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ANTI-BACTERIAL ACTIVITIES OF *VOLVARIELLA VOLVACEA*

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Abstract: Extracts of twenty- one basidiomycete fungi were tested against the bacteria, *Staphylococcus aureus*, *Streptococcus*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Klebsiella*. Among them *Volvariella volvacea* showed promising activity against *E. coli*. Ethyl acetate extract of *Volvariella volvacea* was fractionated to hexane and purified on normal phase silica gel column to obtain ergosterol peroxide which showed comparable activity against *E. coli* as that of the crude extract.

Key words: Basidiomycete, ergosterol peroxide, *Escherichia coli*, mushroom, *Volvariella volvacea*.

INTRODUCTION

Fungi belonging to the subdivision Basidiomycotina possess a great variety of fruit bodies having different shapes and colours.[1] Some of these basidiomycete fungi have been shown to have anti-bacterial, anti-fungal, anti-viral, anti-tumour, hypercholesterolaemic and hyperglycemic activities.[2,3,4,5,6,7] Work has also shown that gram-positive bacteria are more sensitive to the basidiomycete fungi than gram-negative species.[8] Another important observation is that *Micrococcus pyogenes*, a penicillin resistant bacterium, is sensitive to the inhibitory action of a large number of basidiomycetes.[8]

A number of anti-microbial compounds have been isolated from basidiomycete fungi.[9a] They include illudin, collybial, melleolide B, 5-methoxy-p-toluquinone, linoleic acid, pleurotin, crinipellin A, drosophilin A and frustulosin all of which are antibacterial compounds. Furthermore about sixteen antifungal compounds have been isolated from basidiomycetes.[9] Thus basidiomycetes appear to be a good source for new anti-microbial compounds.

Several basidiomycete fungi isolated from different localities in Sri Lanka were screened for anti-bacterial activity and among them *Volvariella volvacea* showed promising activity against *E. coli*. *V. volvacea* is an edible mushroom, belonging to the order Agaricales. The vitamin and mineral content of *V. volvacea* is comparable to other edible mushrooms.[10] Bioassay guided fractionation was done to isolate and characterize the biologically active compounds from *V. volvacea*.

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METHODS AND MATERIALS

Organisms: Fruiting bodies (21) of basidiomycetes were collected from different localities in Sri Lanka. The fungi were identified using the characters of fruiting bodies.[11] Some were identified upto generic level while a few were identified only upto the order level.

Screening of basidiomycete specimens for antibacterial activity: Five grams of each fruit body (fresh weight) was crushed and soaked in 50% methanol (MeOH) in methylene chloride (CH_2Cl_2) for 3 days at room temperature. Thereafter the solvent was filtered and rotary-evaporated under vacuum. Stock solutions (10000 ppm in acetone) were prepared using these extracts.

The bacteria *Staphylococcus aureus* (NCTC 8532), *Streptococcus, Pseudomonas aeruginosa* (NCTC 10662), *E.coli* (NCTC 10148) and *Klebsiella* were inoculated to separate nutrient broths and incubated at 37^{0}C for 24 hours. Thereafter, broth of the test bacterium (0.1 ml).was evenly spread on a nutrient agar plate under sterile conditions.

The required stock solution (20μl) was absorbed onto sterile filter paper discs (7 mm) and allowed to dry for a few minutes in a sterile Petri dish. Each disc was placed at the center of a nutrient agar plate, which was earlier inoculated with the appropriate bacterium. Filter paper discs having 20μl of acetone were used as the control. The Petri dishes were incubated at 37^{0}C. After 24 hours the diameter of any clear inhibition zone around the discs was measured. All experiments were triplicated.

Antibacterial activity of *Volvariella volvacea:* Fruiting bodies of *V. volvacea* were collected from the Export Development Board Research Center, Ratmalana, Sri Lanka.

V. volvacea (900 g) was crushed and soaked in ethyl acetate (EtOAc) for five days at room temperature. Thereafter, the solvent was filtered and the filtrate evaporated to dryness in a rotavapor to obtain 30 g of EtOAc extract. The EtOAc extract was re-extracted to hexane (50 mg) and purified on a normal phase silica gel column packed in hexane and eluted with a gradient of hexane and EtOAc to obtain 10ml fractions which were numbered from 1- 50.

Crystalline 46th fraction, which is active against *E.coli* (15 mg), eluted with 20% EtOAc in hexane was purified on a normal phase preparative silica gel TLC using 33% hexane in EtOAc as the solvent. Band at R_f = 0.3 was extracted to CH_2Cl_2,dichloro methane. Evaporation of CH_2Cl_2 gave a crystalline compound.

The residue was soaked in methyl alcohol (MeOH) for five days, filtered and rotary-evaporated to obtain 80g of MeOH extract. The MeOH extract was reextracted into hexane to remove the non-polar compounds. The remaining residue (79.6 g) was fractionated by passing through a column of Sephadex, and eluted with MeOH.

One fraction active against *E. coli* was chromatographed over a column of reverse phase (C_{18}) silica gel packed in acetonitrile and eluted with acetonitrile, acetone, MeOH, H_2O gradient.

To facilitate the characterization, fractions were acetylated with 1ml pyridine and 1 ml acetic anhydride for 24 hours. Acetylated reaction mixture was dried by nitrogen flushing. The dried reaction mixture was dissolved in CH_2Cl_2 and concentrated. The concentrated CH_2Cl_2 extract (135mg) was chromatographed over a normal phase silica gel column packed in CH_2Cl_2 and eluted with CH_2Cl_2, EtOAc solvent gradient.

Compounds 2, 3, 4, 5, 6 were isolated and characterized using spectroscopic data.

RESULTS

Antibacterial activity of the basidiomycete extracts:

The first four specimens in figure 1 belongs to the order, Tullasnellales, the next 10 specimens belongs to the order Agaricales and the last 7 specimens belongs to the order Aphyllophorales.

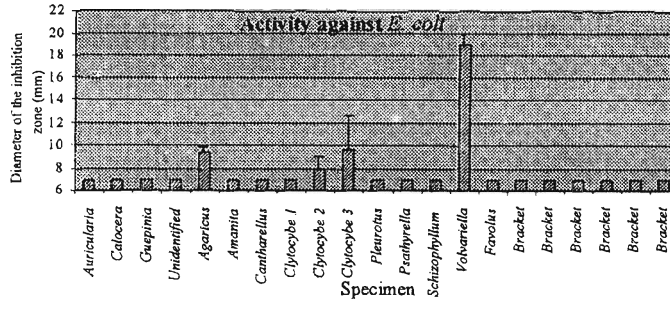


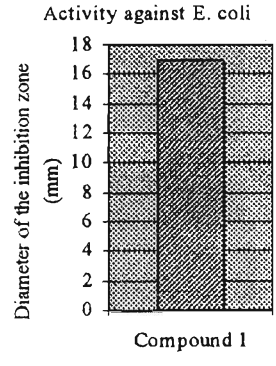
Figure 1: Antibacterial activity of the collected basidiomycetes: specimen (along x axis) against the diameter of inhibition zone (mm). Bars denote standard deviation.

The activities of all 21 specimens against *Klebsiella*, *Pseudomonas*, *Staphylococcus*, and *Streptococcus* are reported elsewhere.[12]

The extract of *V. volvacea* was the most active extract against *Escherichia coli*. Extract of *Agaricus* and *Schizophyllum* possessed significant antibacterial activity against *Klebsiella*. *Pseudomonas aeruginosa* was not significantly inhibited by any of the fungal extracts. *Staphylococcus aureus* was moderately inhibited by the extracts of *Cantharellus*, *Clytocybe* and two bracket fungi. *Streptococcus* was sensitive to the extracts of *Amanita*.

Antibacterial activity of *V. volvacea*

Bio-assay guided fractionation of the ethyl acetate extract of *V. volvacea* resulted in the isolation of compound 1, which showed promising activity against *E.coli*.



Compound 1: ^{1}H NMR (CDCl3, 200 MHz) δ 6.51 (1H, d, J = 8.6 Hz, H-6), 6.25 (1H, d, J =8.6 Hz, H-7), 5.25 (1H, dd, J = 6.8, 15.3 Hz, H-23), 5.13 (1H, dd, J = 6.6, 15.3 Hz, H-22), 3.96 (1H, h, J = 5.4 Hz, H-3), 2.19-1.10 (21H, m), 0.99 (3H, d, J = 6.6 Hz, H-21), 0.90 (3H, d, J = 7.9 Hz, H-28), 0.88 (3H,s, H-19), 0.84 (3H,d, J = 3.2 Hz, H-26/27), 0.81 (3H, s, H-18), 0.80 (3H, d, J = 3.3 Hz, H-26/27) ^{13}C NMR (CDCl3, 200 MHz) δ 135.4 (C-7), 135.2 (C-22), 132.3 (C-23), 130.7 (C-6), 82.1 (C-5), 79.4 (C-8), 66.5 (C-3), 56.2 (C-17), 51.7 (C-14), 51.1 (C-9), 44.5 (C-13), 42.8 (C-24), 39.7 (C-20), 39.3 (C-12), 36.9 (C-4, C-10), 34.7 (C-1), 33.0 (C-25), 30.1 (C-2), 28.6 (C-16), 23.4 (C-15), 20.9 (C-21), 20.6 (C-11), 19.9 (C-27), 19.6 (C-26), 18.2 (C-19), 17.5 (C-28), 12.8 (C-18) HRMS (FAB, thioglycerol + Na) m/z (calcd for $C_{28}H_{44}O_3$, 428.6471; observed 428.3290).

Compound 1 was identified as ergosterol peroxide and the assignments were made using ^{1}H NMR, ^{13}C NMR (BB, DEPT), COSY, Hetcorr and HMQC.

Compound 2 was identified as a mixture of α and β -1, 2, 3, 4, 6 - pentaacetyl glucose. ^{13}C NMR data of the **compound 2** and the known α and β-glucosepentaacetates were compared[13] and the data found to have good correlation.

Compound 3 :^{1}H NMR ($CDCl_3$) 5.50 (1H, dd, J = 9.5, 9.9 Hz, H-3), 5.29 (1H, d, J = 4.0 Hz, H-1), 5.05 (2H, m, H-2, H-4), 4.24 (2H, m, H-6', OH), 4.04 (2H, m, H-5, H-6), 2.09 (3H, s, OAc), 2.08 (3H, s, OAc), 2.06 (3H, s, OAc), 2.04 (3H, s, OAc); ^{13}C NMR ($CDCl_3$) δ 170.6 (OAc), 169.9 (OAc), 169.6 (2 OAc), 92.2 (C-1), 69.9 (C-2, C-3), 68.4 (C-4), 68.2 (C-5), 61.7 (C-6), 20.6 (OAc); CI-MS m/z 331 (M^+-OH), 289 (M^+-OH - Ac), 271, 229; HRMS (FAB, positive, m/z) calcd, for $C_{28}H_{39}O_{19}$ (M+1) 679.2086, found 679.2092
The **compound 3** was identified as octaacetyl trehalose.

Compound 4: ^{1}H NMR ($CDCl_3$) δ 8.88 (1H, s, broad, NH), 7.39 (1H, d, J = 8.2 Hz, H-6), 6.04 (1H, dd, J = 4.9, 2.0 Hz, H-1′), 5.80 (1H, dd, J = 8.2, 1.3 Hz, H-5), 5.33 (2H, m, H-2 ′, H-3 ′), 4.35 (3H, s, H-4 ′, H-5 ′), 2.15 (3H, s, OAc), 2.13 (3H, s, OAc), 2.11 (3H, s, OAc); ^{13}C NMR ($CDCl_3$) δ 170.1 (OAc), 169.6 (2 OAc), 162.7 (C-4), 150.1 (C-2), 139.3 (C-6), 103.4 (C-5), 87.5 (C-1′), 79.9 (C-4 ′), 72.7 (C-3 ′), 70.1 (C-2 ′), 63.1 (C-5 ′), 20.7 (OAc), 20.4 (OAc), 20.3 (OAc); HRMS (CI, m /z) Calcd. For $C_{15}H_{19}N_2O_9$ (M+1) 371.1085, found 371.1078, 259 ($C_{11}H_{15}O_7$, 100) 741 ($C_{30}H_{37}N_4O_{18}$, 17)
The **compound 4** was identified as 2 ′,3 ′,5 ′-Triacetyl uridine.

Compound 5: ^{1}H NMR ($CDCl_3$) δ 5.30 (1H, dt, J = 1.9, 7.4 Hz, H-4), 4.67 (1H, m, H-5), 4.40 (1H, dd, J = 3.3, 12.3 Hz, H-6 ′), 4.30 (1H, dd, J = 3.5, 12.3 Hz, H-6'), 3.02 (1H, dd, J = 7.4, 18.7 Hz, H-3 ′), 2.63 (1H, dd, J = 2.1, 18.7 Hz, H-3'), 2.10 (6H, s, (2 OAc); ^{13}C NMR δ 210.0 (C-2), 173.7 (C-1), 170.3 (OAc), 170.0 (OAc), 82.0 (C-4), 71.1 (C-5), 63.3 (C-6), 34.8 (C-3), 20.8 (OAc), 20.6 (OAc).
Compound 5 was identified as 5,6-acetyl-3-deoxyascorbic acid.

Compound 6: ^{1}H NMR ^{1}H NMR ($CDCl_3$) δ 8.73 (1H, s, broad, NH), 8.69 (1H, s, H-2), 8.15 (1H, s, H-8), 6.22 (1H, d, J = 5.3 Hz, H-1′), 5.96 (1H, t, J = 5.3 Hz, H-2 ′), 5.66 (1H, dd, J = 4.4 Hz, 5.4 Hz, H-3 ′), 4.44 - 4.42 (3H, m, H-4′, H-5′, H-5 ″), 2.63 (3H, s, NHAc), 2.16 (3H, s, OAc), 2.12 (3H, s, OAc), 2.09 (3H, s, OAc); ^{13}C NMR δ 170.4 (OAc), 170.3 (OAc), 169.3 (OAc), 167.7 (NHAc), 152.7 (C-2), 151.0 (C-6), 149.4 (C-4), 141.2 (C-8), 122.2 (C-5), 86.5 (C-1′), 80.4 (C-4′), 73.1 (C-2′), 70.6 (C-3′), 63.0 (C-5′), 25.7 (NHAc), 20.7 (OAc), 20.5 (OAc), 20.4 (OAc). HRMS (CI, m/z) Calcd. for $C_{18}H_{22}N_5O_8$ (M+1) 436.1463, found 436.1467, 259 ($C_{11}H_{15}O_7$, 80), 178 ($C_7H_8N_5O$, 27)

Compound 6 was identified as acetylated adenosine. However identification of the stereochemistry at C-1′, C-2′, C-′, C-4′ cannot be done using the available data. Hence the assignment was indirectly confirmed based on pseudorotational analysis.[14]

Pseudorotational analysis can be applied to comment on the conformation of the ribose ring. ^{1}H NMR data of the **compound 6** shows $J_{1',2'} + J_{3',4'} = 5.3 + 4.4 = 9.7$ and $J_{2',3'} = 5.3$ Hz. Hence $\rho = 18 - 162$ and $\tau_m = 42$ confirming that the compound is in anti conformation. Since the value of ρ and τ falls in the normal range[15] one

can indirectly confirm that the configuration of carbons in the furanose, **6** is similar to ribose.

DISCUSSION

Extract of *V. volvacea* had significant antibacterial activity, but the activity was confined to one bacterium, *E. coli*. The non-polar fraction of the extract of *V. volvacea* was subjected to bio-assay guided fractionation and the most active fraction contained ergosterol peroxide. This is the first report on the isolation of ergosterol peroxide from *V. volvacea* and the activity against *E. coli* of ergosterol peroxide. Linoleic acid and some triglycerides were also isolated from the biologically active fractions.[12]

The polar fraction of the extract of *V. volvacea* was also fractionated and towards the final stages in identification of the structures of active compounds, compounds were acetylated to facilitate the purification and structure elucidation.

It was noted that the most active fraction contains a mixture of sugars and nucleosides; α and β-D-glucose, trehalose, uridine and adenosine. Moreover this fraction contains 3-deoxyascorbic acid. This compound is not available in the un-acetylated form to test against *E. coli*.

According to the available literature this is the first report on isolation of 3-deoxyascorbic acid from a basidiomycete fungus.

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