ISOLATION OF SYMBIONTS AND GC-MS ANALYSIS OF LICHENS COLLECTED FROM OBUDU MOUNTAIN RESORT, SOUTH-SOUTH, NIGERIA.

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ABSTRACT

In Nigeria, a good number of lichen species have been recorded and so far not much work has been done to isolate or identify the symbionts. The utility of lichen comes from a range of secondary compounds produced by them. In view of this, two lichen samples, foliose (*Parmalia reticulata* Taylor) and fruticose (*Usnea subflorida* Zahlbr. Motyka) were collected from Obudu Mountain Resort, Calabar, Nigeria and their symbionts successfully isolated using standard method. The foliose lichen, *Parmelia reticulata* was identified to consist of mycobiont (fungus) and photobiont *Trebouxia* (green alga). The fruticose lichen; *Usnea subflorida* consist of mycobiont (fungus), *Stigonema* (cyanobacterium) and *Trebouxia*. Major components of these two lichens were identified by Gas Chromatography-Mass Spectrometry analysis. Result showed presence of 3,5-Dihydroxytoluene (84.67%) and Benzoic acid 2,4-dihydroxy-3,6-dimethyl-methyl ester (6.23%) in *Parmelia reticulata*. In *Usnea subflorida,* Benzoic acid 2,4-dihydroxy-3,6-dimethyl-methyl ester (32.62%) was the major compound followed by urs-12-en-24-oic acid, 3-oxo- methyl ester, (+)- (21.56%) and 12-Oleanen-3-yl acetate,(3.alpha.) (7.97%). The presence of Resorcinol, (a dye-producing compound) in *Parmelia reticulata* is a pointer to the various ways in which this lichen can be utilized in Nigeria for the production of dyes for use in the manufacturing industry.

Keyword: Lichen, *Permalia reticulata, Usnea,* symbionts, GC-MS, Algae, Fungi.

INTRODUCTION

Lichens are poikilohydric, composite organisms consisting of a fungus (mycobiont) and a photosynthetic partner (photobiont) growing together in a symbiotic relationship. Erickson *et al.* (1947) classified lichens into three major groups based on their thallus structure; crustose, foliose and fruticose. Crustose lichens grow holding tightly to their substrates, which may often be rocks or trees and their colours can range from green, orange, yellow to black (Nash, 1996).

Foliose lichens appear like leaves; they grow on their substrates loosely and can be easily removed intact without damaging the substrates. Fruticose lichens are often shrub-like lichens with plant-like growth pattern and can hang from the substrate from one point (Santis, 1999). Dobson (2000) reported that the photobiont is usually either a green alga or cyanobacterium. According to Nash (1996), lichens are capable of surviving in extremely low levels of water content compare to either the fungus or alga growing independently. Purvis, (2000) gave evidence that lichens inhabit extremely harsh environment on earth. In many species of lichens, the fungus penetrates the algal cell wall, forming haustoria, which is similar to those produced by pathogenic fungi (Hawksworth, 1976; Honegger, 1988, 2000).

However, lichens are abundant as epiphytes on leaf surfaces, trunk, branches, and twigs in rain-forests, temperate woodland, on bare rock, including walls, gravestones and on exposed soil surfaces. Kirk *et al.* (2008) stated that symbiont mode of living of lichens appears to be a very successful way for fungus to derive essential nutrients, as about 20% of all fungal species acquired this mode of life. They found out that a larger number of lichenized fungi occur in the Ascomycota, with about 40% of basidiomycota. Lichen associations may occur in the form of mutualism, commensalism or even parasitism depending on the species.

According to Purvis (2000), the algal or cyanobacteria cells are photosynthetic which reduce atmospheric carbon (iv) oxide into organic carbon sugars to feed both symbionts. Separation and isolation of lichen symbionts have been done by some researchers. Fontaniella *et al.* (2000) reported the separation and isolation of the symbionts by density gradient centrifugation while Gasulla *et. al.* (2012) developed a method, which involves homogenization of lichen thalli, centrifugation, washing with Tween 20 and

sonication.

Müller, (2002) reported that lichens contain metabolites which exert a wide variety of biological actions such as antibiotic, anti-mycobacterial, antiviral, anti-inflammatory, analgesics, antipyretic, anti-proliferative and cytotoxic effects, Lichens are economically useful as a source of food and medicine because of their antibiotic properties. Their harmful effects as reported by dermatology include the presence of Usnic acid, which may be a photosensitizer and a cause of respiratory allergy. Although about 141 species of lichens have been recorded in Nigeria (*BirdLife International,* 2010), not much work has been done to isolate or identify the symbionts which are partners in these lichens.

***Parmelia reticulata* Taylor**

Parmelia species is a large genus of lichenized fungus with a global distribution, extending from the Arctic (Skult, 1985) to the Antarctic continent (Lindsay, 1973) but concentrated in temperate regions. *Parmelia,* a foliose lichen of the division Ascomycota; Class Lecanoromycetes; Order Lecanorales and Family Parmeliaceae. It is popularly referred to as shield lichen.

***Usnea* Species**

Usnea is of the Division Ascomycota, Class Lecanoromycetes, Order Lecanorales and Family Parmeliaceae according to Motyka (1936). This species generally grow hanging from tree branches, resembling grey or greenish hair. It is sometimes referred to as Old Man's Beard, Beard Lichen, Tree's Dandruff, Woman's Long Hair or Tree Moss (Jellin *et al.,* 2000). As a fruticose lichen, *Usnea* appears as a shrub-like growth on host trees. It reproduces via vegetative means through fragmentation, and asexual means through soredia or sexual means through ascogonium and spermatogonium (Marand, 2010).

The usefulness of lichens comes from a range of secondary compounds produced by them, and there is need to conduct extensive research in this regard, in order to exploit the secondary metabolites in them in Nigeria.

The objectives of this work therefore are to isolate and study symbionts partners forming lichens and to analyze for the presence of secondary metabolites presence by using Gas Chromatography-Mass Spectrometry.

MATERIALS AND METHODS

Description of Study Site

Obudu Cattle Ranch as known at present is Obudu Mountain Resort (Fig. 1). It is located on the Obudu Plateau, close to the Cameroon border in the North Eastern part of Cross River State, Nigeria, West Africa with Coordinates (5°34'50"N - 5.580451°N ; 8°44'54"E - 8.748379°E; alt 1,765 m a.s.l.). It is located approximately 110 km east of the town of Ogoja and 65 km from the town of Obudu in Obanliku Local Government Area of Cross River State. It is about 30 minutes' drive from Obudu town and about 332 km drive from Calabar, the Cross River State capital. The climate of the Obudu Mountain Resort is semi-temperate mountane climate, which is the general weather condition experienced on the Obudu plateau due to its altitude.

Figure 1: Obudu Mountain Resort Location. (Source: Google Map**)**

Collection of Lichen Samples

Samples of *Parmelia* and *Usnea* (foliose and fruticose) growing on *Bridelia* tree were collected using scraping tool. Samples were placed on old newspapers and pressed between herbarium presses before transporting to laboratory. Authentication of the lichen samples was done by Mr. Alfred Ozioko (Curator) at the Department of Botany, University of Nigeria, Nsukka.

Isolation of Symbionts

Isolation procedure was carried out at the Department of Botany Laboratory, University of Lagos, to separate the fungus partner and algal and cyanobacteria partner growing as a lichen. Fontaniella *et. al.* (2000) technique was adopted. With this method, sample of dry thallus (0.5 g) of each lichen sample was rinsed in distilled water to remove contamination. The cleaned samples were then macerated in three separate mortars with 10 ml distilled water. The homogenate was filtered through six layers of muslin cloth and the filtrates were centrifuged at 1000 rpm for 10 minutes.The resulting supernatant for each of the samples was discarded, the pellet resuspended in 8.0ml 0.25M Sucrose and 4.0ml of the suspension gently overlayed on the top of three separate 5.0ml of 80 per cent (w/v) Potassium Iodide in centrifuge tubes which was then centrifuged at 200rpm for 45s. Algal and hyphal fragments were found in a broad layer in the Sucrose solution above the KI, while large fragments of non-disrupted thalli form sediments. The layers containing algal cells and hypha fragments were recovered with micropipette and placed in 5.0ml KI solution. Then, 2.0ml of 10mM Phosphate buffer was added and centrifuged at 800rpm for 90s. Algal cells formed an interphase between Phosphate buffer and Sucrose whereas small fragments of fungal hyphae were retained in the bulk of the Sucrose solution. Large hypha fragments were deposited at the bottom of the centrifuge tube as pellets. For each of the samples, the interphase containing algal cells was recovered with a micropipette, deposited on 5ml KI and then, 3.0ml of Phosphate buffer added and centrifuged

at 1000rpm for 3minutes. For purer samples of algal cells, this last step was repeated twice. The fungal fraction obtained as a pellet during the second centrifugation was also recovered and 80% KI was added to a final volume of 4.0ml. The mixture was strongly stirred and 4.0ml 10mM Potassium Phosphate buffer, pH 7.2 was added. The gradient was then centrifuged at 1000rpm for 3minutes. Small algal cells were recovered from the interphase between the buffer and KI solution while fungal hyphae formed sediment at the bottom of the centrifuge tube. Algal contaminants were removed with a micropipette. This process was also repeated twice to yield a pure preparation of fungal cells.

Identification of Symbionts

Algae: After isolation, the algal cells were viewed and identified using Olympus XSZ-N107 microscope.

Fungi: The fungal cells were cultured in Potato Dextrose Agar (PDA). Inoculation was done by incubating the petri-dishes at room temperature for 5 days. Two sets of inoculation were done for each sample and identification carried out upon growth of fungus.

Gas Chromatography-Mass Spectrometer (GC-MS) Analysis

Bioactive compounds present in the lichen samples were identified with the use of Gas Chromatography-Mass Spectrometry (GC-MS), in the Department of Chemistry, University of Lagos. The Gas chromatography analysis was carried out with a 6980N Network GC System, using Elite-5MS column. Helium was used as carrier gas at a flow of 1ml per minute. The injection port was maintained at 250°C and the split ratio was 10:1. Oven temperature programming was done from 5°C to 280°C at 10°C per minute and it was kept at 280°C for 9 minutes. Interface temperature was kept at 250°C. The ionization mode was electron impact ionization and the scanning range from 45 to 450 (m/z). Mass spectra of the compounds were matched with NIST version year 2005 library.

RESULTS

Symbionts: *Parmelia reticulata* and *Trebouxia* were identified growing in partnership to form the foliose type while *Usnea* species were observed with *Trebouxia* and *Stigonema*.

GC-MS Studies

Gas Chromatography-Mass Spectrometry studies revealed six compounds in *Usnea subflorida* with higher composition of benzoic acid, 2,4-dihydroxy-3, 6-dimethyl-, methyl ester (32.62%) followed by urs-12-en-24-oic acid, 3-oxo-, methyl ester, (+)- (21.56%) (Table 1) while *Parmelia reticulata* recorded five compounds with 3, 5-Dihydroxytoluene also known as 5-methyl-resorcinol has highest (84.67%) percentage composition (Table 2). Mass spectrum and structures of bioactive important phytochemical constituents of *Usnea subflorida* is shown in Figure 2a while *Parmelia reticulata* mass spectrum is represented in Figures 3a and 3b. Chemical structures of some of the bioactive compounds are presented on Figures 4a-c.

Table 1: Suspected Bioactive Compounds Identified in the Thallus of *Usnea subflorida*

S/No.	RT	Name of Compound	Percentage composition
1.	17.97	1,3-Benzenediol 4, 5-dimethyl-	1.24
2.	24.24	Benzoic acid 2, 4-dihydroxy-6-methyl-methyl ester	2.24
3.	25.49	Benzoic acid 2, 4-dihydroxy-3, 6-dimethyl-methyl ester	32.62
4.	42.04	1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl)ester	0.94
5.	47.96	12-Oleanen-3-yl acetate, (3.alpha.)	7.97
6.	49.80	Urs-12-en-24-oic acid, 3-oxo-, methyl ester, (+)-	21.56
7.	49.92	.alpha.-Amyrin	6.13

*RT – Retention time

Table 2: Suspected Bioactive Compounds Identified in the Thallus of *Parmelia reticulata*

S/No.	RT	Chemical Compound	Percentage composition
1.	17.08	3,5-Dihydroxytoluene	84.67
2.	22.35	Cyclotetradecane	0.21
3.	25.46	Benzoic acid 2,4-dihydroxy-3, 6-dimethyl-methyl ester	6.23
4.	26.10	Ethyl 2,4-dihydroxy-6-methylbenzoate	2.72
5.	31.03	n-Hexadecanoic acid	0.34

*RT – Retention time

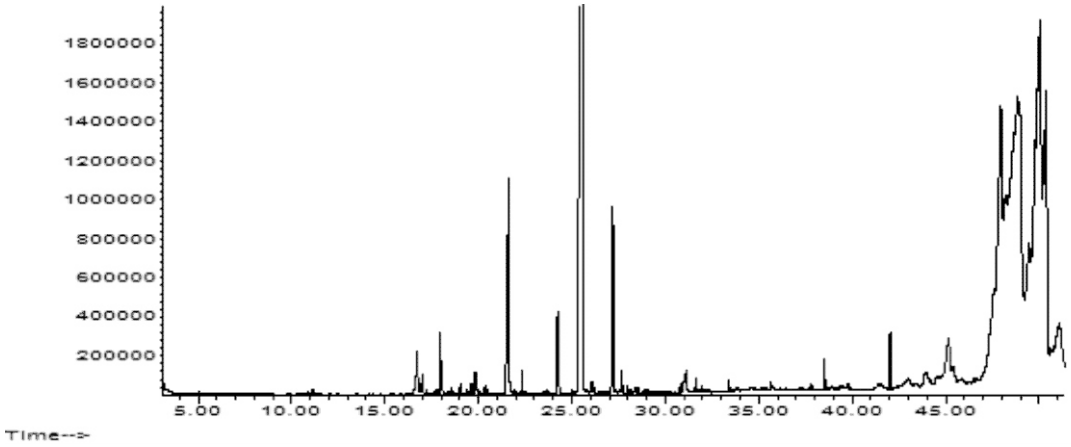

Figure 2a: GC-MS Chromatogram of the Hexane Extract of *Usnea subflorida*

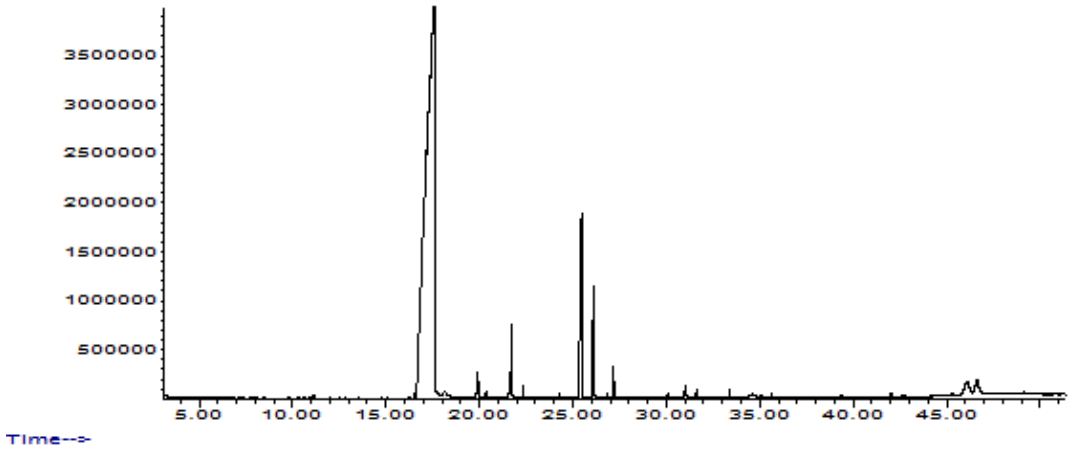

Figure 3a: GC-MS Chromatogram of the Hexane Extract of *Parmelia reticulata*

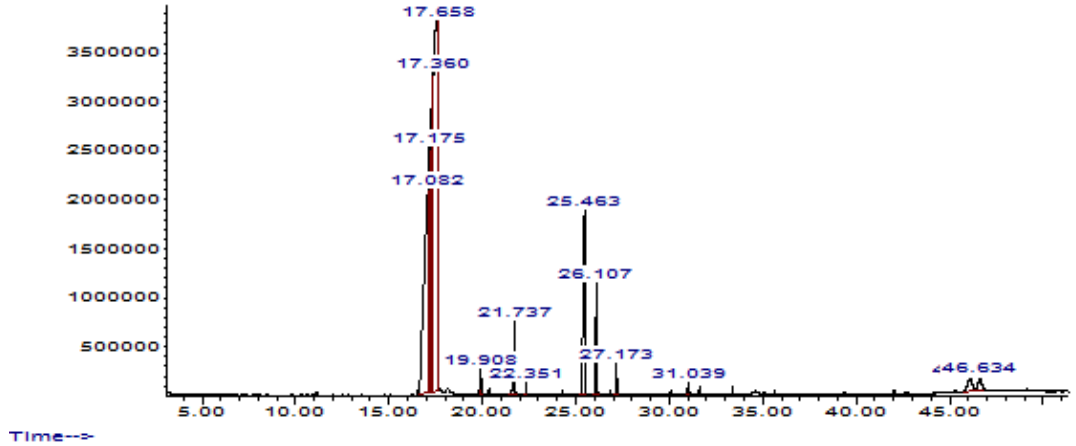

Figure 3b: GC-MS Chromatogram of the Hexane Extract of *Parmelia reticulata* Showing Retention Time

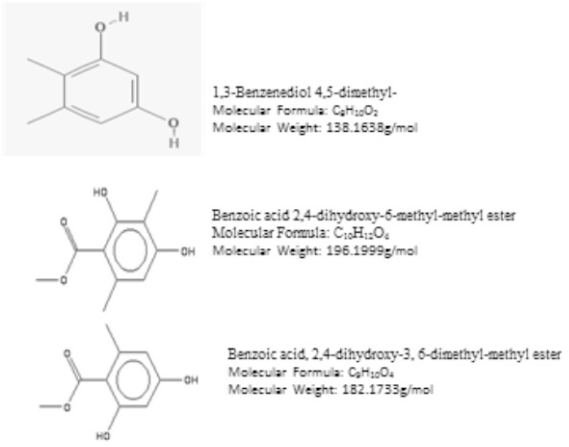

Figure 4a: Chemical structures of some of the bioactive compounds

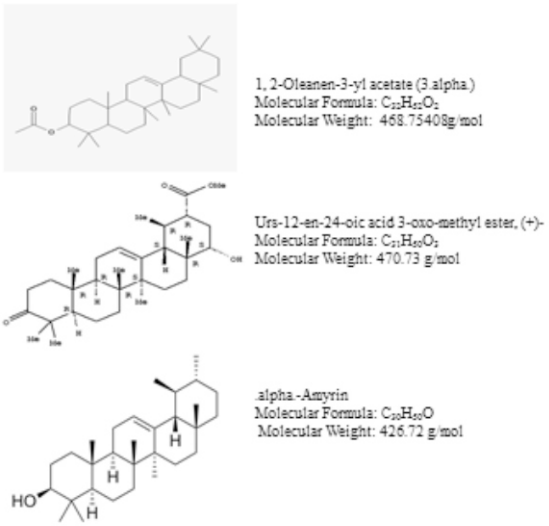

Figure 4b: Chemical structures of some of the bioactive compounds

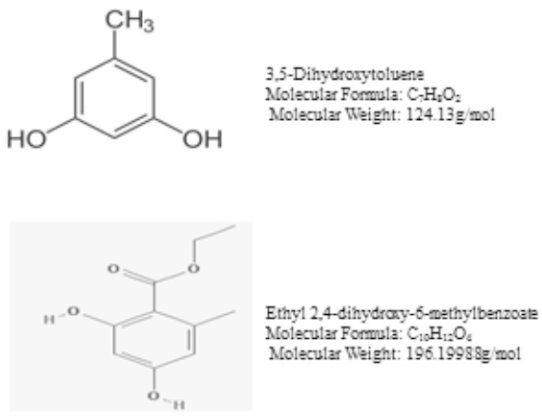

Figure 4c: Chemical structures of some of the bioactive compounds

Source: National Institute of Standards and Technology (2011)

DISCUSSION

He and Zhang (2012) provided new insight into the biological composition of *Usnea*, highlighting its microbial diversity while Grube and Muggia (2010) reported that lichen fungi may internalize more than one algal symbiont, which is in conformity with *Usnea subflorida* in this study. Identification of *Treuboxia* in both genera (*Parmelia* and *Usnea*) is in line with Baurley (2013) who reported that *Trebouxia* is the most common alga occurring in about 40% of all lichens. *Stigonema* has also been suggested as one of the common algal components of the dark-brown lichens such as *Usnea* according to Hawksworth and Francis (1976). The dominance of 3,5-Dihydroxytoluene which is used to produce Orcein, a food dye according to Musso (1960) is an indication of importance of *Parmelia reticulata*.

The presence of Benzoic acid 2,4-dihydroxy-3,6-dimethyl-methyl ester which has been reported by Warth (1991) as compound that can inhibit the growth of mold, yeast and some bacteria with its salts used as food preservatives and also being reported by Wilson *et. al.,* (2004) as one of the constituents of Whitfield's ointment which is used for the treatment of fungal skin diseases such as tinea, ringworm, and athlete's foot is of great importance in this study. *Usnea* species contained Urs-12-en-24-oic acid, 3-oxo methyl ester (+)- which has medicinal applications. Usnic acid ($C_{18}H_{16}O_7$) is a potent antibiotic and antifungal agent found in all species (Buhner, 1999). This, combined with the hair-like structure of the lichen made *Usnea* useful in treating surface wounds when steriled gauze and modern antibiotics were unavailable (Tilford, 1997). Therefore, this study has established the potentials of *Parmelia reticulata* in dye manufacturing industry and *Usnea subflorida* in phytochemistry and medicine which can be exploited in Nigeria.

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