al-Bari, A.M., M. Abu Sayeed, M.S. Rahman and M.A. Mossadik, 2006. Characterization and antimicrobial activitiesof a Phthalic acid derivative produced by *Streptomyces bangladeshiensis*, a novel species collected in Bangladesh. ResearchJournal of Medicine and Medical Sciences, 1(2): 77-8.
8. Ab Zitounii, F.B., F. Mathieu, A. Lebrihi and N. Sabaou, 2011. Taxonomic study and partial characterization of antimicrobial compounds from a moderately halophilic strain of the genus Actinoalloteichus Braz. J. Microbiol., 42 (4): 835-845.
9. Khatiwora, E., V.B. Adsul, M. Kulkarni, N.R. Deshpande and R.V. Kashalkar, 2012. Antibacterial activity of Dibutyl Phthalate: A secondary metabolite isolated from *Ipomoea carnea* stem. Journal of Pharmacy Research, 5(1): 150-156.
10. El-Sayed, M.H., 2012. Di-(2-ethylhexyl) Phthalate, a major bioactive metabolite with antimicrobial and cytotoxic activity isolated from the culture filtrate of newly isolated soil streptomyces (*Streptomyces mirabilis* Strain NSQu-25). World Applied Sciences Journal, 20(9): 1202-1212.
11. Shanmugam, V., 2005. Role of extracytoplasmic leucine rich repeat proteins in plant defence mechanisms. Microbiological Research, 160(1): 83-94.
12. Mandels, M. and E.T. Reese, 2003. Inhibition of Cellulases. Annual Review of Phytopathology, 3(1): 85-102
13. Wu, H.J. Luo, W. Raza and Q. Shen, 2010. Effect of exogenously added ferulic acid on *in vitro Fusarium oxysporum* f. sp. niveum. Scientia Horticulturae, 124(4): 448-453.
14. Ximenes, E., Y. Kim, N.S. Mosier, B. Dien and M. Ladisch. 2011. Deactivation of cellulases by phenols. Enzyme Microb. Technol., 48(1): 54-60.
15. Booth,C., 1971. The genus *Fusarium.* Commonwealth Mycological Institute, Kew, Surrey, England pp: 235.
16. Sneh B., L. Burpee and A. Ogoshi, 1991. Identification of *Rhizoctonia* species. The APS Press, St. Paul, MN, USA, pp: 133.
17. Al-Askar, A.A. and Y.M. Rashad, 2010. Efficacy of Some Pant Extracts Against *Rhizoctonia solani* on Pea. Journal of Plant Protection Research, 50(3): 239-243.
18. Liu, M.W. Zhang, L. Qiu and X. Lin, 2011. Synthesis of butyl-isobutyl-phthalate and its interactionwith a-glucosidase *in vitro*. J. Biochem., 149(1): 27-33.

19. Kremers, E.N. Wakeman and R.M. Hixon, 1941. Organic synthesis of thymoquinone. Organic Syntheses Coll., 1: 511-513.
20. Kaori, Y., X. Xie, H. Sekimoto, Y. Takeuchi, S. Ogasawara, K. Akiyama, H. Hayashi and K. Yoneyama, 2008. Strigolactones, host recognition signals for root parasitic plants and arbuscular mycorrhizal fungi, from Fabaceae plants. New Phytologist, 179: 484-494.
21. Riou, C., G. Freyssinet and M. Fever, 1991. Production of cell wall degrading enzymes by the phytopathogenic fungus *Sclerotinia scelrotiorum*. Applied and Environmental Microbiology, 57(5): 1478-1484.
22. Miller, G.L., 1959. Use of dinitrosalicylic acid for estimation of reducing sugar. Anal. Chem., 31: 426- 428.
23. Ahsan, T., J. Chen, X. Zhao, M. Irfan and Y. Wu, 2017. Extraction and identification of bioactive compounds (eicosane and dibutyl phthalate) produced by *Streptomyces* strain KX852460 for the biological control of *Rhizoctonia solani* AG-3 strain KX852461 to control target spot disease in tobacco leaf. AMB Expr., 7: 54.
24. Akhtar, N., O.M. Alakloby, S.H. Aljabre, A.M. Alqurashi and M.A. Randhawa, 2007. Comparison of Antifungal Activity of Thymoquinone and Amphotericin B Against *Fusarium solani in-vitro*. Basic and Applied Sciences, 8(2): 137-145.