International Journal of Pharmacognosy and Life Science

E-ISSN: 2707-2835
P-ISSN: 2707-2827
IJPLS 2020; 1(1): 59-67
Received: 18-01-2020
Accepted: 22-03-2020

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In vitro antifungal activity and phytochemical analysis of lichen *Parmelia perlata* (L.) Ach.

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DOI: https://doi.org/10.33545/27072827.2020.v1.i1a.51

Abstract
Fungal diseases like candidiasis and cryptococcosis are the commonest opportunistic infections. Since the difficulties associated with the management of antifungal infections, there is an urgent need for the development of new and effective antifungal agents. In this perspective, natural products are a viable alternative source. The lichen *Parmelia perlata*, commonly known as stone flower, is traditionally used in the Indian system of medicine. The aim of the present study was to evaluate *in vitro* antifungal activity of petroleum ether extract of *Parmelia perlata* against *Candida albicans*, *Candida glabrata*, *Candida krusei*, *Candida tropicalis*, and *Cryptococcus neoformans* and its phytochemical analysis. The anti-fungal activity of the extract of *Parmelia perlata* was determined by measuring the Minimal Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) by microbroth dilution method as per the CLSI (NCCLS M27-A2) guidelines. The extract of *Parmelia perlata* showed the MIC and MFC against *Candida* species between 600±28µg/ml to 690±16µg/ml and between 750±90µg/ml to 820±45µg/ml, respectively. In addition, the respective values against *Cryptococcus neoformans* were 550±17µg/ml and 690±34µg/ml, respectively. Further, *Parmelia perlata* has yielded an acid named- (+)-6-deacetyl-9b-carbmethoxy-9b-dimethylusnic acid and, a quinine named -6-acetyl-11-carbmethoxy-1,4,7,10-tetrahydroxy-2,8-dimethyl naphthacene-5,12-quinone, which were identified on the basis of spectroscopic studies as 2-acetyl-9b-carbmethoxy-7,9-dihydroxy-8-methyl-1,3(2H,9bH)-dibenzofurandione and naphthacene-5,12-quinone derivative, along with four well known compounds-tridecyl myristate, 3-ketooleanane, arachidyl alcohol and (+) usnic acid, respectively. The results of this study showed that *Parmelia perlata* is a promising antifungal agent.

Keywords: *Parmelia perlata*, candidiasis, cryptococcosis, antifungal agent, phytochemicals

1. Introduction
Candida species are otherwise commensals of approximately one-half of human oral cavities and the genitourinary tract of females. Diseases caused by these yeasts are known as Candidiasis, which is mainly caused by *Candida albicans* (*C. albicans*) but recently other species such as *C. krusei*, *C. glabrata*, and *C. tropicalis* have also been reported as potential etiological agents. The diseases are commonly seen in immunocompromised patients such as AIDS patients [1]. In India, the disease appears in approximately 66.7% of AIDS patients as the first opportunistic infection [2].
Cryptococcus neoformans (*C. neoformans*) is encapsulated yeast-like fungus, which causes the life-threatening disease cryptococcosis. It is a highly pathogenic organism and has the potential to cause severe disease in both immunocompromised and immunocompetent individuals [3]. The mortality rate of HIV-associated cryptococcosis in developed countries is 10-30% while in developing countries, it is 13-44%. In India, recently its prevalence rate was reported to be 6-8% among AIDS patients [3, 4].
The existing drugs for the treatment of cryptococcosis include amphotericin B, fluconazole, clotrimazole, ketoconazole, 5-flurocytocin, miconazole, and azole derivatives. However, these drugs are costly, have several moderate to severe side effects, and recently resistance to these drugs has been reported in *Candida* as well as in *Cryptococcus* [5, 6]. Hence there is an urgent need for the discovery and development of new antifungal agents, which are cost-effective and have the least side effects. In this direction, World Health Organisation (WHO) has recognized the potential of traditional health care and estimates that about 70-80% of the world population trust traditional health care practices which are mainly based on the use of medicinal plants and their derivatives [7].

Recently, the Government of India has also started promoting research on phytotherapeutic products [8].

The lichen *Parmelia perlata* (*P. perlata*), which belongs to the family Parmeliaceae is a symbiotic association between an alga and a fungus that grows on trees and rocks, especially in the Himalayan region of India. It has a unique morphological form that is entirely different from either partner, chemically as well as physiologically [9]. In India, it is used in the Indian folk medicine system as a demulcent, tonic, febrifuge, diuretic, emollient, stomach disorders, dyspepsia, vomiting, headache, and pain in the liver and for curing the wounds [10]. *P. perlata* contains an acidic substance that has been used as an antibiotic in several countries as a topical antibacterial agent for human skin diseases and is also known to have antibacterial and anti-viral activities [11, 12]. These previously reported significant medicinal properties of this lichen prompted us to evaluate the anticandidal and anticryptococcal activities of petroleum ether extract of *P. perlata* against potentially pathogenic fungal species, the *C. albicans*, *C. glabrata*, *C. krusei*, *C. tropicalis*, and *C. neoformans*. The phytochemical analysis of the active extract was done to investigate the chemical ingredient of the extract which may be responsible for the antifungal activity.

2. Material and Methods

2.1. Plant material

The plant material *P. perlata*(lichen) was collected from the hills of Uttarakhand (India) and the authenticity of the lichen was confirmed by Dr. Vishal Kaushik, In-charge of Herbarium, Department of Botany, NREC College, Khurja, District- Bulandshahr 203 131, U.P., India.

2.2. Preparation of extract

The lichen- *P. perlata* was shade dried and pulverized. The powder was packed into a Soxhlet apparatus and subjected to hot continuous percolation using petroleum ether (AR grade) as a solvent for 72 hours at 58- 60 ºC. The extract was filtered and evaporated to dryness under reduced pressure in a rotary evaporator (Labmate, Asia Pvt. Ltd.) at < 40 ºC, yielded (6.4% $^{w}/_{w}$), which yielded a semisolid yellowish-brown mash. The concentrated mass was treated with acetonitrile for the removal of fats. Acetonitrile solvent was evaporated to dryness and the resulting mass was stored at -20 ^{0}C until further use. The solvent-free extract was suspended in the dimethyl sulfoxide (DMSO) to prepare desired concentration of the extract for evaluation of the antifungal activity. The remaining extract was used for fractionation to isolate and purify the compounds.

2.3. Microbial strains

Four standard strains of *Candida* and one standard strain of *Cryptococcus* were studied including *Candida albicans* (ATCC 10231), *C. glabrata* (ATCC 15126), *C. krusei* (ATCC 14243), *C. tropicalis* (ATCC 20336) and *Cryptococcus neoformans* (ATCC 90112). The culture stock was maintained on Sabouraud's Dextrose Agar (SDA) at 4 ^{0}C. The organisms were sub-cultured on SDA and incubated at 37 ^{0}C for 24 hours. The active cultures for the experiment were prepared by streaking on SDA and incubated at 37 ^{0}C for 24 hours. The suspension was adjusted to 70% transmittance by a spectrophotometer at 530 nm. This should result in a suspension containing about 1 x 10^{6}CFU/ml.

2.4. Test methods

The anti-candidal and anti-cryptococcal activities of petroleum ether extract of *P. Perlata* were determined by measuring the Minimal Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) by microbroth dilution method according to the CLSI, NCCLS M27-A2 guidelines [13]. Geometric dilutions ranging from 0.25 to 2.0 mg/ml of the *P. perlata* extract were prepared in a 96-well micro titter plate. The final concentration of each well was adjusted to 1×10^{6} CFU/ml spores of *C. albicans*, *C. glabrata*, *C. krusei*, *C. tropicalis*, and *C. neoformans* and incubated at 37 ^{0}C for 24 hours (*Candida* species) and 48 hours (*C. neoformans*). The MIC was defined as the lowest concentration of the test sample that resulted in complete inhibition of visible growth. The microbial growth was indicated by the presence of a white pellet on the well bottom. Nystatin was used as a reference standard drug. The petroleum ether extract of *P. perlata* was dissolved in DMSO. The control experiments were performed using DMSO as solvent at the same concentration (1%) which was used to test for *P. perlata* extract. Aliquots from each of the wells were sub-cultured onto SDA plates and incubated for 24 h (*Candida* spp.) and 48 hours (*C. neoformans*). MFC was read as the lowest concentration of the test sample at which the organisms were killed and showed no growth on sub-culturing the broth on SDA plates. All experiments were repeated three times and the mean ± standard deviation (SD) was calculated by statistical analysis.

2.5. Isolation and purification of compounds

Fat-free extract (30g) was subjected to repeated column chromatography for the separation and isolation of pure compounds over a silica gel column. For this, a column of 1.5 meters in height with 4 centimeters in diameter and 550g silica gel G (60-120 mesh) was used. The column was eluted with different solvents using petroleum ether and the mixture of petroleum ether-benzene in order of increasing polarity. After isolation and purification, the six compounds were obtained which were labeled as A to F.

2.6. Identification and structure elucidation of compounds

The structure of the purified compounds was identified by mass spectrometry (Agilent 1100 Series LC/MSD API-ES spectrometer), infrared analyses (FTIR-8400S Spectrophotometer, Shimadzu, Japan), and Nuclear Magnetic Resonance (NMR) (JEOL AL-300 MHz Spectrometer) using ^{1}H NMR (300.40 MHz) and ^{13}C NMR (75.45 MHz) analyses in $CDCl_3$ and by comparison of spectral data with those available in the literature.

2.6.1. Compound A

It was obtained when column was eluted with petroleum ether. After removal of solvent, a yellow solid mass was obtained which on crystallization with ethyl acetate yielded shining yellow crystals. It showed single spot-on TLC examination (R_f = 0.35) in benzene as a mobile phase. The melting point of this compound was found to be 131°C.MS (m/z): 346(M^+), 347[M^++H], 330, 260, 233, 165 etc.; molecular formula: $C_{17}H_{14}O_8$; IR (KBr, cm^{-1}): 3410 (O–H stretching), 1780 (>C=O, str.), 1625 (>C=C< str.), 1055 (C–O str.) etc.; ^{1}H NMR (d ppm, $CDCl_3$): 2.50 (s, 3H, C-2, -$COCH_3$), 5.13 (s, 1H, C-4, -H), 6.20 (s, 1H, C-6, -H), 13.32 (s, 1H, C-7, -OH), 2.11 (s, 3H, C-8, -CH_3), 12.04 (s, 1H, C-

9, -OH), 4.01 (s, 3H, C-9b, -$COOCH_3$), 18.84 (s, 1H, C-3, -OH, enolic form); ^{13}C NMR (d ppm, $CDCl_3$): 197.24 (C-1), 110.53 (C-2), 200.38 (-$COCH_3$ at C-2), 32.14 (-$COCH_3$ at C-2), 165.11 (C-3), 101.58 (C-4), 179.11 (C-4a), 140.16(C-5a), 99.13 (C-6), 163.16 (C-7), 108.49 (C-8), 7.69 (-CH_3 at C-8), 158.02 (C-9), 105.14 (C-9a), 59.87 (C-9b), 172.62 (-$COOCH_3$ at C-9b), 51.84 (-$COOCH_3$ at C-9b).

2.6.2. Compound B

A yellow solid mass was obtained when column was eluted with petroleum ether. After removal of the solvent, it was redissolved in acetone, and acetone soluble part was crystallized. Its R_f value was found to be 0.73 in benzene and chloroform (8:2) as a solvent system and decomposed > 200°C melting point. MS (m/z): 450 (M^+) ; molecular formula: $C_{24}H_{18}O_9$; IR (KBr, cm^{-1}): 3362 (hydroxy absorption), 1733, 1762 (>C=O, stretching), 2924 (C–H, str.), 1573 (>C=C<, str.), 1123, 1043 (C–O–C, str.), 996 etc.; ^1H NMR (d ppm, $CDCl_3$): 12.50 (s, 1H, C-1, -OH), 2.55 (s, 3H, C-2, -CH_3), 6.53 (s, 1H, C-3, -H), 12.60 (s, 1H, C-4, -OH), 2.75 (s, 3H, C-6, -$COCH_3$), 10.40 (s, 1H, C-7, -OH), 2.17 (s, 3H, C-8, -CH_3), 6.47 (s, 1H, C-93, -H), 11.98 (s, 1H, C-10, -OH), 4.01 (s, 3H, C-11, -$COOCH_3$); ^{13}C NMR (d ppm, $CDCl_3$): 169.09 (C-1), 112.85 (C-2), 9.37 (C-2, -CH_3), 111.55 (C-3), 169.71 (C-4), 116.77 (C-4a), 186.31 (C-5), 132.11 (C-5a), 143.13 (C-6), 193.87 (-$COCH_3$ at C-6), 25.62 (-$COCH_3$ at C-6), 108.53 (C-6a), 167.48 (C-7), 134.91 (C-8), 24.06 (-CH_3 at C-8), 110.24 (C-9), 162.88 (C-10), 116.01 (C-10a), 127.51(C-11), 172.24 (-C00CH_3 at C-11), 52.37 (-$COOCH_3$ at C-11), 139.89 (C-11a), 189.93 (C-12), 152.46 (C-12a).

2.6.3. Compound C

It was obtained when the column was eluted with petroleum ether and benzene in a ratio of 3:1. After the removal of the solvent, a colorless solid was obtained. It showed a single spot-on TLC examination (R_f = 0.86) in benzene and chloroform (7:3) as a solvent system. The melting point of this compound was found to be 63.5°C. MS (m/z): 410 (M^+); molecular formula: $C_{27}H_{54}O_2$; IR (KBr, cm^{-1}): 2925, 2850 (C-H, stretching), 1730 (>C=O, str.), 1260 (C-O, str.), 715, 710 etc.; ^1H NMR (d ppm, $CDCl_3$): 2.30 (t, 2H, J=7.32, 7.50 Hz, C-2, -CH_2COO-), 1.65 (q, 2H, C-3, -CH_2-), 4.1 (t, 2H, J=6.75, 6.78 Hz, C-1', -$COOCH_2$-), 1.55 (q, 2H, C-2', -CH_2-), 0.90 (t, 6H, J=6.62, 6.93 Hz, C-14 and C-13', -CH_3), 1.24-1.45 (s, 40H,C-4 to C-13 and C-3' to C-12'); ^{13}C NMR (d ppm, $CDCl_3$): 175.20 (C-1), 70.10 (C-1'), 34.42 (C-2,), 31.93 (C-2'), 25.93 (C-3), 28.64 (C-3'), 32.11 (C-12), 22.50 (C-13), 14.13 (C-13'), 14.10 (C-14), 29.15-29.70 (C-4 to C-11 & C-4' to C-10').

2.6.4. Compound D

It was isolated as colourless solid when elution of column by petroleum ether with benzene in the ratio 1:1 and it showed single spot-on TLC examination. Its R_f value was observed at 0.63 (benzene as mobile phase). Melting point of the compound was found to be 235°C. MS (m/z): 426 (M^+), 427[M^++H], 302, 205, 125, 97, 69, 55 etc.; molecular formula: $C_{30}H_{50}O$; IR (KBr, cm^{-1}): 1720 (>C=O, stretching), 2930, 2910 (C-H, stretching), 1440 (C=C str.), 1370 etc.; ^1H NMR (d ppm, $CDCl_3$): 0.71 (s, 3H, C-26, -CH_3), 0.85 (s, 3H, C-25, -CH_3), 0.94 (s, 3H, C-28, -CH_3), 0.98 (s, 3H, C-30, -CH_3), 1.03 (s, 3H, C-29, -CH_3), 1.07 (s, 3H, C-27, -CH_3), 1.16 (s, 3H, C-24, -CH_3), 1.25 (s, 3H, C-23, -CH_3), 0.89 (m, 2H, C-1, -CH_2-), 0.84 (m, 1H, C-13, >CH-), 1.32-2.41 (complicated pattern for remaining twenty three protons); ^{13}C NMR (d ppm, $CDCl_3$): 35.10 (C-1), 27.50 (C-2), 213.83 (C-3), 59.41 (C-4), 58.16 (C-5), 40.87 (C-6), 17.93 (C-7), 40.60 (C-8), 53.05 (C-9), 38.26 (C-10), 35.31 (C-11), 30.48 (C-12), 38.26 (C-13), 39.22 (C-14), 31.77 (C-15), 29.68 (C-16), 32.38 (C-17), 39.48 (C-18), 35.58 (C-19), 29.97 (C-20), 32.07 (C-21), 35.98 (C-22), 28.15 (C-23), 22.26 (C-24), 18.20 (C-25), 14.64 (C-26), 18.65 (C-27), 20.24 (C-28), 35.02 (C-29), 32.74 (C-30).

2.6.5. Compound E

This compound was isolated when the column was eluted with petroleum ether and benzene in a ratio of 1:3. It showed a single spot-on TLC examination (R_f = 0.72) in benzene and chloroform (7:3) as a solvent system. The melting point of compound E (colorless solid) was found to be 66°C. MS (m/z): 298 (M^+); molecular formula: $C_{20}H_{42}O$; IR (KBr, cm^{-1}): 3400-3250 (-OH, stretching), 1062 (C-O, stre.), 734, 724 etc.; ^1H NMR (d ppm, $CDCl_3$): 0.87 (t, 3H, J = 6.2, 6.9Hz, C-20, -CH_3), 2.34 (t, 2H, J = 7.3, 7.6Hz, C-1, -CH_2-), 2.89 (br, s, 1H, C-1, -OH), 1.63 (m, 2H, C-2, -CH_2-), 1.25 (br, s, 34H, C-3 to C-19).

2.6.6. Compound F

It was isolated as yellow solid by eluting the column with petroleum ether and benzene (1:3). This mass was redissolved in acetone and acetone soluble part was purified and analysed. Its R_f value was 0.80 in chloroform and methanol (95% + 5%) system. The melting point of this crystalline yellow compound was 204°C. MS (m/z): 344 (M^+), 345 (M^++H), 261, 260, 234, 233, 232, 217, 215 etc.; molecular formula: $C_{18}H_{16}O_7$; IR (KBr, cm^{-1}): 3150 (-OH stretching), 1800 (C=O, str.), 1550 (C=C, str.) etc.; ^1H NMR (d ppm, $CDCl_3$): 2.67 (s, 3H, -$COCH_3$ at C-2), 5.97 (s, 1H, C-4, -H), 2.66 (s, 3H, -$COCH_3$ at C-6), 13.30 (s, 1H, C-7, -OH), 2.11 (s, 3H, C-8, -CH_3), 11.01 (s, 1H, C-9, -OH), 1.76 (s, 3H, C-9b, -CH_3), 18.84 (s, 1H, C-3, -OH, enolic form); ^{13}C NMR (d ppm, $CDCl_3$): 198.02 (C-1), 109.24 (C-2), 200.38 (-$COCH_3$ at C-2), 32.08 (-$COCH_3$ at C-2), 163.90 (C-3), 101.58 (C-4), 179.32 (C-4a), 155.31 (C-5a), 98.37 (C-6), 200.50 (-$COCH_3$ at C-6), 31.22 (-$COCH_3$ at C-6), 163.56 (C-7), 105.33 (C-8), 7.50 (-CH_3 at C-8), 157.38 (C-9), 104.03 (C-9a), 59.13 (C-9b), 27.83 (-CH_3 at C-9b).

3. Results and Discussion

3.1. Antifungal activity

The petroleum ether extract of *P. perlata* showed considerable anti-candidal activity. The MIC values were determined by the broth dilution method. The mean of MIC against *C. albicans*, *C. glabrata*, *C. krusei*, and *C. Tropicalis* was found at 630±19µg/ml, 600±28µg/ml, 690±16µg/ml, and 650±22µg/ml, respectively. The MFC values were determined by using the growth visibility on SDA plates. Accordingly, the MFC values were found at 780±75µg/ml, 750±90µg/ml, 820±45µg/ml, and 790±68µg/ml. The MIC values of nystatin (standard drug) against *C. albicans*, *C. glabrata*, *C. krusei*, and *C. Tropicalis* were found 1.86±0.11µg/ml, 1.67±0.10µg/ml, 1.92±0.12µg/ml and 1.54±0.14µg/ml. Accordingly, the MFC values were found at 3.24±0.16µg/ml, 3.14±0.21µg/ml, 3.32±0.22µg/ml and 3.16±0.12µg/ml (Table 1).

The extract of *P. perlata* showed highly encouraging anti-cryptococcal activity with MIC and MFC values of 550±17µg/ml and 690±34µg/ml, respectively. The MIC and MFC values of nystatin against *C. neoformans* were found 1.48±0.13µg/ml and 2.96±0.14µg/ml, respectively (Table 1).

3.2. Characterization of purified compounds

Compounds A to F (Fig.1-6) were isolated and characterized from the petroleum ether extract of *P. perlata*. The known compounds were identified on the basis of physical, chemical, and spectral data. Identification of known compounds was done by comparing their melting point, IR, ^{1}H NMR, ^{13}C NMR, and mass spectral data with the reported values in the literature and also by comparing the spectra with the spectrum of authentic samples earlier isolated in our laboratory.

3.2.1. Compound A

In the mass spectrum of compound A, the prominent signal was observed at m/z 347[M^{+}+H] and 346 [M^{+}], and other prominent ions were observed at m/z: 330, 260, 233, 165, etc. The ^{1}H NMR & ^{13}C NMR spectrum indicated that it contains fourteen protons and seventeen carbon atoms in the skeleton. On the basis of these observations, the molecular formula of compound A was calculated as $C_{17}H_{14}O_8$. The IR spectrum (cm^{-1}, KBr) showed strong absorption at 3410 (br), indicating the presence of a hydroxyl group. The absorption at 1625 confirmed the presence of the olefinic (>C=C<, stretching) group whereas the absorption at 1055 was assigned for C-O stretching.

In the ^{1}H NMR spectrum (δppm, $CDCl_3$), the presence of two hydroxy groups was observed at 12.04 (*s*, 1H, C-9) and 13.32 (*s*, 1H, C-7), both the hydroxy signals are comparable with (+) usnic acid molecule. The presence of two protons attached at C-4 and C-6 positions were observed as two singlets at 5.13 and 6.20, respectively, while in (+) usnic acid, the vinylic proton at the C-4 position was observed at 5.97 as a singlet. A singlet at 2.11 was assigned for the methyl group at the C-8 position which is similar to the methyl group in (+) usnic acid attached at the C-8 position. In (+) usnic acid, two acetyl groups were observed as two singlets at 2.66 and 2.67 for three protons each at C-2 and C-6 positions, but in compound A, a sharp singlet for three protons was observed at 2.50 (*s*, 3H), which indicated the presence of only one acetyl group and its possibility of attachment at the C-2 position. The spectrum showed the absence of a singlet at 1.76 for a methyl group at position C-9b, as in (+) usnic acid, but in compound A, an additional sharp singlet for three protons was observed at 4.01 which indicated the presence of carbmethoxy group at C-9b position in place of a methyl group which was further confirmed by its ^{13}C NMR spectrum by observing at the 172.62 (–$\underline{C}OOCH_3$) and 51.84 (–$COO\underline{C}H_3$) carbons. In solution, compound A exists in two tautomeric forms i.e., ketonic and enolic. The enolic proton shows an intense PMR absorption peak at 18.84.

Absorptions at 158.02, 163.16, and 165.11 in the ^{13}C NMR spectrum (δppm, $CDCl_3$) also confirmed the presence of three phenolic groups, and the attachment of these three hydroxyl groups was assigned at C-9, C-7, and C-3 positions as compared with usnic acid. The carbon atoms at C-4 and C-6 positions were confirmed by the absorption at 101.58 and 99.13, respectively. The absorption appearing at 197.24 indicated the presence of the carbonyl group at position C-1. A signal at 172.62 indicated the presence of carbmethoxy group (-$\underline{C}OOCH_3$) and attachment of this group was established at the C-9b position. The absorption for the methoxy group (–$COO\underline{C}H_3$) was observed at 51.84. Other signals in ^{13}C NMR were obtained at 110.53 (C-2), 200.38 (C-2, -$\underline{C}OCH_3$), 32.14 (C-2, -$CO\underline{C}H_3$), 179.11 (C-4a), 140.16 (C-5a), 108.49 (C-8), 7.69 (C-8, -CH_3), 105.14 (C-9a) and 59.87 (C-9b) and their assignment have shown in parenthesis [14-16]. On the basis of above observation, compound A (Fig.1) was identified as (+)-6-diacetyl-9b-carbmethoxy-9b-dimethyl-usnic acid (2-acetyl-9b-carbmethoxy-7,9-dihydroxy-8-methyl-1,3 (2H,9bH)-dibenzofurandione) [17-20].

3.2.2. Compound B

The mass spectrum of compound B showed a molecular ion peak at m/z 450[M^{+}] and the molecular formula was determined as $C_{24}H_{18}O_9$ with the help of ^{1}H NMR and ^{13}C NMR spectral analysis. The IR spectrum (cm^{-1}, KBr) of compound B confirmed the presence of hydroxy group/groups by showing a broad absorption at 3362. The presence of the carbonyl group was established by the absorption at 1733 and 1762. Absorption at 1043 confirmed the presence of C-O-C linkage.

The ^{1}H NMR spectrum (δppm, $CDCl_3$) showed sharp singlets for ten different types of protons. Presence of four sharp singlets at 12.60, 12.50, 11.98, and 10.40 for one proton and each assigned for hydroxyl groups at positions C-4, C-1, C-10, and C-7, respectively. The singlet observed at 2.75 for three protons was ascertained to be acetyl group (-$CO\underline{C}H_3$) and its attachment at the C-6 position was assigned with the maximum possibility. The presence of two singlets for two methyl groups was observed at 2.55 and 2.17 for three protons, each at C-2 and C-8 positions, respectively. The presence of three protons of the carbmethoxy group attached to carbon atom C-11 was observed at 4.01 as a sharp singlet. The downfield shift of the proton of the hydroxyl group is due to intramolecular hydrogen bonding. The presence of the carbmethoxy group (–$COOCH_3$) was also confirmed by the ^{13}C NMR spectrum by locating the absorptions.

On the basis of the ^{1}H NMR and ^{13}C NMR spectrum, the location of the substituents like phenolic hydroxyl, methyl, acetyl, and carbmethoxy groups was ascertained. The presence of the remaining two aromatic protons in the molecule (Compound B) was confirmed by the absorption at 6.53 and 6.47 as two singlets for one proton each and assigned to the C-3H and C-9H, respectively.

The ^{13}C NMR spectrum (δppm, $CDCl_3$) showed absorption at 189.93 (C-12), and 186.31 (C-5) which indicated the presence of two carbonyl groups, and these values were assigned on the basis of reported chemical shift for the naphthacenequinone derivatives [21]. The hydroxy groups attached at carbon atom C-1, C-4, C-7, and C-10 positions showed absorptions at 169.09, 169.71, 167.48, and 162.88, respectively. The carbon atom of the acetyl group showed absorption at 193.83 and the attachment of the acetyl group at the C-6 position was confirmed by the absorption at 143.13. The absorption at 9.37 and 24.06 confirmed the presence of two methyl groups at C-2 and C-8 positions, respectively. The appearance of a typical methyl signal at 52.37 was assigned to methoxy carbon of the carbmethoxy group. The carbonyl carbon of the carbmethoxy group

appeared at 172.24 and its location was assigned at the C-11 position. Other absorption in the spectrum were observed at 112.85 (C-2), 111.55 (C-3), 116.77 (C-4a), 132.11 (C-5a), 108.53 (C-6a), 134.91 (C-8), 110.24 (C-9), 116.01 (C-10a), 127.51 (C-11), 139.89 (C-11a) and 152.46 (C-12a) and their assignment have been given in the parenthesis. On the basis ^{13}C NMR spectrum, the position of all the twenty-four carbon atoms was established. The spectral studies established the structure for compound B (Fig.2) as 6-acetyl-11-carbmethoxy-1,4,7,10-tetrahydroxy-2,8 dimethylnaphthacene-5,12-quinone, a purified compound.

3.2.3. Compound C

The mass spectrum of compound C exhibited the molecular ion peak at m/z 410 [M^+]. On the basis of ^1H NMR and ^{13}C NMR, the molecular formula was calculated as $C_{27}H_{54}O_2$. The IR spectrum (cm^{-1}, KBr) displayed the significant absorption at 2925, 2850 for C-H stretching, 1730 for C=O stretching and 1260 for C-O stretching of the ester group. The other important absorptions were observed at 715 and 710 ($-(CH_2)_n-$ bending). On the basis of these observations, compound C seems to be a long-chain saturated aliphatic ester.

The ^1H NMR spectrum (δppm, $CDCl_3$) showed two triplets at 4.1 (*J= 6.75, 6.78 Hz*) $\left(-\underset{\underset{O}{\|}}{C}-O-\underline{C}H_2-\right)$ and 2.30 (*J=7.32, 7.50 Hz*) $\left(-\underline{C}H_2-\underset{\underset{O}{\|}}{C}-O-\right)$ which corresponded to the methylene group attached at ester oxygen C-1' and ester carboxyl group at C-2, respectively. The signals observed at 1.65 (*m*, 2H) and 1.55 (*m*, 2H) were assigned to two protons, each at C-3 and C-2´ positions, respectively. The presence of six protons of terminal methyl groups was assigned as a triplet at 0.90 (*J = 6.62, 6.93 Hz*). Methylene protons were observed at 1.24 as a broad singlet and calculated for forty protons. In the ^{13}C NMR spectrum (δppm, $CDCl_3$), the ester linkage was confirmed by the absorption at 175.20 and 70.10 for the carboxyl function of the ester C-1 and methylene proton attached at C-1´ to the ester oxygen. The signals for terminal methyl groups were observed at 14.10 and 14.13. The absorptions for other carbon atoms were assigned as 34.42 (C-2), 25.93 (C-3), 32.11 (C-12), 22.50 (C-13), 31.93 (C-2´), 28.64 (C-3´) and besides these signals, many other overlapped peaks were observed at 29.15 to 29.70 for remaining carbon atoms (C-4 to C-11 & C-4´ to C-10´ positions) in the title compound. On the basis of the above evidence, compound C (Fig.3) was identified as tridecyl myristate (tridecyl tetradecanoate).

3.2.4. Compound D

The mass spectrum of compound D displayed [M^++H] at 427 and [M^+] at m/z 426. Other characteristic ions were observed at m/z 302, 205, 125, 97, 69, 55, etc. The compound gave a positive test with Libermann – Burchard reagent which indicated its triterpenoid nature. It also responded to Zimmermann's test suggesting the presence of the keto group at position C-3 (3-ketone), which was further confirmed by the fragment at m/z 205 in the mass spectrum. Thus, the molecular formula for compound D was established as $C_{30}H_{50}O$. In the IR spectrum [cm^{-1}, KBr], characteristic strong absorption was observed at 1720, suggesting the presence of a carbonyl group. Strong absorptions observed at 1440 and 1370 were characterized by bending vibration of the gem dimethyl group ($-CHMe_2$). The ^1H NMR spectrum [δppm, $CDCl_3$], showed the presence of eight methyl groups at 0.71 (*s*, 3H, C-26), 0.85 (*s*, 3H, C-25), 0.94 (*s*, 3H, C-28), 0.98 (*s*, 3H, C-30), 1.03 (*s*, 3H, C-29), 1.07 (*s*, 3H, C-27), 1.16 (*s*, 3H, C-24) and 1.25 (*s*, 3H, C-23). The signals at 0.89 (*m*, 2H) & 0.84 (*m*, 1H) were observed for C-1 and C-13 protons, respectively. A complicated pattern from 1.32 to 2.41 was observed and accounted for the remaining twenty-three protons in the title compound.

In the ^{13}C NMR spectrum [δppm, $CDCl_3$], an absorption at 213.83 confirmed the presence of the keto group at the C-3 position. Other absorptions at 14.64, 18.20, 18.65, 20.24, 22.26, 28.15, 32.74, and 35.02 were due to eight methyl groups and their positions were assigned as C-26, C-25, C-27, C-28, C-24, C-23, C-30, and C-29, respectively. The assignments of other carbon atoms and their position were established as 35.10 (C-1), 27.50 (C-2), 59.41 (C-4), 58.16 (C-5), 40.87 (C-6), 17.93 (C-7), 40.60 (C-8), 53.05 (C-9), 38.26 (C-10), 35.31 (C-11), 30.48 (C-12), 38.26 (C-13), 39.22 (C-14), 31.77 (C-15), 29.68 (C-16), 32.38 (C-17), 39.48 (C-18), 35.58 (C-19), 29.97 (C-20), 32.07 (C-21), 35.98 (C-22), by comparing the data with the literature values. The identity of the title compound was further confirmed by comparing the spectral data with reported values [22]. On the basis of the above spectral analysis of compound D (Fig.4) was characterized as 3-ketooleanane.

3.2.5. Compound E

The molecular formula of compound E was found to be $C_{20}H_{42}O$ by mass spectral studies [M^+, 298]. An intense peak was observed at m/z 31corresponding to the to the CH_2OH^+ ion, which confirmed the primary alcoholic nature of the compound. The characteristic ion at m/z 280 appeared due to the loss of water molecule from the molecule ion i.e. (M^+ -H_2O). Besides this, the spectrum showed the peaks at 280, 266, 252, etc. at the interval of 14 mass units i.e., regular loss of methylene (CH_2) groups suggesting that this is long-chain alcohol. The IR spectrum [cm^{-1}, KBr] showed a broad absorption peak at 3400-3250 (-OH stretching, due to polymeric association), 1062 (C-O stretching) for primary alcohol, and a doublet at 734 and 724 [C-H bending for straight chain methylene groups [-$(CH_2)_s$-], further confirmed it to be a long chain primary alcohol [23].

The ^1H NMR spectrum (δppm, $CDCl_3$) showed a triplet for the methyl group at 0.87 (*t*, 3H, *J = 6.2, 6.9 Hz*, C-20) and a broad signal with sidebands integrating for thirty-four protons of methylene groups was accounted at 1.25 (br, *s*, 34H, C-3 to C-19). A triplet for two protons at 2.34 (*t*, 2H, *J =7.3, 7.6Hz*, C-1) was observed along with a broad singlet at 2.89 for one proton of the hydroxyl group. A multiplet for two protons was observed at 1.63 for C-2 protons. On the basis of the above spectral studies, compound E was identified as Icosan-1-ol (m.p. 66ºC) (Fig.5).

3.2.6. Compound F

The mass spectrum of this compound showed a molecular ion peak at m/z 344[M^+]. Other prominent ions were

observed at m/z: 345 [M^++H], 261, 260, 234, 233, 232, 217, and 215 etc. The spectral analysis and molecular weight determination established its molecular formula as $C_{18}H_{16}O_7$. In the IR spectrum (KBr, cm^{-1}), the presence of the phenolic (OH) group was observed at 3150 and the absorptions at 1800 confirmed the presence of the carbonyl group whereas the absorption at 1550 was assigned for >C=C< stretching.

The ^{1}H NMR spectrum (δppm, $CDCl_3$) showed two sharp singlets at 1.76 (*s*, 3H) and 2.11 (*s*, 3H) for two methyl groups, and their positions were assigned as C-9b and C-8, respectively as reported [14]. The absorption at 2.66 (*s*, 3H) and 2.67(*s*, 3H) as two sharp singlets confirmed the presence of two acetyl groups at C-6 and C-2 positions, respectively. Two sharp singlets were observed at 11.01 (*s*, 1H) and 13.30 (*s*, 1H) for phenolic groups at positions C-9 and C-7 correspondingly. Vinylic proton present at the C-4 position showed the absorption at 5.97 as a singlet for one proton. In solution form, this compound exists in two tautomeric forms i.e., ketonic and enolic. The enolic proton showed intense ^{1}H NMR absorption at 18.84.

The absorptions at 163.90, 163.56, and 157.38 in the ^{13}C NMR spectrum (δppm, $CDCl_3$) also confirmed the presence of three phenolic groups linked at C-3, C-7, and C-9 positions, respectively. Vinylic carbon atom was confirmed by the absorption at 101.58 which was assigned to the C-4 carbon atom. The absorption appearing at 198.02 clearly indicated the presence of the carbonyl group at the C-1 position. The values of other carbon atoms in compound F were established as 109.24 (C-2), 200.38 (-$\underline{C}OCH_3$ at C-2), 32.08 (-CO$\underline{C}H_3$ at C-2), 179.32 (C-4a), 155.31 (C-5a), 98.37 (C-6), 200.50 (-$\underline{C}OCH_3$ at C-6), 31.22 (-CO$\underline{C}H_3$ at C-6), 105.33 (C-8), 7.50 (-CH_3 at C-8), 104.03 (C-9a), 59.13 (C-9b), and 27.83 (-CH_3 at C-9b). On the basis of the above spectral analysis, compound F (Fig.6) was identified as (+) usnic acid. The identity of this compound was confirmed by comparing its spectral data with reported values [14-20].

Candidiasis and cryptococcosis are opportunistic fungal infections caused by *Candida* species and *Cryptococcus* species. The very high prevalence of candidiasis and cryptococcosis has been reported in Indian AIDS patients by various workers [1-4]. Moreover, treating physicians have encountered a new challenge with the appearance of non-albicans *Candida* species as common human pathogens [24]. Failure of currently available antifungal agents against these non-albicans species such as *C. glabrata* and *C. krusei* and *C. tropicalis* is now a therapeutic challenge. Therefore, there is an urgent need for new antifungal agents for the effective management of these fungal infections.

Herbal and alternative medicines are popular in the general population worldwide. A high number of modern drugs are still derived from herbs. Most of the commercially available drugs are synthetic and for that reason, their cost is unaffordable to most of the affected patients from developing and poor countries. These synthetic drugs also carry several side effects. Therefore, herbal products could be an inspiration for a new prototype for the drug development against candidiasis and cryptococcosis. From ancient times in India, herbal products are clinically used for curing various ailments, but unfortunately, this treasure has not been fully explored for the containment of candidiasis and cryptococcosis. Therefore, more attention is desired for the exploration of plant species commonly used in traditional herbal medicine for treating fungal diseases. Recently, other workers have also reported some herbal products to show anticandidal and anti-cryptococcal activities [25, 26]. These reports established that the herbal drugs hold promise and source of chemical leads for the development of novel therapeutic agents against fungal diseases.

The present study shows that the petroleum ether extract of *P. perlata* possesses significant anticandidal and anticryptococcal activity. The phytochemical analysis of *P. Perlata* revealed that it contains (+)-6-diacetyl-9b-carbmethoxy-9b-dimethylusnic acid, 6-acetyl-11-carbmethoxy-1,4,7,10-tetrahydroxy-2,8-dimethylnaphth-acene-5,12-quinone, Tridecyl myristate, 3-ketooleanane, Icosan-1-ol, and (+) Usnic acid. These above-mentioned compounds might be responsible for their antifungal activity either alone or synergistically. The literature study reveals that various constituents such as atranorin, leconoric acid, usnic acid, chrysophanic acid, flavonoids, tannins, glycosides, steroidal, aglycone, and saponins are also reported in *P. Perlata* [10, 11]. The depsides and benzofuran derivatives might be responsible for its antifungal activity and two new terpenes parmelanostene and permelabdone are reported to have antibacterial activity [27]. It is postulated that probably the saponins exert antifungal activity via membranolytic actions, following the enzymatic (glycosidase) conversion of the saponins to their corresponding aglycones in the cell membranes vicinity [28]. The tannins are also known to inhibit extracellular microbial enzymes, cause deprivation of the substrate required for microbial growth, and are also known to exert direct action on microbial metabolism through inhibition of oxidative phosphorylation. It is also possible that iron deprivation is involved in such a type of antimicrobial activity [29].

In our study, we used MIC and MFC instead of disk diffusion methods. Although the agar-cup diffusion method is commonly employed in preliminary susceptibility studies, it is not always dependable for accurate assessment and comparison. This is because of the high degree of interference, which is inherent in this method due to drug diffusion in the medium [30]. A more generally accurate method of assessment is the broth dilution technique. In this study, therefore, the broth dilution method was used in determining the activities measured as MIC. In using this method, higher degrees of inter-strain differences in susceptibility were observed and then Standard Cultural Method on the SDA plate was used for the determination of MFC. The gross difference in the MIC and MFC values of crude extract and the standard antifungal agent was on the expected line, as the crude extract has several impurities and further purification of the compounds from the extract would be expected to enhance the observed activity. Therefore, we have isolated and characterized A to F compounds from the petroleum ether extract of *P. perlata* which might be responsible for the anticandidal and anticryptococcal activity. The purified compounds were obtained in a very less quantity and have been utilized in structure elucidation and characterization. Further, isolation and purification of active compounds of the extract in larger amounts and its antifungal activity are in progress in our laboratory.

Table 1: Anti-fungal activity expressed as minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of petroleum ether of *Parmelia perlata* extract and standard drug Nystatin by Microbroth Dilution Method

Strains	Petroleum ether extract of *Parmelia perlata*		Antifungal Agent (Nystatin)	
	Mean MIC values ± SD (µg/ml)	Mean MFC values ± SD (µg/ml)	Mean MIC values ± SD (µg/ml)	Mean MFC values ± SD (µg/ml)
Candida albicans (ATCC 10231)	630 ± 19	780 ± 75	1.86 ± 0.11	3.24 ± 0.16
Candida glabrata (ATCC 15126)	600 ± 28	750 ± 90	1.67 ± 0.10	3.14 ± 0.21
Candida krusei (ATCC 14243)	690 ± 16	820 ± 45	1.92 ± 0.12	3.32 ± 0.22
Candida tropicalis (ATCC 20336)	650 ± 22	790 ± 68	1.54 ± 0.14	3.16 ± 0.12
Cryptococcus neoformans (ATCC 90112)	550 ± 17	690 ± 34	1.48 ± 0.13	2.96 ± 0.14

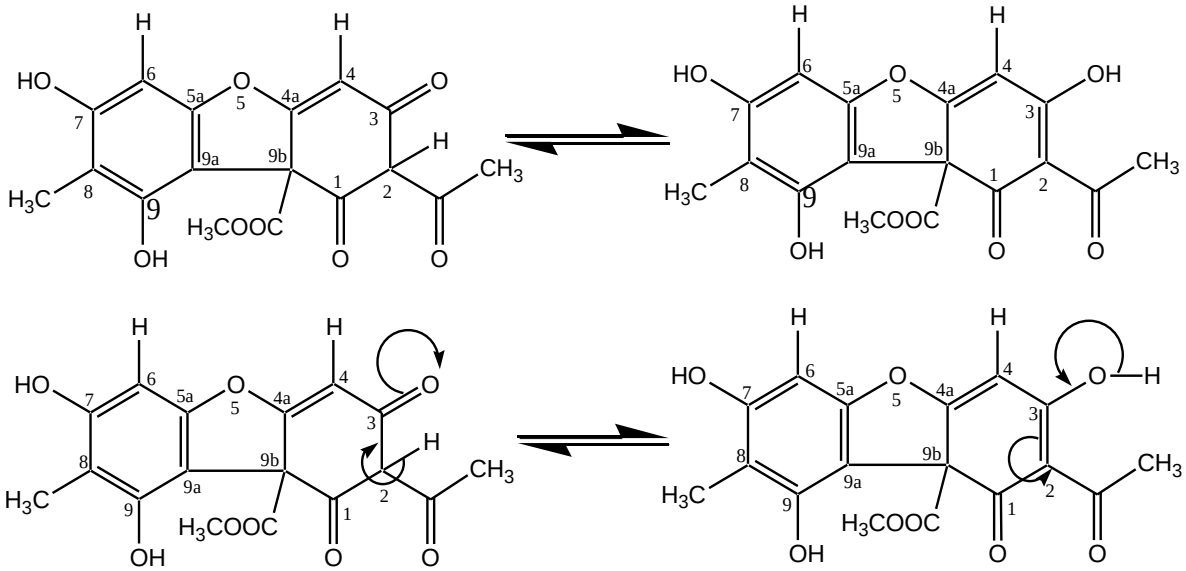

Fig 1: Structure of Compound A

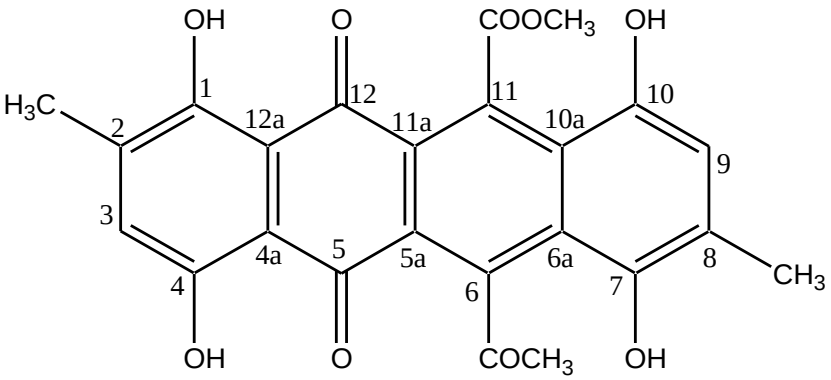

Fig 2: Structure of Compound B

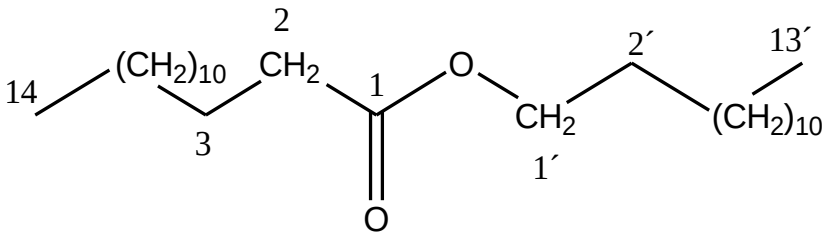

Fig 3: Structure of Compound C

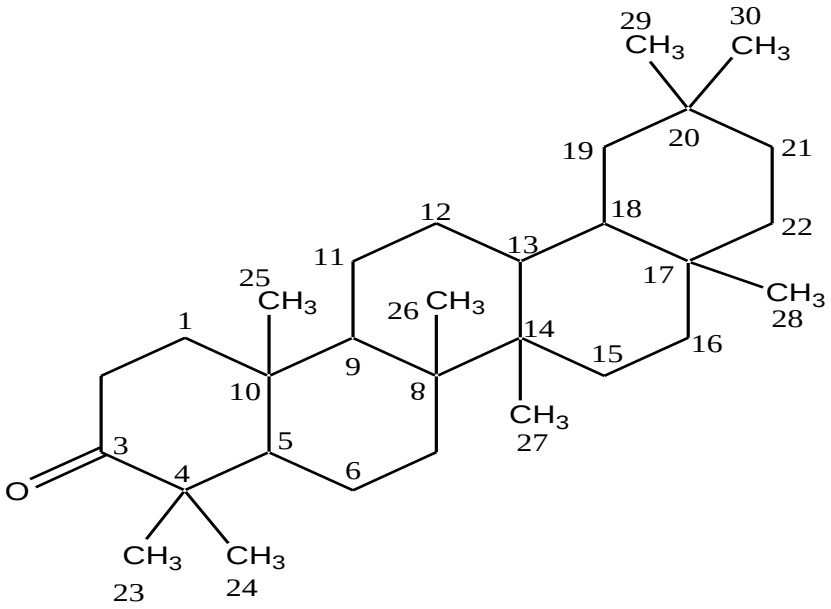

Fig 4: Structure of Compound D

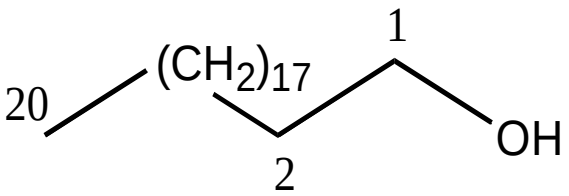

Fig 5: Structure of Compound E

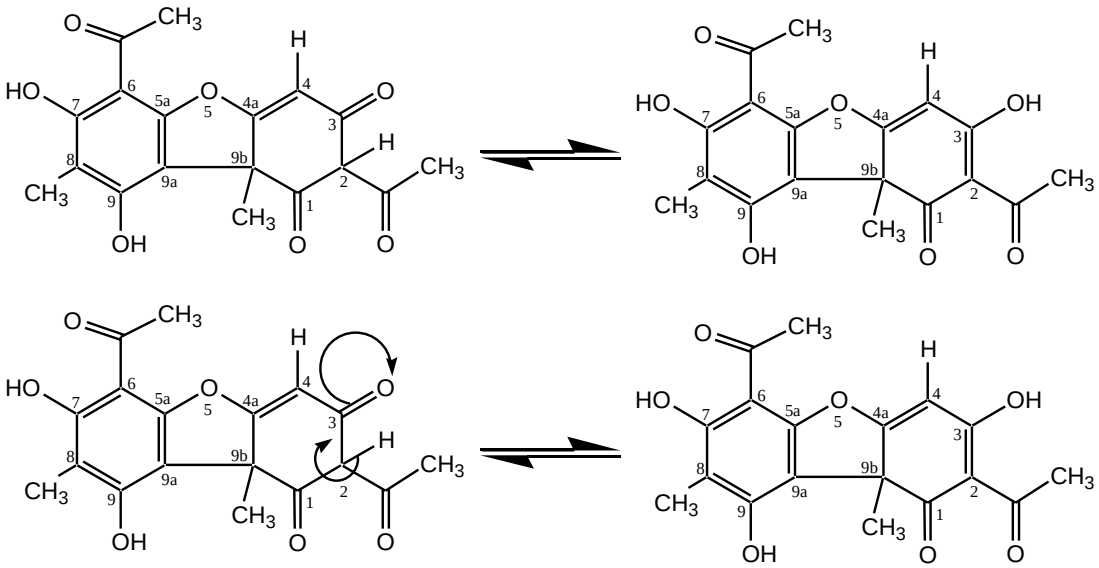

Fig 6: Structure of Compound F

4. Conclusion

The results of our study indicate that *P. perlata* is a promising antifungal agent which provides a veritable basis for further evaluation for possible use as an herbal treatment for candidiasis and cryptococcosis patients.

5. Conflicts of Interest

The authors declare no conflict of interest.

6. Acknowledgments

The authors are thankful to the respective authorities for providing the necessary facilities and support.

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