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Secondary Metabolites of *Aureobasidium Pullulans* Isolated from Egyptian Soil and Their Biological Activity

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Abstract: The cell free extract of *Aureobasidium pullulans*, isolated from Wadi Houf soil showed significant antibacterial activity against Gram negative; *Salmonella typhi*, *Proteus vulgaris*, *Escherichia coli*, *Pseudomonas aeurginosa*, *Klebsiella pneumonia* and Gram positive; *Bacillus subtilis*, *Staphylococcus aureus*, and *Sarcina ventricull*. However, the successive extraction of the fungal broth showed that the diethyl ether extract was the best antibacterial activity against *K. pneumonia* and *P. aeruginosa*. Four compounds; 2-propylacrylic acid (1), 8,9- dihydroxy-2-methyl-4H,5H-pyrano[3,2-c]chromon-4-one (2), 2-methylenesuccinic acid (3), hexane-1, 2, 3,5, 6-hexol (4), were isolated from the this extract and identified using ^{1}HNMR, ^{13}CNMR, Heteronuclear multiple bond connectivity (HMBC), Heteronuclear multiple quantum Correlation (HMQC) and EI-MS. The results of the antibacterial activity of the isolated compounds revealed that the activity was attributed to compounds 1 and 3. Side effects on liver and kidney function in addition to histopathology for the ether extract and isolated compounds were carried out; it revealed that there is some side effects for the extracts but no similar effects were shown with the isolated compounds.

Key word: Aureobasidium pullulans, fungal secondary metabolites, biological activity

INTRODUCTION

The fungi are prolific producers of secondary metabolites. Turner [20] determined almost 1000 fungal products but by 1983 this had more than doubled[21] and today it is realistic to anticipate in excess of 6000 different metabolites produced by fungi[25]. However, fungal secondary metabolites have a wide range of chemical structure and biological activities; they are derived from many different intermediates by special enzymatic pathways encoded by specific genus[5].

The production of secondary metabolites in fungi is frequently associated with differentiation and sporulation and is affected by various kinds of environmental stress. Secondary metabolites occur as growth rate declines and during the stationary phase[19,1,12]. The fungal secondary metabolites, or biochemical indicators of fungal development, are of intense interest to humankind due to their pharmaceutical and/or toxic properties[4].

The penitrems, roquefortines, terrestric acids and viridicatols and andrastin A were isolated from Arctic and non-Arctic isolates of *Penicillium crustosum*[17]. However, once its mechanism of action was determined, a search for other naturally occurring HMG-CoA reductase inhibitors led to the discovery of lovastatin, a metabolite of the fungus *Aspergillus terreus*[2] that was first introduced to the market in 1989 followed by pravastatin in 1991. Many analogs, both semi-synthetic and totally synthetic, were later prepared, and from these several have become important drugs, including simvastatin (1991), pravastatin (1991), and atorvastatin (1997). The latter, a chiral totally synthetic compound, has become the drug of choice in this therapeutic category based on its superior ability to reduce cholesterol at low doses.

Sclerotia produced by *Asperigillus* spp. have proven to be a rich source novel antiinsectan compounds with activity against the corn earthworm (*Helicoverpa zea*) and/or the dried fruit beetle (*Carbophilus hemipterus*). Like the aspergilli, molds possessing the *penicillium anamorph* state have historically yielded a wealth of biologically active fungal metabolites[23].

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MATERIALS AND METHODS

Fungal Material: The investigated fungi were obtained from soil samples collected from Wadi-Houf, Cairo, Egypt. The soil samples were collected in clean plastic bags, at the depth of 5-20 cm, from different sites including wadi-bed, terases, and upstream; with various habitats including rocky, clay, and sandy soils. The direct inoculation method was used for sampling and isolation of fungal isolates. For isolation, culturing, maintenance of stock cultures, and experimental studies the following range of media were used: Czapek-Dox Agar (Sucrose, 30 g; Sodium nitrate, 2 g; Potassium hydrogen orthophosphate, 1 g; Potassium chloride, 0.5 g; Magnesium sulphate, 0.5 g; Ferrous sulphate, 0.002 g; Agar, 20 g; Distilled water, 1 L), Malt Extract Agar (Malt extract, 20 g; Peptone, 1 g; Glucose, 20 g; Agar, 20 g; Distilled water, 1 L), Potato Dextrose Agar (Potato extract, 4 g; Glucose, 20 g; Agar, 20 g; Distilled water, 1 L), Yeast Extract Sucrose Agar (Yeast extract, 20 g; Sucrose, 150 g; Agar, 20 g; Distilled water, 1 L). The bacterial test organisms were cultured on nutrient agar (Pepton, 5 g; Beef extract, 3 g; Sodium chloride, 3 g; Agar, 20 g; Distilled water, 1 L). The liquid medium was prepared by using the same ingredients without agar.

Ultrastructural Study: About 5 millimeter squares of agar with fungal material were fixed in 2% (w/v) aqueous osmium tetroxide for 12 hr at 4°C in dark. Fixed materials were washed twice in distilled water and dehydrated through a graded (10% steps) ethanol series from 10% to 90%, then 95%, and finally absolute ethanol. Materials were retained in each concentration for 15 minutes at room temperature. The absolute alcohol was replaced with acetone via a stepwise series (ethanol: acetone 3:1, 1:1, 1:3) and then finally maintained in water-free acetone for 1 hr with 3 changes. Specimens dehydrated to acetone were critical-point-dried using a Polaron E3000 apparatus with liquefied carbon dioxide as the drying agent. Dried materials were mounted on aluminum stubs using double-sided tape, gold-palladium (60:40) coated using a Polaron diode sputtering system (E5000 unit), and examined using the high-vacuum mode of a JEOL JSM-5500LV scanning electron microscope.

Apparatus: For identification of the isolated compounds, melting points were determined on a Kofler hot-stage apparatus and are uncorrected; mass spectra (Electrospray negative ion) sample dissolved in acetonitriale on a Micromass Quattro spectrometer. ^{1}H and ^{13}C NM spectra, using external electronic referencing through the deuterium resonance frequency of the solvent, were determined at 600.17 or 150.91 MHz, respectively with a JEOL ECA 600 spectrometer fitted with an auto 5mm X/H probe. Carbon atom types were established in the ^{13}CNMR spectrum by employing a combination of broad- and proton-decoupled and distortionless enhancement by polarization transfer(DEPT) experiments with 64 K data points over a spectrum width of 17,605.6Hz.[$^{1}J_{C-H}$] and $^{2}J_{C-H}$ and $^{3}J_{C-H}$]. ^{1}H-^{13}C correlations were established by using HMQC and HMBC pulse sequences respectively. ^{1}H-^{1}H correlations were by double quantum filtered COSY.

Extraction and Isolation: The mycelia mat (700 g) was harvested, washed with distilled water, then extracted by refluxing in boiled methanol (2 liter) for 2 hours and then filtered off. The mycelia residue was re-extracted again for three times. The combined filtrates were concentrated under reduced pressure at temperature not exceeding 35˚C. The obtained residue (67 g) was kept for investigation and symbolized as R1. The growth medium (4 L) was extracted by n-butanol (8 L). This step was repeated until complete extraction. The butanol extract was filtered on anhydrous sodium sulphate. The combined filtrates were subjected to concentration using reduced pressure at temperature not exceeding 35˚C. The obtained residue (40 g) was kept for investigation and symbolized as R2. Both R1 and R2 were subjected to Antimicrobial and chromatographic examination. R1 showed better activity higher number of spots.

The obtained residue of mycelia mat extract (60.5 g) was diluted with distilled water (200 ml) and extracted successively by diethyl ether, chloroform, ethyl acetate and n-butanol. Each extract was dried over anhydrous sodium sulphate and concentrated using reduced pressure. The obtained residues were 6, 10, 20 and 35 g, respectively. All fungal extracts (diethyl ether, chloroform, ethyl acetate and n-butanol) were chromatographically investigated on pre-coated silica gel GF plates in (Chloroform- diethyl ether 50:50 v/v) a, (Chloroform- methanol 96:4 v/v) b, and (Ethyl acetate- Methanol- water 30:5:4 v/v/v) solvents systems. Isolation of the active compounds was carried out using ethyl acetate and chloroform extracts because they showed highest number of spots (using system a, b &c) and higher antimicrobial activity. According 20 gram from the combined extracts was applied on silica gel (400 g) column (diameter 5 cm x 120 cm length) and eluted chloroform gradually increased with methanol. Hundred fractions (50 ml each) were collected reduced into 4 mean fractions (according to number, colure and R_f of the spots), each fraction was concentrated under reduced pressure to yield (0.9, 3.0, 5.0 and 1.1 g. respectively). The residue of each fraction group was separately reapplied on Silica gel

columns (30, 60, 100 and 40 g. respectively) and eluted with $CHCl_3$ gradually increased by methanol as from which 6 compounds were isolated in Simi purified condition, therefore they purified by preparative TLC and re-purified by applying on column packed with sephadex LH 20 and eluted with methanol-water 1:1. Crystallization procedure was performed from chloroform to get compounds 1-4.

Prppylacrylic acid 1: (2 g), colorless crystals from methanol, (m.p. 66-68 ˚C) R_f 0.39 in system b.^{1}H-NMR (CD_3OD); d 3.66 ppm (3H, S, H-1),d 5.80 (2H, S, H-3), d 6.43 (2H, S, H-6).^{13}C-NMR(CD_3OD): C1-52.24, C2-171.24, C3-37.13,C4-133.30, C5-130.96, C6-171.58 **dihydroxy-2-methyl-4H,5H-pyrano[3,2-c]chromen-4-one 2:** (500 mg), yellow crystals from methanol, (m.p. 260-262 ˚C) R_f 0.149 in system e. UV kmax in MeOH : (nm) (225, 260, 300,370), NaOAc (220, 260, 300,375) and NaOMe (225,235,440). ^{1}H-NMR (DMSO-D_6): d 2.32(3H, S, CH_3-17), d 4.96 (2H, S, H-14), d 6.13 (1H, S, H-11), d 6.38(1H, S, H-6), d 6.99(1H, S, H-3).^{13}C-NMR(DMSO-D_6): C2-155.33, C3-103.63,C4-150.63, C5-150.37,C6- 103.86, C7-106.16,C8-140.74,C10-164.34, C11-113.21,C12-174.74, c13-110.18, c14-62.24,c17-19.05 EI-MS m /z (% re. int): 247.1(M^+), 245.1(M^-),

Methyenesuccinc Acid 3: (3 mg), colorless crystals from methanol, and chloroform (m.p. 148-150 ˚C), R_f 0.15 in system e.^{1}H-NMR(methanol-d_6): d 3.28 (2H,S, H-3), d 5.72 (2H, d, J= 1.20, H-2), d 6.25(2H, d, J= 1.37,H-2).^{13}C-NMR: 136.45(C-1), 128.83(C-2), 38.37(C-3), 169.62(C-4), 174.80(C-4). EI-MS m /z (% re. int): 130 (M), 129 (M^-)

Hexane-1, 2, 3, 4, 5, 6-hexol 4: (0.3 mg), obtained as light brown crystals from methanol (m.p. 165-166˚C). It showed colorless color under UV light, R_f 0.20 in system e. ^{1}H-NMR (DMSO-D_6): d 3.57 (1H, m, H-2), 3.50(1H, t, J=7.56, 7.56 Hz, H-5), 3.43(1H, m, H-7), 3.35(2H, m, H-3). ^{13}C-NMR (DMSO-D_6); 70.26(C-2), 64.37(C-3), 71.88(C-5), 71.88(C-7), 70.26(C-9), 64.37(C-17).EI-MS m /z (% re. int): 182.2(M^+) 181.0 (M-1).

Biological Activities:

1) Antimicrobial Activity: Representatives of Gram-negative bacteria; namely, *Salmonella typhi*, *Proteus vulgaris*, *Escherichia coli*, *Pseudomonas aeurginosa*, *Klebsiella pneumonia* and Gram- positive bacteria; namely, *Bacillus subtilis*, *Staphylococcus aureus*, *Sarcina ventricull* and unicellular fungi; namely, *Candida albicans*, and filamentous fungi; namely, *Aspergillus niger* and *A. ochraceous* were used as test organisms.

The filter paper disc method[14] was used for the antimicrobial screening of the investigated isolates. The fungal mat and broth filtrate were extracted as before, left to evaporate till dryness, and then dissolved in 3 ml distilled water. The disc diffusion method was employed for the determination of antimicrobial activities of both intra- and extra-cellular extracts of the investigated fungal isolates. A standard blank paper discs (12.7 mm in diameter) were separately soaked in the extracts and transferred onto the surface of growth media seeded with the test organism. After incubation period, following suitable conditions for the test organism, the diameter of the inhibition zone around the discs was measured in millimeters. The agar-well diffusion method was used for determination of the minimum inhibitory concentration (MIC). The test organisms were separately seeded in the agar medium. The wells (10 mm in diameter) were cut from the agar and 0.1 of extract solution (different concentrations) was transferred into them. After incubation, the plates were examined and the inhibition zones were determined.

2) Pharmacological Studies:

Toxicological study: LD_{50} of the tested extract was determined in mice as described by Kerber[9].

Infection and Treatment:

a) Preparation of bacterial inoculum: Two different strains of bacteria (*Klebsiella pneumoniae*, and *Pseudomonas aeruoginosa*) were prepared after an incubation period (18 h) on nutrient agar medium at 37˚C and diluted in PBS buffer. The number of viable organism/ml was determined by the plate count method [7]. Opacity tubes: McFarland No. 2 barium sulphate tubes were prepared as follows: Two-tenth ml of 1% barium chloride solution was mixed with 9.8 ml of 1% sulfuric acid in a test tube and the mixture was shaken well before use for matching. The tube was sealed and wrapped in aluminum foil (darkness) and stored for use to standardize bacterial inoculum in sensitivity testing. Fresh tubes were prepared every six months[7] this opacity tube corresponds to 6 × 108/ml.

McFarland No. 3 barium sulphate tubes were prepared as follows: Three-tenth ml of 1% barium chloride solution was mixed with 9.7 ml. of 1% sulfuric acid in a test tube and the mixture was shaken well before use for matching. The tube was sealed and wrapped in aluminum foil (darkness) and stored for use to standardize bacterial inoculum in sensitivity testing. Fresh tubes were prepared every six months[7] this opacity tube corresponds to 9 × 108/ml.

b) Experimental Infection of Mice: Pathogen-free female albino mice aged 6 weeks and weighing 25 ±2

g was used. They were classified into groups each of five animals. Mice were kept in cages with free access to sterile food and water in a biosafety containment facility. The inoculum of 2 × 103 CFU per mouse for *Klebsiella pneumonia*[8] and 7.9 × 104 per mouse for *Pseudomonas aeruginosa* were prepared[18]. Inoculum of *Klebsiella pneumonia* was given intranasally to anaesthetized mice by short exposure to ether, ethanol, and chloroform; (3: 2: 1), in a dose of 50 µl/animal[8], while the inoculum of *Pseudomonas aeruginosa* was given intrapretonialy to mice in a dose of 0.2 ml/animal[18].

c) Treatment: The isolated compounds, C1 and C3, were suspended in sterile normal saline (0.9 % NaCl) with the aid of few drops of Tween 80 immediately before use. About 1% of each compound, C1 and C3, were prepared. A suspension of total extract was administrated to rats by intrapretonial at a dose of 0.187 ml / rat. The compounds C1 and C3 were separately given to rats by intrapretonial injection in a dose of 0.75 ml/mouse/day each of which is alone. Four groups, each of five rats (120 – 150 g), were used. The first group was used as a control. The 2nd, 3rd and 4th groups were intrapretonialy injected with the fungal extract (125), C1 (50) and C3 (50) mg/kg, respectively, and blood samples were collected from the orbital plexus of each rat, 7 days post-treatment. Serum was separated from each sample.

Effect on liver and kidney:

a) Liver and kidney: The effect of extract and isolated compounds on serum activities and levels of urea according to the method described by [3], creatinine, The serum transaminases (ALT and AST) were carried out according to the method described by Reitman and Frankel [15]. The results obtained were statistically analysed by using of "t" test method explained by Snedecor and Cochran[16].

b) Histopathological Investigation: The histopathological changes of the liver, kidney, and lung of the infected mice that medicated with the fungal extract or gentamicine for 5 days were investigated. For this purpose, 195 mice were divided into thirteen equal groups (15 animals each). They were kept in cages with free access to sterile food and water in a biosafety containment facility. They were divided into the following groups: (1) Control without any medication, (2) Given fungal suspension only, (3) Given gentamicin only, (4) Given *Klebsiella pneumonia* only, (5) Given fungal suspension followed by *Klebsiella pneumonia*, (6) Given *Klebsiella pneumonia* followed by fungal suspension, (7) Given *Klebsiella pneumonia* with fungal suspension, (8) Given *Klebsiella pneumonia* followed by gentamicin, (9) Given *Pseudomonas aeruginosa* only, (10) Given fungal suspension followed by *Pseudomonas aeruginosa*, (11) Given *Pseudomonas aeruginosa* followed by fungal suspension, (12) Given *Pseudomonas aeruginosa* with fungal suspension, (13) Given *Pseudomonas aeruginosa* followed by gentamicin.

Fungal suspension and gentamicine sulphate were given to mice along five days by intrapretonial injection in a dose of 3.125 and 0.75 ml/mouse/day, respectively. - Five mice from each group were killed (cervical dislocation) one, two, and five days post treatment. Tissue samples of the lung, kidney, and liver were removed using forceps and scissors (flaming) from each mouse in 10% formalin solution for histopathological examination.

Samples were fixed in neutral formation for 24 hour. Then they were washed by running water over night. The washed samples were dehydrated by using graded increased concentration of ethyl alcohol starting with 80% and ending with absolute alcohol. The dehydrated samples were immersed in xylol for 4-6 hours (till clearance). Then sample was placed in a crucible containing soft paraffin and kept in an oven at 56°C for 8-12 hours. Samples were blocked in hard paraffin and cut into sections of about 5 micron in thickness.

Paraffin was removed from the sections by two changes, 5 minutes each, in xylol. Xylol was removed by two changes of absolute alcohol (5 minutes in each) which was removed by washing with tap water. Sections were stained with hematoxylin for 5-10 min[11,6]. Then sections were stained with eosin for 1-2 min. Sections were dehydrated with two changes of absolute alcohol, 5 minutes each, then cleared with xylol (2 changes, 5 minutes each). Sections were mounted with Canada balsam and covered with cover slides to be ready for microscopic examination.

RESULTS AND DISSECTIONS

Antimicrobial Screening of Fungal Isolates: Screening of the antimicrobial activities of twelve fungal strains, isolated from soil samples, collected from different localities in Wadi-Houf, showed variable effects (Tables 1, 2). All showed no activity against the all tested fungi. However, there was a significance activity against Gram-negative and Gram-positive bacteria. On the other hand, the extract of fungal isolate symbolized WH9 was the only extract that showed an activity against all the tested Gram-negative and Gram-positive bacteria. The highest activity of WH9 was determined against *Pseudomonas aeurginosa* and *Klebsiella pneumonia*. The minimum inhibitory concentration (MIC) of WH9 total alcohol extract

against *Klebsiella pneumonia* and *Pseudomonas aeurginosa* was 12.5 and 6.25 mg/ml, respectively (Table 3).

Identification of the Fungal Isolate (WH9): The isolated fungus was cultivated on malt agar and incubated for 7 days at 28±2°C. Colonies grew moderately well, attained 3-4 cm diameter. Colonies were yellow in colour, composed of low, loose mat of hyphae. The reverse of the colonies was yellow-brown to reddish-brown.

The microscopic features were observed using booth light and scanning electron microscopy. The vegetative hyphae were erect, branched, hyaline, 2–12 mm in diameter, and septate. The septum was of the simple type that distinguishing the Ascomycetes. The conidia were of blastic type, produced in dense groups from undistinguished conidiogenous cells in tufts and all over the hyphae. The conidia were single-celled, oval to spherical in shape, 3-4 X 5-7 mm in diameter (Fig. 1).

Terminal and intercalary, 8–10 x 14-16 mm in diameter chlamydoconidia and secondary blastoconidia, varied in diameters, were produced. The main descriptive features of the isolate WH9 indicated that the isolate belongs to the genus *Auerobasidium*. However, the specific description and diameter of the diagnostic features of the isolate matched the diagnostic features of species *pullulans*.

Chemistry Results: Spectrum of compound 1. From ^{13}C-NMR it could be concluded that the compound contains 6 carbon atoms. DEPT 135 experiment exhibited one tertiary carbon, two secondary and the rest (three) are quaternary carbons. HMQC showed; 2H at d 3.31 ppm protons linked to the carbon(3) at 37.13, 3H at d 3.66 protons cross peak with carbon (1) at 52.24, H at d 5.80 proton linked to the carbon (5) at 130.96, H at d 6.43 proton cross peak with carbon (5) at 130.96. HMBC showed; 2H at d 3.31 ppm coupled to the carbons (3,5,4,2,6) at (37.13, 130.96, 133.30, 171.24, 171.58), 3H at d 3.66 coupled to carbons (1,2,6) at (52.24, 171.24, 171.58), H at d 5.80 coupled to the carbons (3,5,6) at (37.13, 130.96, 171.24), H at d 6.43 coupled to carbons (3,5,4,2) at (37.13, 130.96, 133.30, 171.58) (Fig. 2).

^{13}C-NMR of Compound 2 showed 13 carbon atoms, three primary, one secondary, one tertiary, and eight quaternary carbons, this was confirmed from dept experiments at 135 and 90. In HMQC spectrum their was 3H at d 2.31 ppm protons linked to the carbon 17 at 19.05, 2H at d 4.96 protons cross peaked with carbon 14 at 62.24, H at d 6.13 proton linked to the carbon 11 at 113.21, H at d 6.38 proton cross peaked with carbon 6 at 103.86, H at d 6.99 proton cross peaked with carbon 3 at 108.63. HMBC confirmed that 3H-17 at d 2.31 ppm long rang coupled with carbons (17, 11, and 10) at 19.05, 113.21, and 164.34. 2H-14 at d 4.96 long rang coupled with carbons (14, 13, 4, 5, 2, and 12) at 62.24, 110.18, 150.37, 150.63, 155.33, and 174.74. H-11 at d 6.13 long rang coupled with carbons (17, 13, and 10) at 19.05, 110.18, and 164.34. H-6 at d 6.38 long rang coupled with carbons (6, 7, 8, 4, 5 and 2) at 103.86, 106.16, 140.74, 150.37, 150.63, and 155.33. H-3 at d 6.99 long rang coupled with carbons (3, 8, 4, 5, and 2) at 105.63, 140.74, 150.37, 150.63, and 155.33 (Fig. 2).

Compound 3; ^{13}C-NMR spectrum of compound MS5 is carried out in methanol-D and data was listed in table (103). From ^{13}C-NMR it could be concluded that the compound contains 5 carbon atoms, two of which is secondary (one olefenic and one metheylinic), and the rest (three) are quaternary carbons. Dept 135 exhibited 2(CH_2). HMQC showed 2H at d 3.28 ppm protons linked to the carbon 3 at 38.37, H at d 5.72 protons cross peaked with carbon 2 at 128.83, H at d6.25 protons linked to the carbon 2 at 128.83. HMBC showed 2H at d 3.28 ppm coupled with carbons (3,2,1,4,5) at (38.37, 128.83, 136.45, 169.62, 174.80), H at d 5.72 coupled with carbons (3,2,1,4,5)at (38.37, 128.83, 136.45, 169.62 and174.80), H at d 6.25 proton long rang coupled with carbons (3,2,1,4,5)at (38.37, 128.83, 136.45, 169.62, 174.80) (Fig. 2).

^{13}C-NMR of Compound 4 revealed the present 6 carbon atoms, four of which are primary, and the rest (two) are secondary carbons, this was confirmed that from dept 135 and 90 experiments. HMQC showed: H at d 3.57 ppm proton linked to the carbon at 64.37, H at d 3.35 proton cross peaked with carbon at 64.37, H at d 3.50 proton linked to the carbon at 70.27, H at d 3.43 proton cross peaked with carbon at 71.88. Signals at d 4.07, 4.26 and 4.34 are proposed to be hydroxyl groups. HMBC showed: H at d 3.35 ppm long rang coupled with carbons at (64.37,70. 27, 71.88), H at d 3.43 long rang coupled with carbons at (64.37,70.27 ,71.88), H at d 3.50 long rang coupled with carbons at (64.37,70.27, 71.88), H at d 3.57 long rang coupled with carbons at (64.37,70.27 ,71.88), H at d 4.07 long rang coupled with carbons at (64.37,70.27,71.88), H at d 4.26 long rang coupled with carbons at (64. 37, 70. 27, 71.88), H at d 4.34 long rang coupled with carbons at (64. 37, 70.27, 71.88). DQF-COSY showed: H at d 3.57 ppm correlated to protons at (3.43,3.35), H at d 3.50 correlated to proton at (3.43), H at d 3.43 correlated to protons at (3.50,3.57,3.35), H at d 3.35 correlated to protons at (3.43,3.57) (Fig. 2).

Biological results:

Antimicrobial activity: The successive extracts of fungal showed that both petroleum ether and n-butanol

have activities, while diethyl ether extract gave the highest effect (23 & 21 mm against *K. pneumonia* and *P. aeruginosa* respectively) followed chloroform (17 & 18 mm) an finally ethyl acetate (16 & 15). The antibacterial activity of the collective fractions was tested against *K. pneumonia* and *P. aeurginosa*. The obtained results revealed that the fraction I has no activity against the two tested organisms. Fraction II showed the highest activity (35 & 28 mm against *Klebsiella pneumonia& Pseudomonas aeurginosa* respectively), Fraction III (21 & 25 mm) and Fraction IV (15 &17). The isolated compounds 1 and 3 showed activity against the two tested microorganisms while no activity were shown with compounds 2 and 4. The minimum inhibitory concentration of compounds 1 and 3 against *K. pneumonia* and *P. aeurginosa* were determined (Table 3).

Determination of LD_{50}: The total alcohol extract failed to kill mice within 24 h in doses up to 4 g/kg. This indicated that the extract is safe for human use (LD_{50} is very high). In this respect, Van den Heuvel *et al.*, (1990) recorded that substance with LD_{50} more than 2000 mg/kg are considered non-toxic.

Effect on Liver and Kidney Functions: The effect of I/P injection of the fungal extract and the isolated compounds to the rats for 7 successive days on the serum activity of ALT, AST, creatinine and urea was recorded in tables (4 and 5). The results revealed that all activities were increased with administration of total extract only but no similar activity was shown with the isolated compounds.

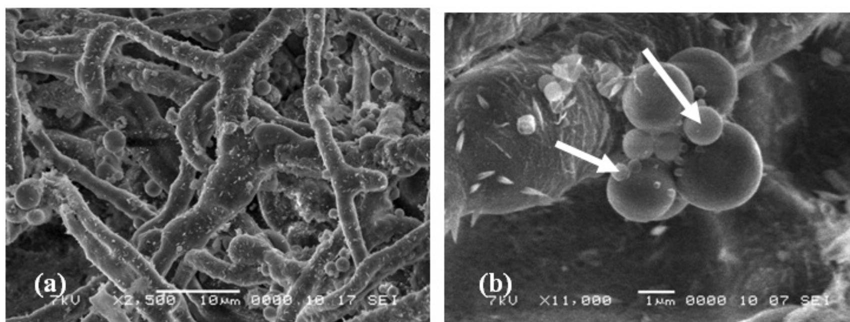

Fig. 1: The blastoconidia all over the hyphae (a) and in tuft (b). Note the secondary blastoconidia (arrowed).

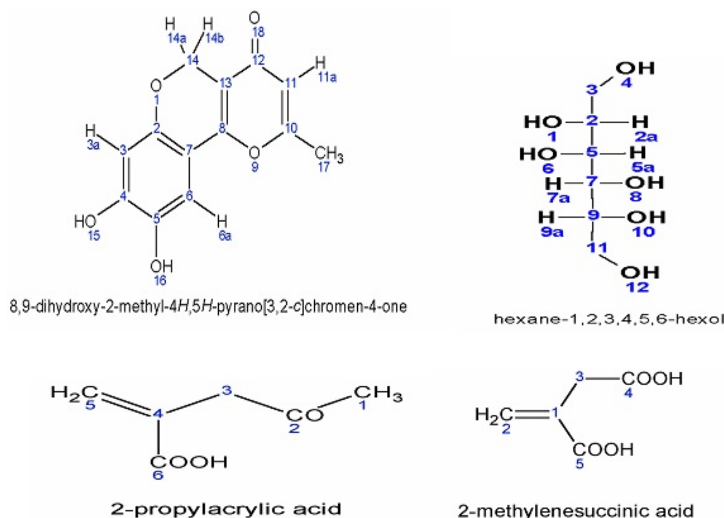

Fig. 2: The structures of compounds (1 - 4) isolated from Aureobasidium pullulans

Table 1: Antimicrobial activity of the fungal isolates grown on malt extract broth medium.

Fungal	Extract	Diameter of inhibition zone (mm)										
		Sal. typ.	*Pr.vul.*	*E.coli*	*Ps. aeu.*	*Kl. pn.*	*Bac.sub.*	*Sta. aur.*	*Sar. ven.*	*Cand. alb.*	*Asp. nig.*	*Asp. och.*
WH1	Int.	00	00	00	00	00	00	00	00	00	00	00
	Ext.	00	00	00	23	19	16	17	16	00	00	00
WH2	Int.	00	17	18	00	00	00	00	00	00	00	00
	Ext.	17	18	21	00	26	00	00	26	00	00	00
WH3	Int.	00	00	00	00	00	00	00	00	00	00	00
	Ext.	00	17	16	16	28	00	00	19	00	00	00
WH4	Int.	00	00	00	00	00	16	15	17	00	00	00
	Ext.	00	00	00	00	00	16	25	21	00	00	00
WH5	Int.	16	14	00	15	16	17	00	16	00	00	00
	Ext.	16	16	00	17	18	18	14	20	00	00	00
WH6	Int.	00	00	00	00	00	15	18	00	00	00	00
	Ext.	00	00	00	00	00	17	00	25	00	00	00
WH7	Int.	00	00	00	00	00	00	00	00	00	00	00
	Ext.	00	00	16	00	00	16	17	23	00	00	00
WH8	Int.	00	00	00	00	00	19	18	00	00	00	00
	Ext.	00	00	00	00	00	19	25	23	00	00	00
WH9	Int.	15	00	00	21	19	15	19	17	00	00	00
	Ext.	23	23	17	31	27	19	23	18	00	00	00
WH10	Int.	19	00	00	27	25	19	25	18	00	00	00
	Ext.	19	00	00	25	25	19	23	19	00	00	00
WH11	Int.	00	00	00	19	19	14	19	16	00	00	00
	Ext.	00	00	00	19	18	14	17	17	00	00	00
WH12	Int.	00	00	00	00	00	00	17	19	00	00	00
	Ext.	00	00	00	00	00	00	15	16	00	00	00

Sal. typ., *Salmonella typhi*; *Pr. vul.*, *Proteus vulgaris*; *E. coli*, *Escherichia coli*; *Ps. aeu.*, *Pseudomonas aeurginosa*; *Kl. pn.*, *Klebsiella pneumonia*; *Bac. sub.*, *Bacillus subtilis*; *Sta. aur.*, *Staphylococcus aureus*; *Sar. ven.*, *Sarcina ventricull*; *Cand. alb.*, *Candida albicans*; *Asp. nig.*, Aspergillus niger; *Asp. och.*, *Aspergillus ochraceous*; Int., internal secondary metabolites; Ext., External secondary metabolites.

Table 2: Antimicrobial activity of the fungal isolates grown on yeast extract sucrose medium.

Fungal	Extract	Diameter of inhibition zone (mm)										
		Sal. typhi	*Prot.vulg.*	*E.coli*	*Ps. aeu.*	*Kl. pneu.*	*Bac.sub.*	*Staph. aureus*	*Sar. vent.*	*Cand. alb.*	*Asp. niger*	*Asp. ochr.*
WH1	Int.	00	00	00	00	00	00	00	00	00	00	00
	Ext.	15	00	00	19	00	16	18	17	00	00	00
WH2	Int.	00	00	00	00	00	00	00	00	00	00	00
	Ext.	00	00	17	00	19	00	00	23	00	00	00
WH3	Int.	00	00	00	00	00	00	00	00	00	00	00
	Ext.	00	00	00	14	24	00	00	17	00	00	00

Table 2: Continue

WH4	Int.	00	00	00	00	00	17	00	15	00	00	00
	Ext.	00	00	00	00	00	19	17	23	00	00	00
WH5	Int.	00	00	00	00	00	17	00	18	00	00	00
	Ext.	00	00	00	00	15	19	00	18	00	00	00
WH6	Int.	00	00	00	00	00	00	00	17	00	00	00
	Ext.	00	00	00	00	00	17	21	27	00	00	00
WH7	Int.	00	00	00	00	00	15	16	16	00	00	00
	Ext.	00	00	00	00	00	18	21	23	00	00	00
WH8	Int.	00	00	00	00	00	17	00	00	00	00	00
	Ext.	00	00	00	00	00	19	27	24	00	00	00
WH9	Int.	00	00	00	17	17	00	15	17	00	00	00
	Ext.	18	21	00	26	23	16	23	18	00	00	00
WH10	Int.	00	00	00	17	18	19	21	00	00	00	00
	Ext.	00	00	00	21	21	17	23	21	00	00	00
WH11	Int.	00	00	00	00	00	00	16	16	00	00	00
	Ext.	00	00	00	17	20	17	18	19	00	00	00
WH12	Int.	00	16	19	00	00	17	19	23	00	00	00
	Ext.	00	00	18	15	00	21	22	17	00	00	00

Sal. typ., *Salmonella typhi*; *Pr. vul.*, *Proteus vulgaris*; *E. coli*, *Escherichia coli*; *Ps. aeu.*, *Pseudomonas aeurginosa*; *Kl. pn.*, *Klebsiella pneumonia*; *Bac. sub.*, *Bacillus subtilis*; *Sta. aur.*, *Staphylococcus aureus*; *Sar. ven.*, *Sarcina ventricull*; *Cand. alb.*, *Candida albicans*; *Asp. nig.*, Aspergillus niger; *Asp. och.*, *Aspergillus ochraceous*; Int., internal secondary metabolites; Ext., External secondary metabolites.

Table 3: Minimum inhibition concentration of the fungal isolate (WH9) alcohol extract.

Concentration (mg/ml)	Diameter of inhibition zone (mm)	
	Klebsiella pneumonia	*Pseudomonas aeurginosa*
100.00	26	27
50.00	23	24
25.00	18	20
12.50	16	17
06.25	00	15
3.125	00	00
1.562	00	00

Table 3: The minimum inhibitory concentration of compounds C1 and C3.

Compound concentration (mg/ml)		Diameter of inhibition zone (mm)	
		Klebsiella pneumonia	*Pseudomonas aeurginosa*
C1	10.00	28	25
	5.00	21	19
	2.50	16	15
	0.125	-ve	-ve

Table 3: Continue

C3	10.00	19	23
	5.00	15	19
	2.50	-ve	16
	0.125	-ve	-ve

Table 4: Effect of I/P injection of total extract, C1 and C3 for 7 successive days on the serum activities of ALT and AST in rats (M+ SE, n=5).

Groups	Dose (mg/kg)	ALT (U/l)	AST (U/l)
Control	0	3.66 + 0.30	8.96 + 0.77
Fungal extract	125	3.73 + 0.07	15.04*** + 0.64
C1	50	5.19 +1.33	9.92+ 1.05
C3	50	4.40 + 0.27	5.85 + 0.39

Significance: * P < 0.05, ** P < 0.01, *** P < 0.00.

Table 5: Effect of I/P injection of total extract, C1 and C3 for 7 successive days on the level of urea and creatinine in serum of rats (M+ SE, n=5).

Groups	Dose (mg/kg)	Blood Urea (mg/dl)	Creatinine (mg/dl)
Control	0	20.60 + 0.75	0.41 + 0.07
Fungal extract	125	29.80*** + 1.16	0.82*** + 0.07
C1	50	21.80 + 1.69	0.45 + 0.04
C3	50	22.20 + 1.16	0.45 + 0.02

Significance: * P < 0.05, ** P < 0.01, *** P < 0.00.

Histopathological Findings: in liver and kidney showed that their were some alteration in hepatocytes parenchyma and focal aggregation of mononuclear cells in addition to sub capsular hemorrhage, these effect occurred with the total extract only but no effect for the isolated compounds

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