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MICROSCOPIC EVALUATION OF THE ANTIMICROBIAL ACTIVITY OF SEED EXTRACTS OF *MORINGA OLEIFERA*

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Abstract

Seed extracts of *Moringa oleifera* were assayed for the evaluation of antimicrobial activity against bacterial (*Pasturella multocida, Escherichia coli, Bacillus subtilis* and *Staphlocuccus aureus*) and fungal (*Fusarium solani* and *Rhizopus solani*) strains. The crude, supernatant, residue and dialyzed samples inhibited the growth of all microbs to various extents. The zones of growth inhibition showed greater sensitivity against the bacterial strains as compared to the fungal strains. The extracts worked in dose dependent manner and resulted in crippled and distorted hyphae and apical branching in fungi. Minimum inhibitory concentrations (MIC) extracts revealed that *Pasturella multocida and Bacillus subtilis* were most sensitive strains. However, the activity of the extracts was antagonized by cations (Na^+, K^+, Mg^{2+} and Ca^{2+}). Maximum activity was found between temperature 4 -37 ^OC and pH 7.

Introduction

Previous studies have reported that various parts of *Moringa* roots, flowers, bark, and stem including seeds possess antimicrobial properties (Lockett *et al*., 2000; Anwar and Rashid, 2007). Seed of *Moringa oliefera* are also known for *Moringa oleifera* coagulation properties for treating water and wastewater due to presence of flocculent protein/ peptides (Katayon *et al*., 2005). Seed extracts of *Moringa oleifera* have been found to have antimicrobial properties (Kebreab *et al*, 2005; Jamil *et al*., 2007).

Structural morphological study of microbes after treatment with antimicrobial agents is an important parameter in understanding the mechanism of action of these agents (Kitajim *et al*., 1998). However, a little review is available on the mechanistic microscopic study of *Moringa oleifera*. Therefore, microscopic evaluation of the some fungal and bacterial strains was carried out after treatment with seed extracts of the plant that demonstrate the antimicrobial effect of the optimized medicinal plant extracts on the structural deformities of the microbes. Effect of temperature, pH and different ionic concentrations on antimicrobial activity of *Moringa oleifera* seed extracts was also investigated and reported in this paper.

Materials and Methods

Plant materials: Seed of *Moringa oleifera* was purchased from the local market of Faisalabad and taxonomically identified from the Department of Botany, University of Agriculture Faisalabad, Pakistan.

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Microbial strains: Pure cultures of fungal strains, *Fusarium solani*, *Aspergillus niger*, *Metarhizium aniscoplae* and *Rhizopus solani* and bacterial strains *Pasturella multocida*, *Escherichia coli*, *Bacillus subtilis* and *Staphlocuccus aureus* were obtained from Department of Microbiology, University of Agriculture, Faisalabad, Pakistan.

General procedures: Protein contents were determined by Bradford method (Bradford, 1976). Antimicrobial activity was determined by disc diffusion method (NCCLS, 2002). Streak method (Hancock, 1997) and disc diffusion methods were used for minimum inhibitory concentrations (MIC) values. Microscopy was performed on Nikon (Japan) microscope with Nikon FDX-35 fitted camera.

Extraction and partial purification : *Moringa oleifera* seed were ground and extracted in 10 m*M* potassium phosphate buffer (pH: 7) by a ratio of 1:2 (w/v), phenyl methyl sulfonyl flouride 10 m*M* (PMSF) was added as protease inhibitor. The extract was centrifuged at 10,000 x*g*, 4 ºC for 20 min. The supernatant was $(NH_4)_2SO_4$ precipitated at 80% saturation level (Huynh *et al*., 2001) followed by centrifugation at 10,000 x*g*, 4 ºC for 10 min. The supernatants were stored at 4 ^{O}C and residues were resuspended in minimum quantity of the extraction buffer, and dialyzed for 24 hours against distilled water (Huynh *et al.*, 2001).

Antimicrobial assay: Strain of bacteria and fungi were grown on nutrient agar (Oxoid) and potato dextrose agar (Oxoid) growth media respectively. Inocula for each strain with 1×10^5 colony forming unit /mL were used. Streptomycin sulphate (Pharmacia) was used as positive control. Antimicrobial activities of seed extracts were determined by disc diffusion method. The zones of inhibition (mm) were measured on zone reader (NCCLS, 2002).

Determination of minimum inhibitory concentrations (MIC) of *Moringa oleifera* buffer extract: Crude extract of *Moringa oleifera* was diluted in microdilution plate, 10 *u*L spores were added to each well and incubated at 28 C for 48 h for fungi and at 37 C for 24 h for bacteria. MIC was determined by streak method for bacteria and by microscopy for fungi. Rose Bangal stained slides were examined under microscope (Nikon, Japan) and photographs of these slides were captured by camera (Nikon FDX-35).

Effect of different pH, temperature and ionic concentration on the antimicrobial activity of plant extracts (*Moringa oleifera*): The activity of seeds extracts at pH (3, 5, 7, 9, 11), temperatures (pH 7) 0, 37, 60, 100, 121 ^{O}C was determined by streaking method. The activity at various ionic concentrations of Na^+, K^+, Mg^{2+}, Ca^{2+} was determined by disc diffusion method (NCCLS, 2002).

Results

Antifungal and antibacterial activities of seed extracts of *Moringa oleifera*: The results of antifungal activity of extracts of *Moringa oleifera* are shown in Table 1. Fig 1(A and B) show the inhibition zones of different extracts of *Moringa oleifera* against *Bacillus subtilis* and *Staphlococcus aureus*, respectively.

Crude samples showed very strong activity against *Fusariam solani*, *Bacillus subtilis* and *Staphlococcus aureus;* but showed almost no activity against *Rhizopus solani* and less activity against *Pasturella multocida, Aspergillus niger*, *Metarhisium aniscoplae* and *Escherichia coli* whereas poor activity of supernatant was found against *Rhizopus solani*, *Pasturella multocida, Staphlococcus aureus* and *Bacillus subtilis* and moderate activity against *Escherichia coli, Aspergillus niger* and *Metarhisium aniscoplae*. Dialyzed samples showed moderate activity against all of the four species of bacteria and *Aspergillus niger* of fungas, and no activity was found in dialyzed sample against

Table 1 Antimicrobial activity of crude, supernatant and dialyzed samples of *Moringa oleifera* against selected microbial strains

Selected organisms		Crude		Residue		Dialyzed		Positive control		Negative control	
		DD	MIC	DD	MIC	DD	MIC	DD	MIC	DD	MIC
Bacterial strains	***Pasturella multocida***	21	26.0 ±2.05	13.5	22.4 ±1.51	20	20.2 ±0.05	36.5	20.1 ±0.09	0	N.D
	Escherichia coli	20	28.0 ±1.50	20.5	24.2 ±1.04	20	21.4 ±0.07	18	26.0 ±0.02	0	N.D
	Bacillus. subtilis	36	22.2 ±1.15	18.75	19.4 ±1.06	25.25	16.7 ±0.06	38	17.4 ±0.03	0	N.D
	Staphlococcus aureus	31	24.0 ±1.50	18	21.2 ±0	26	18.2 ±0.02	28.75	26 ±0.06	0	N.D
Fungal strains	***Fusarium solani***	37.5	26.0 ±0.03	30.5	28.0 ±0.09	32.5	22.4 ±0.06	32.5	18.02 ±0.07	0	N.D
	Rhizopus solani	9	1073.35	17.5	102.72	0	N.D	15	644	0	N.D
	Aspergillus niger	22	38.0 ±0.02	21	36.0 ±0.02	23	28 ±0.04	24.0 ±0.4	26 ±0.07	0	N.D
	Metarhizium aniscoplae	26	32.0 ±0.9	27.01 ±1	30 ±0.09	30.01 ±0.06	26 ±0.05	30 ±0.06	25 ±0.06	0	N.D

DD = Diameter of inhibition zone (mm) including disc diameter of 6 mm.
MIC = Minimum Inhibitory Concentration (mg/mL)

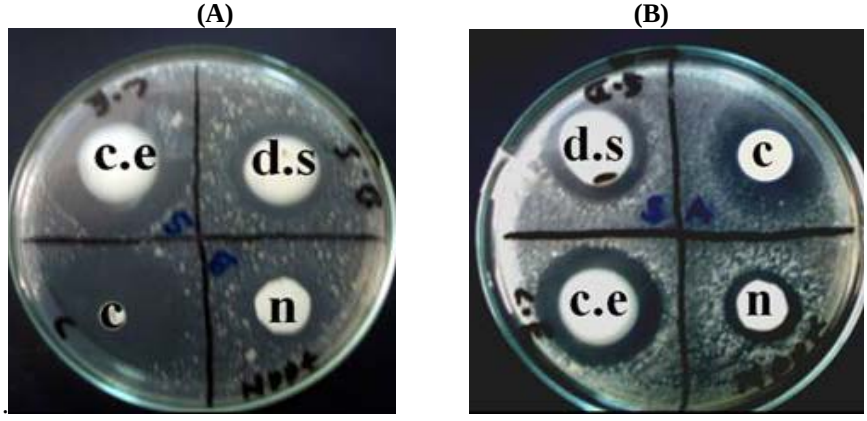


Figure: 1 Antibacterial activity of *Moringa oleifera* extracts against *Bacillus subtilis* (A) and *Staphlococcus aureus* (B) by disc diffusion method. (c) Standard control (c.e) Crude extract (d.s) Dialyzed sample (n) Supernatant (ammonium sulphate precipitation)

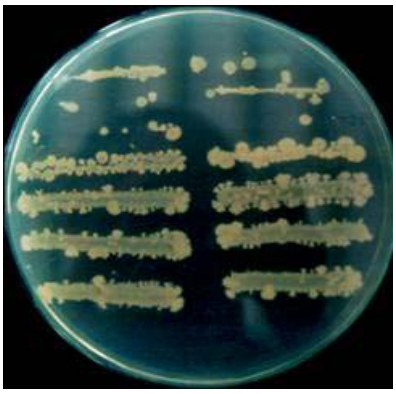


Fig: 2: Minimum inhibitory concentration assay against *Pasturella multocida.* Left column in the figure is for dialyzed sample and right column for crude sample of *Moringa oleifera* extracts. Streaks 1, 2, 3, 4, 5, 6, 7 (top to bottom) in each column are from micro plate well No 1, 3, 5, 7, 9, 11, 12 respectively from micro dilution plate, in which well No "1" served as control and 12th as blank. It is a representative diagram; results for other species are not shown.

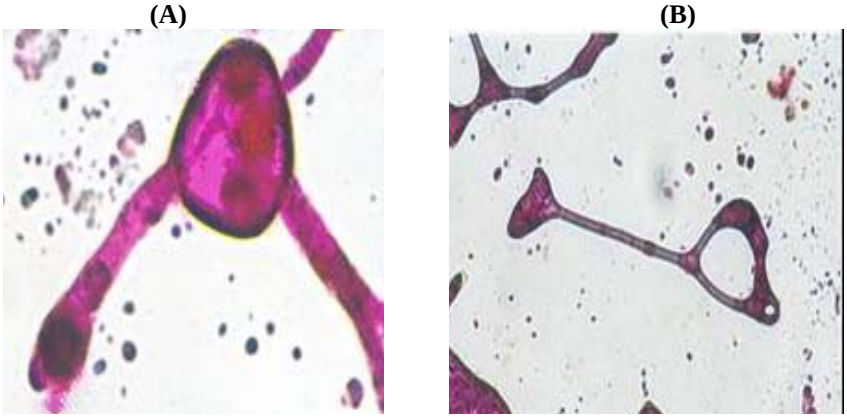


Figure: 3 (A and B) Microscopy of *Rhizopus solani* from first well (A) and 11th well (B) having diluted crude extract of *Moringa oleifera.* Magnification (20 x 10) x.

Rhizopus solani. Only *Fusariam solani* and *Metarhisium aniscoplae* were the most sensitive species inhibited by dialyzed sample strongly.

MIC values demonstrated that in bacterial species the most sensitive strains were *Pasturella multocida* and *Bacillus subtilis. Staphlococcus aureus* had moderate sensitivity and *E. coli* was found to be comparatively least sensitive strain. *Fusarium solani* was more sensitive than *Rhizopus solani*, *Aspergillus niger* and Metarhisium *aniscoplae* against the extract.

The growth pattern of *Pasturella multocida* is presented in Figure 2. It showed that *Pasturella multocida* growth increased as concentration of the extract decreased in each dilution and it gave MIC value of the crude extract at 26.0±2.05 mg/mL protein

concentration, whereas it was 20.2±0.05 mg/mL protein concentration of the dialyzed sample.

Microscopic evaluation of activities of seed extracts of *Moringa oleifera:* *Rhizopus solani* was selected for microscopy due to comparatively rapid growth and least sensitivity. The slides of the fungi were prepared from different wells of microtiter plate and observed under microscope. The growth of *Rhizopus solani* treated with crude extract of *Moringa oleifera* showed that growth of hyphea was inhibited.

There was direct relation between concentration and damaging of the cell wall/ membrane of microorganism as shown in Figure 3 (A and B). A comparative effect of the different concentrations of protein extract of *Moringa oleifera* is demonstrated in Figure 4. It showed that high concentration of protein ruptured the cell wall of hyphea and damaged the conidia, and resulted in broken hyphea. Low concentrations of protein extract had less effect.

Effect of pH and temperature on activities of seed extracts of *Moringa oleifera*: Due to the greatest sensitivity in the bacterial strains, *Bacillus subtilis* was selected for further study for pH and temperature effect on activity of *Moringa* extracts. Figure 6 shows the effect of pH on the activity of the extract, by growth rate of *Bacillus subtilis*. Minimum growth was found at pH 7. Maximum activity was found at 0 ^{O}C and pH 7.

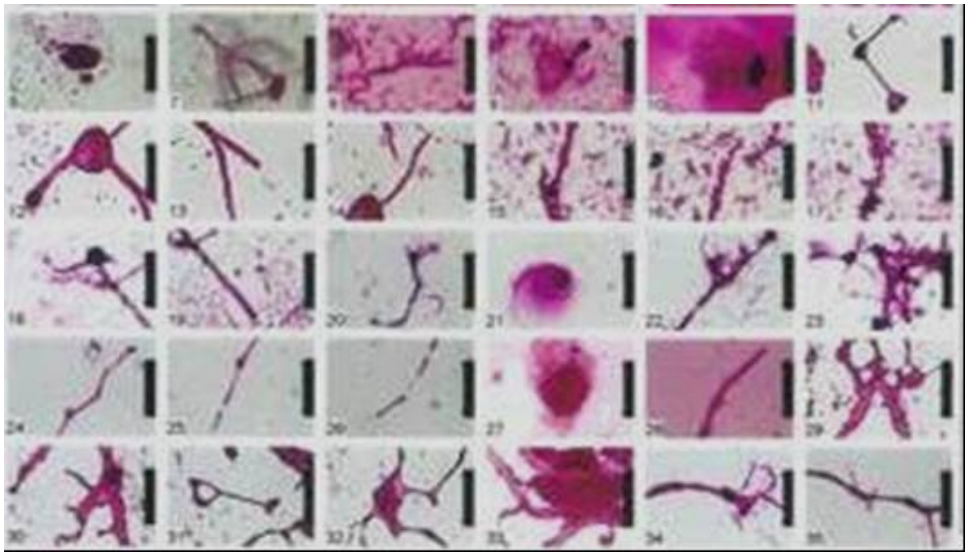
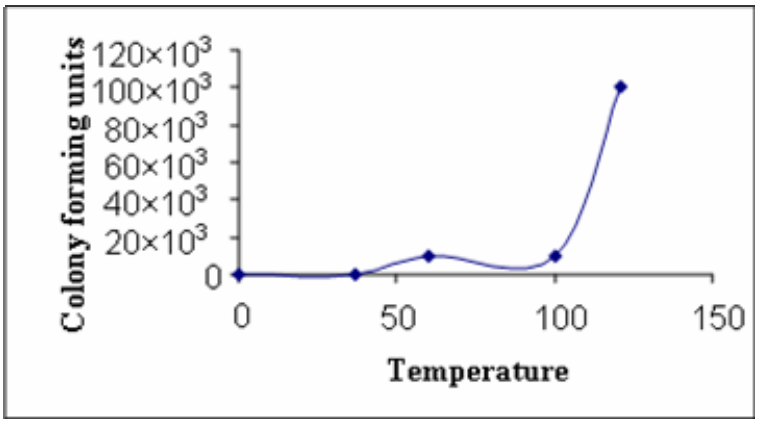


Figure: 4 Microscopy of *Rhizopus solani* at different protein concentrations effecting the growth of *Rhizopus solani*. In this index picture, pictures 4 to 11 are from blank, 12 to 14 from 1st well, 15 to 17 from 3rd , 18 to 21 from 5th , 22,23 from 7th , 24 to 29 from 9th and 30 to 36 from 11th well. Magnification pictures (20 x 10) x.

5 (A)



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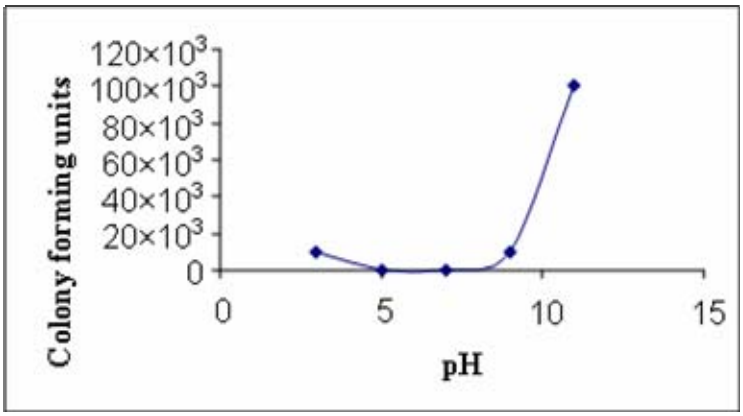

Figure 5: The effect of temperature (A) and pH (B) on activity of crude extract of *Moringa oleifera* tested agaist *Bacillus subtilis.*

Effect of ionic concentration on on activities of seed extracts of *Moringa oleifera*: The ionic (Na^+, K^+, Ca^{2+}, Mg^{2+}) concentration effects resulted in same zone size for all molarities. From these results we may conclude that the ionic concentration have some effect on the plant extracts but the molarity difference may not have much impact or it may be effective on higher or lower dozes than those evaluated in this work.

These ions significantly decreased the inhibitory activity on *Staphlococcus aureus.* This might be due to effect of these ions at coagulation properties of *Moringa oleifera* extracts (Okuda *et al*., 2001), or by stabilization of membrane phospholipid structures (Thevissen *et al*., 1999).

Discussion

Our results demonstrate that the antimicrobial activity of the extracts of *Moringa oleifera* affected predominantly bacterial species. The antimicrobial activity of the crude extract and supernatant might be due to the presence of lipophilic compounds that might bind within or internal to the cytoplasmic membrane (Body & Beveridge, 1979, 1981), and affect the growth of filamentous fungi mainly by causing membrane permeabilization (Huang *et al*., 2000). Same type of results were described by Wong & Ng (2006) in which seed of shelf bean potently suppressed mycelial growth of *Botytis cinerea*, *Fusarium oxysporum* and *Mycosphaerella arachidicola*. The main target might be some important enzymes, bacterial cell wall or membrane (Theis *et al*., 2003). The production of antibiotic metabolites, such as carboxylic acid (Thomasshow & Weller, 1988) and 2, 4 –diacetyl phloroglucinol (Vincent *et al*., 1991) may also be involved in the elimination of fungal pathogens. Some researchers demonstrated that cell wall degrading enzymes and chitinases could be involved in antagonism towards phyto-pathogenic fungi (Budi *et al*., 2000). Therefore, it can be suggested that may be these metabolites had an antagonistic activity in our results. Our extracts worked in doze dependent manner, as the concentration of the extract decreased the activity also decreased, indeed different minimum inhibitory concentrations (MIC) values were observed against different microbial species. This is due to susptibility of the species towards concentration of the extracts, after which this extract damage that species which is not tolerable for it (Ordonez *et al*. 2006).

The results of our work showed that the extract of *Moringa oleifera* was more effective under low temperature, or moderate temperature conditions (4 °C or 37 °C). But at high temperature (70°C or more) the activity was lost, which pointed us that the antibacterial compounds might be some protein which may result in membrane permeabilization resulting from binding of cationic proteins to the negatively charged membrane surface and subsequent pore-formation (Thevissen *et al*., 1996). With increase

Figure: 6 Effect of different pH on activity of extract of *Moringa oleifera* against *Bacillus subtilis.* Streaks 1, 2, 3, 4, 5, 6, (top to bottom) are for blank/without protein extract (at 37 °C, pH 7), and pH 3, 5, 7, 9, 11 respectively

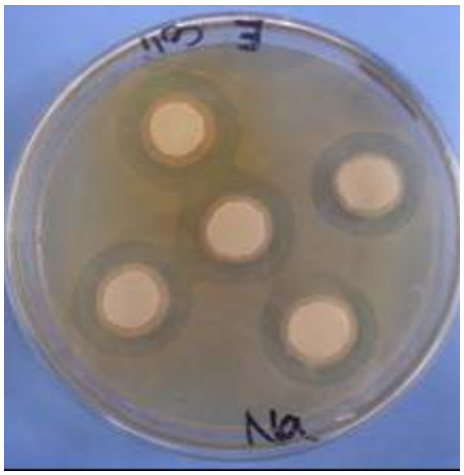

Figure 7: Effect of different ionic concentrations of Na^+ on the antibacterial activity of *Moringa oleifera* extract against *Escherichia coli* by disc diffusion method.

of temperature these proteins might degrade. In the same way the pH suitable of this extract was 7, at which it yielded maximum antimicrobial activity.

Our results also demonstrate that the extracts (proteins/ peptides) were sensitive to different ions and their concentrations. Na^+, K^+, Ca^{2+}, Mg^{2+} decreased the activity of the crude extract of *Moringa oliefera* on *Staphlococcus aureus*. This might be due to effect of these ions at coagulation properties of *Moringa oleifera* extracts (Okuda *et al*., 2001), or by stabilization of membrane phospholipid structures (Thevissen *et al*., 1999). Some researcher found that small amount of mono-and divalent cations (up to 50 m*M*) were shown to severely decrease the potency of antifungal plant proteins, possibly by stabilization of membrane phospholipid structures (Osborn *et al*., 1995; Thevissen *et al*., 1999).

Changes in morphology were observed with microscopic study of the fungi when cultivated in the extract of *Moringa oleifera* containing liquid medium. The affected hyphae swelled and formed very short hyphae with multiple branchs leading to a disordered appearance, demonstrating that the target for extract might be cell wall or cell membrane.

However, the specificity of *Moringa oleifera* extracts towards the species can satisfactorily be explained by the idea of a more specific interaction of extracts with their target organisms, in which ions may also have significant role (De Samblanx *et al*., 1997; Thevissen *et al*., 1997). It was found that the generation of reactive oxygen species could also be the reason of membrane permiabilization which were found interacellularly, they may as oxidative radicals disintegrate the phospholipid residues of membranes by peroxidation (Moore *et al*., 2000).

We may conclude that *Moringa oleifera* extract inhibited the germination of conidia and the growth of germinated hyphae, which implies that conidia, or germinated conidia, and hyphae contained the same target structures necessary for extract (protein) activity. Similar effects were reported for the antifungal protein AFPI from *Streptomyces tendae* and for class of morphogenic plant defensins (Bormann *et al*., 1999). It was demonstrated

that factors that establish apical growth e.g. Ca^{2+} fluxes, cell wall synthesis, the transport of cell wall material-containing vesicles by the cytoskeleton, and actin polymerization, might represent targets for such antimicrobial extracts (Bormann *et al.*, 1999).

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References

Anwar, F. and U. Rashid. 2007. Physico-chemical characteristics of *Moringa Oleifera* seeds and seed oil from a wild provenance of Pakistan *Pak. J. Bot.*, 39(5): 1443-1453.

Bormann, C., D. Baier, I. Horr, C. Raps, J. Berger, G. Jung and H. Schwarz.1999. Characterization of a novel, antifungal, chitin-binding protein from *Streptomyces tendae* Tu901 that interferes with growth polarity. *J. Bacteriol.*, 181: 7421-7429.

Boyd, I. and E.G. Beveridge. 1979. Relationship between the antibacterial activity towards *E. coli* NCTC 5933 and the physicochemical properties of some esters of 3, 4, 5-trihydrobenzoic acid (*gallic acid*). *Microbios*, 24:173-184.

Boyd, I. and E.G. Beveridge. 1981. Antimicrobial activity of some alkyl esters of gallic acid (3, 4, 5-trihydrobenzoic acid) against *E. coli* NCTC 5933 with particular reference to n-propil gallate. *Microbios*, 30:73-85.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Ann. Biochem.*, 72: 248–254.

Budi, S.W., D. van Tuinen, C. Arnould, E. Dumas-Gaudor, V. Gianinazzi-Pearson and S. Gianinazzi. 2000. Hydrolytic enzyme activity of Paenibacillus sp. Strain B2 and effects of the antagonistic bacterium on cell integrity of two soil-borne pathogenic fungi. *App. Soil Ecol.*, 15: 191-199.

De Samblanx, G.W., I.J. Goderis, K. Thevissen, R. Raemaekers, F. Fant, F. Borremans, D.P. Acland, R.W. Osborn, S. Patel and W. Broekaert. 1997. Mutational analysis of a plant defensin from radish (*Raphanus sativus L.*) reveals two adjacent sites important for antifungal activity. *J Biol Chem.*, 272: 1171-1179.

Hancock, R.E. 1997. Peptides antibiotics. *Lancet*, 349: 418-422.

Haung, X., W.J. Xie and Z.Z. Gong. 2000. Characterization and antifungal activity of a chitin binding protein from *Ginkgo biloba. FEBS Lett*., 478: 123-126.

Huynh, Q.K., J.R. Borgmeyer C.E. Smith, L.D. Bell and D.M. Shah. 2001. Isolation and characterization of a 30 kDa protein with antifungal activity from leaves of engelmannia pinnatifida. *J. Bio. Chem*., 316: 723-727.

Jamil, A., M. Shahid, M. M. Khan and M. Ashraf. 2007. Screening of some medicinal plants for isolation of antifungal proteins and peptides. *Pak. J. Bot.*, 39 (1): 211-221.

Katayon, S., M.J. Noor, M. Asma, L.A. Ghani, A.M. Thamer, I. Azni, J. Ahmad, B.C. Khor and A. M. Suleyman. 2005. Effects of storage conditions of *Moringa oleifera* seeds on its performance in coagulation. *Bioresour Technol*., 97(13): 1455-1460.

Kebreab, A.G., K.R. Gunaratna, H. Henriksson, H. Brumer and G. Dalhammar. 2005. A simple purification and activity assay ofthe coagulant protein from Moringa oleifera seed. *Water Res.*, 39: 2338-2344.

Kitajima, Y. 1998. Introduction: electron microscopy for fungal cell ultrastructure. *Nippon Ishinkin Gakkai Zasshi*, 39(3) 121 – 122.

Lockett, C.T., C.C. Calvet and L.E. Grivetti. 2000. Energy and micronutrient composition of dietary and medicinal wild plants consumed during drought. Study of rural Fulani, northeastern Nigeria. *Int. J. Food Sci. Nutr*., 51(3):195-208.

Moore, C.B., N. Sayers, J. Mosquera, J. Slaven and D.W. Denning. 2000. Antifungal drug resistance in *Aspergillus*. *J Infec.*, 41: 203-220.

NCCLS. 2002. Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals. Approved Standard. 19087-1898.

Okuda, T., A. U. Baes, W. Nishijima and M. Okada. 2001. Coagulation mechanism of salt solution-extracted active component in Moringa oleifera seeds. *Water Res*., 35(3):830-4.

Ordonez, R.M., A.A. Ordonez, J.E. Sayago, M.I. Nieva Moreno and M.I. Isla. 2006. Antimicrobial activity of glycosidase inhibitory protein isolated from *Cyphomandra betacea* Sendt. Fruit. *Peptides*, 27:1187-1191

Osborn, R.W., G.W. De Samblanx, K. Thevissen, I. Goderis, S. Torrekens, F. Van Leuven, S. Attenborough, S.B. Rees and W.F. Broekaert. 1995. Isolation and characterization of plant defensin from seeds of *Asteraceae, Fabaceae, Hippocastanaceae* and *Saxifragaceae*. *FEBS Lett*., 368: 257-262.

Theis, T., M. Wedde, V. Meyer and U. Stahl. 2003. Antifungal protein from Aspergillus giganteus causes membrane permeabilization. *Antimicrob. Agents. Therop*., 47:588-593.

Thevissen, K., A. Ghazi, G.W. De Samblanx, C. Brownlee, R.W. Osborn and W.F. Broekaert. 1996. Fungal membrane responses induced by plant defensins and thionins. *J. Biol. Chem*., 271: 15018-15025.

Thevissen, K., F.R. Terras, and W.F. Broekaert. 1999. Permeabilization of fungal membranes by plant defensins inhibits fungal growth. *Appl. Environ. Microbiol*., 65:5451-5458.

Thevissen, K., R.W. Osborn, D.P. Acland. 1997. Specific, high affinity binding sites for an antifungal plant defensin on *Neurospora crassa* hyphae and microsomal membrane. *J. Biol. Chem*. 272: 32176-32181.

Thomashow, L.S and D.M. Weller. 1998. Role of phenazine antibiotic from *Pseudomonas fluorescens* in biological control of *Gaeumannomyces graminis var. tritici*. *J. Bacteriol.*, 170: 3499-3508.

Vincent, M.N., L.A. Harrison, J.M. Brackin, P.A. Kovacevich, P. Mukerji, D.M. Weller and E.A. Pierson. 1991. Genetics analysis of the antifungal activity of a soil-borne *Pseudomonas aureofaciens* strain. *Appl. Environ. Microbiol.*, 57: 2928-2934.

Wang, H. and T.B. Ng. 2005. An antifungal protein from ginger rhizomes. *Biochem. Biophys. Res. Commun.*, 336:100-104.

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