EVALUATION OF PHYTOCHEMICAL CONTENT OF CALOCYBE INDICA ISOLATED IN NIGERIA FOR A POSSIBLE THERAPUTIC USE

Authors: Okorie, Princewill Chimezie 1*, Ofoegbu, V. O., Akinyemi, A. O., Abikoye, E. T. Okere, V.O., Lawal, A. K and Asagbra, A. E.
1, : Federal Institute of Industrial Research Oshodi, Lagos, Nig.

Corresponding author: Okorie, Princewill Chimezie

Email: chimezieokorie@yahoo.co.uk

ABSTRACT

Calocybe indica is a common mushroom species in Nigeria. Oral reports in Nigeria suggest that this mushroom species has some therapeutic applications. This study was therefore designed to evaluate the phytochemical composition of this mushroom species with the intent of determining its possible medicinal uses. Pure culture of this mushroom species obtained from the wild was isolated and cultivated on a cassava based substrate to produce the fruiting body. The produced fruiting body was evaluated for its phytochemical content. Qualitative and quantitative analysis of the following phytochemical components were determined following established protocols; tannins, saponins, flavonoids, anthroquinones, alkaloids, phenolic compounds, glycosides, terpenoids and phlobatannins. Both aqueous and methanolic extracts showed the presence of phenols, terpenoids, saponins, glycosides, and alkaloids while flavonoids, tannins, anthroquinones and phlobatannins were absent. The quantitative analysis of this study indicated varying quantities of tannins, alkaloids, flavonoids and phenols in both aqueous and methanolic extracts of *C.indica,* though flavonoids were not detected in the aqueous extract. DPPH scavenging activities of aqueous and methanolic extracts of this mushroom were 56.2% and 65% respectively. The aqueous extract has a lower anti-radical scavenging power compared to the methanolic extract. This mushroom species indeed has potentials as therapeutic agent for management of some human disease conditions.

Key words: Phytochemical content, *Calocybe indica*, isolated in Nigeria

INTRODUCTION:

Calocybe indica, commonly known as milky white mushroom is a popular mushroom species commonly seen growing on humus rich soil in agricultural fields or along the roads in the tropical areas. They grow every year between the months of May and August, which coincides with the period of sufficient rainfall required by the mushroom for its growth. It is a saprophytic

organism but sometimes are ectomycorrhizal (having a symbiotic relationship with roots of some plants like *Cocos nucifera,, Borassus flabellifer,, Tamarindus indica and Peltophorum ferruginum)*. Small scale mushroom growers prefer to cultivate this mushroom species because it is ideally suited for warm tropical climate, its long shelf life and with short crop cycle.

It has been shown for a long time that some mushroom strains can be used as medicinal herbs for therapeutic purposes. Research has shown that some mushroom species possess anti-tumor properties and could be effectively deployed in the treatment of cancer. Specific polysaccharides, known as beta-D-glucans, have been shown to stimulate the immune system to fight cancerous cells. One of such beta-D-glucan isolated from oyster mushrooms is called pleuran and currently is going through studies to determine its effectiveness in the treatment of cancer. *Auricularia* species is used as poultice for inflame eyes and gargled inflammation of the throat. Others mushroom species are used as remedy for chronic cararrh, disease of the breast and lungs, tuberculosis, rheumatism, jaundice and intestinal worms.

Phytochemical analysis is important in identifying bioactive substances for therapeutic and medicinal benefits. This study was designed to evaluate the phytochemical composition of this mushroom species with the intent of determining its possible medicinal uses. The following phytochemicals were evaluated in this study; tannins, saponins, flavonoids, anthroquinones, alkaloids, phenolic compounds, glycosides, terpenoids and phlobatannins. Records available show that some of these phytochemicals have some beneficial health effect. Although saponins have been reported to be toxic, they may be beneficial since they have been found to lower plasma cholesterol (Oakenfull *et al.*, 1979). Saponins and alkaloids have been found to show prominent antibacterial activity against *Staphylococcus aureus, Bacillus subtilis*, *Bacillus cereus* and *Klebsiella pneumonia* (Khan *et al*., 2012). Sterols have also been found to exhibit antimicrobial activity against *Staphylococcus aureus*. Some of these phytochemicals have been shown to have antioxidant properties (Khan *et al.,* 2012). In some plants, saponins may serve as anti-feedants, and to protect the plant against microbes and fungi. Some plant saponins may enhance nutrient absorption and aid in animal digestion (Foester, 2006). They are being promoted commercially as dietary supplements and nutriceuticals (Francis *et al*., 2002).

Ifeoluwa (2015) reported that one of the values of commercial cultivation of mushrooms, especially in a developing economy like Nigeria, is the availability of large quantities of several agro–industrial wastes which can serve as substrates for their cultivation. Recent research efforts at the Federal Institute of Industrial Research Oshodi, Lagos, Nigeria (FIIRO) have shown that

cassava peels can effectively be deployed in the commercial production of edible mushroom species like the oyster mushrooms and *Calocybe indca*. Results from these research efforts show that cassava peels can also be used in the production of spawn (mushroom seed) and as a casing material for the cultivation of *Calocybe indica*. This research effort deployed cassava peels in the cultivation of the mushroom species used in this study.

MATERIALS AND METHODS

Cultivation of the mushroom species

Pure culture of *Calocybe indica* obtained from the wild and identified at the Biotechnology Department of Federal Institute of Industrial Research, Oshodi, Lagos, Nigeria was grown on potato dextrose agar following standard mycological procedure. The obtained pure culture was used to produce spawn of the mushroom species following established protocol. Produced spawn was used to cultivate the mushroom fruit bodies using cassava peels.

Cassava peels for the mushroom cultivation were first dried to remove hydrogen cyanide because mushrooms pick up the content of their substrates. Prior to usage of the dried peels for cultivation, they were soaked overnight with 2% $CaCO_3$ for further fermentation for complete removal of residual cyanide. The dried cassava peels were composted with $CaCO_3$ and watered at a ratio of 32:2:66 respectively. Composted substrates were dispensed into nylon bags and pasteurized at 100^0c for 3-4hrs depending on the size and volume of the substrates. Pasteurized substrates were allowed to cool to ambient temperature before being inoculated with the spawn. Inoculation was done under aseptic conditions. Incubation stage was done in darkness. Fully ramified substrates were exposed for fruiting.

Phytochemical assay

Fully grown fruit bodies were harvested and subjected to both qualitative and quantitative analysis to determine the phytochemical content.

Preparation of extract

a) The fruiting bodies of *Calocybe indica* were dried, grounded and stored for analysis.

b) 5grams of the grounded mushroom was mixed with 5ml of both methanol and water respectively. These mixtures were left to stand for 24 hours and filtered. The filtrate was then analyzed for phytochemicals.

Qualitative assay

Qualitative analysis of phytochemicals was carried out according to the methods described by Wandati *et al*. (2013) and Vimalkumar *et al*. (2014) with some modifications.

Test for Tannins: This was done following ferric chloride test. Two milliliters (2.5ml) of extract was pipetted into a test tube and 1ml of 0.1% ferric chloride was added to it and mixed thoroughly. Formation of greenish-black precipitate indicated the presence of tannins. However, a milky-brown precipitate in both extracts indicated the absence of tannins.

Test for saponins: This was done according to frothing test method. One milliliter (1ml) of extract was added into 2.5ml of distilled water and the mixture heated while shaking vigorously. The presence of persistent foam lasting for at least 5minutes indicated the presence of saponins in both extracts.

Test for Flavonoids: One milliliter (1ml) of extract was added into 1ml of dilute sodium hydroxide and hydrochloric acid solutions respectively and filtered. Zinc dust and concentrated hydrochloric acid were added to the filtrate, no colour formation indicated the absence of flavonoids.

Test for anthroquinones: Few drops of 10% Hydrochloric acid solution were added to 0.5ml of the extract and boiled for 5 minutes, filtered and cooled. Two milliliter (2ml) of chloroform was added to 2ml of filtrate, and few drops of 10% ammonium solution added and heated. Absence of colour formation showed the absence of anthroquinones in both extracts.

Test for alkaloids: Two milliliters (2ml) of extract was heated, and 2%v/v sulphuric acid solution and 1ml of iodine in potassium iodide solution were added respectively. The presence of brown precipitate indicated the presence of alkaloids in both extracts.

Test for phenolic compounds (phenols): Two milliliter (2ml) of extract was added into 5ml of 95% v/v ethanol; the mixture was boiled in a water bath for 5minutes and filtered. Five milliliter (5ml) of distilled water was added to the filtrate and heated to evaporate the ethanol. Five (5) drops of 1% v/v ferric chloride solution was added to the concentrated mixture and 1% w/v of potassium ferricyanide solution.

Test for glycosides: Few drops of hydrochloric acid solution and sodium hydroxide solution were added to 2ml of the extract, few drops of 5% w/v ferric chloride solution and 1ml of

concentrated sulphuric acid were further added. The formation of reddish brown ring indicated the presence of glycosides.

Test for terpenoids: Two milliliters (2ml) of extract was added into 2ml of chloroform and mixed together. Three milliliters (3ml) of concentrated sulphuric acid was added to the mixture. A deep red colour formation indicated the presence of terpenoids.

Test for phlobatannins: Three milliliters (3ml) of distilled water were added to 2ml of extract and filtered. To the fitrate, few drops of 2% hydrochloric acid solution were added and boiled. The absence of a red precipitate showed the absence of phlobatannis.

Quantitative assay

Determination of tannins: This was done according to Harbone (1973) method with modifications. Five milliliters (5ml) of extract was pipetted into a 50ml volumetric flask and 35ml of distilled water was added. One milliliter (1ml) of Folin-Denis reagent and 2.5ml of saturated sodium carbonate solution were added. Distilled water was added to the mixture to make up to 50ml and mixed well. The mixture was left to stand to for 1hr 30minutes. Samples were filtered and absorbance measured at 760nm against a blank. The quantity of tannins was calculated using the equation below:

$$\text{Tannins (mg/100g)} = \frac{\text{absorbance of sample}}{\text{absorbance of standard}} \times \frac{100}{1}$$

Determination of alkaloids: The quantity of alkaloids in the samples was determined as follows: 2.5ml of the extract was measured into test tubes and 1.25ml of 0.2M sodium nitrate added to the samples. One milliliter (1ml) of 1% v/v sulphuric acid solution and 1ml of 20%ammonia solution were added to the mixtures, shaken to mix well and allowed to stand at room temperature. One point five milliliters (1.5ml) of Nessler's reagent was added and absorbance taken at 560nm using a spectrophotometer. A known standard of alkaloid concentration 5mg/ml was prepared and treated as the samples. The quantity of alkaloids in the samples was calculated using the formula below:

$$\text{Alkaloids (mg/100g)} = \frac{\text{absorbance of sample}}{\text{absorbance of standard}} \times \frac{\text{standard conc.}}{1} \times \frac{100}{1}$$

Determination of total flavonoid content: Total flavonoid content (TFC) of the extracts was determined following the method described by Wandati *et al*. (2013) using rutin as standard. One milliliter (1ml) of extract was diluted with 4ml of distilled water in a 10ml volumetric flask, 0.3ml of 5% w/v sodium nitrite was added to sample solution and left to stand for 5minutes.

0.3ml of 10% w/v aluminuim chloride was added to sample mixture and 6 minutes later, 2ml 2M sodium hydroxide solution was added. Two milliliter (2.4ml) of distilled water was added to the mixture and mixed thoroughly. The absorbance of the reaction mixture was read at 510nm. The total flavonoid content of the extract was expressed as quercertin equivalent (mg/g) of dry weight with the formula below:

$$\text{TFC (mgQE/g)} = \frac{\text{absorbance of sample - absorbance of control}}{\text{Absorbance of control}} \times \frac{100}{1}$$

Determination of total phenolic content: Total phenolic contents (TPC) of the extracts was determined using Folin-Ciocalteu reagent method as described by Sumathy et al. One milliliter (1ml) of extract was put into test tube followed by 5ml of a 10 fold dilution of Folin-Ciocalteu reagent and 4ml of 7.5% w/v sodium bicarbonate; shaken to mix well and left to stand for 30minutes at room temperature. The absorbance was measured at 765nm on a spectrophotometer. The total phenolic content was expressed as gallic acid equivalents (GAE) in milligrams per gram of dry weight/material using the formula below:

$$\text{TPC (mgGAE/g)} = \frac{\text{absorbance of sample}}{1} \times \frac{\text{standard conc.}}{\text{absorbance of standard}}$$

DPPH Radical Scavenging assay: The free radical scavenging activities of both aqueous and methanolic extracts were determined by 1, 1-diphenyl-2-picyl-hydrazil (DPPH) according to the method by Shimada with modifications. 0.1mM solution of DPPH in ethanol was prepared; 2.5ml of extract was added to 1ml DPPH solution. The mixture was shaken vigorously and left to stand for 30minutes at room temperature. The absorbance was read at 518nm using a spectrophotometer. Low absorbance indicated high free radical scavenging activity. The percentage scavenging activity was measured using the formula below:

$$\text{DPPH Scavenging effect} = \frac{\text{absorbance of sample} \times 100}{\text{absorbance of control} \quad 1}$$

Results and Discussion

Mushrooms lack chlorophyll and cannot like green plants, manufacture their own food through photosynthesis. They are saprophytes and grow on dead organic matters of vegetative origin, mainly agro-industrial wastes such as rice straw, wheat straw, sawdust, sugarcane bagasse and cotton waste. Ifeoluwa. (2015) reported that one of the values of commercial cultivation of

mushrooms, especially in a developing economy like Nigeria, is the availability of large quantities of several agro–industrial wastes which can serve as substrates for their cultivation. In this study, cassava peel was effectively deployed in the cultivation of *Calocybe indica* (Plate 1). Anton *et al*. (2015) recorded similar result when they successfully employed cassava peels in the cultivation of Oyster mushroom. Ifeoluwa *et al*. (2015) in their study of "effect of chemical preservative on the shelf life of *Pleurotus ostreatus"* reported a successful deployment of cassava peels in the cultivation of their mushroom species.

Plate 1; *Calocybe indica* grown on cassava-based substrate

Phytochemical analysis is important in identifying bioactive substances present in any mushroom species for possible therapeutic and medicinal benefits. Qualitative analysis of *Calocybe indica* extracts was carried out to evaluate the presence or absence of the following phytochemicals: phenols flavonoids, terpenoids, alkaloids, glycosides, tannins, phlobatannins, anthroquinones and saponins. Both aqueous and methanolic extracts revealed the presence of phenols, terpenoids, saponins, glycosides, and alkaloids while flavonoids, tannins, anthroquinones and phlobatannins were absent (Table 1). The quantitative analysis of this study indicated varying quantities of tannins, alkaloids, flavonoids and phenols in both aqueous and methanolic extracts of *C.indica* though flavonoids were not detected in the aqueous extract (Table 2 and Figures 1and 2). DPPH scavenging activities of aqueous and methanolic extracts were 56.2% and 65% respectively. The aqueous extract has a lower anti-radical power compared to the methanolic extract.

Antioxidants have many applications that are beneficial to human health in relation to prevention and management of diseases (Vimalkumar *et al.*, 2014). Phenols and flavonoids are good natural antioxidants and both were detected in study, suggesting a potential of this mushroom species as possible agent in the management of disease condition. Previous studies report that phenols, flavonoids, tannins and terpenoids have many medicinal/ pharmacological benefits which include anti-inflammatory, anti-cancer and anti-malaria (Wandati and Kenji, 2013, Pandimeena *et al*., 2015). Phenolic compounds are found in antiseptics and disinfectants due to their anti-fungal, therapeutic and antiseptic characteristics (Gill, 1992).

Saponins have been reported to possess anti-diabetic and cytotoxic attributes (Wandati and Kenji, 2013), and they have the ability to lower blood sugar level and also cholesterol levels (Pandimeena *et al*., 2015). They were detected in this study and therefore suggest of a possible use of this mushroom species in the management of diabetics and cholesterol related human disease conditions. Saponins and alkaloids have also been shown to have prominent antibacterial activity against *Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus cereus* and *Klebsiella*

pneumonia (Khan *et al*., 2012). Their presence in the extract of *Calocybe indica* as established in this study also suggest of possible application of this mushroom species in the control of these human infectious agents.

Tannins have astringent properties which enable quick healing of wounds (Okwu, 2004). Alkaloids are used for their basic medicinal benefits which include analgesic, bactericidal and anti-spamodic effects (Stary, 1998). Sterols have also been found to exhibit antimicrobial activity against *Staphylococcus aureus*. These chemical agents were detected in the extract of *Calocybe indica* as this study has revealed. This mushroom species therefore has potentials as anti-microbial agent and therapeutic agent in the management of some disease conditions.

Table 1 Qualitative analysis

Phytochemicals	Aqueous extract	Methanoic extract
Phenols	+	+
Tannins	-	-
Flavonoids	-	-
Saponins	+	+
Terpenoids	+	+
Glycosides	+	+
Anthroquinones	-	-
Phlobatannins	-	-
Alkaloids	+	+

Key: + = Present
- = Absent

Table 2: Quantitative Analysis and Scavenging activity of *C. indica*

Parameters	Aqueous extracts	Methanolic extracts
Tannins	146.5 mg/100g	134.4mg/100g
Alkaloids	2317 mg/100g	2979 g/100g
Flavonoids	-	505 mgQE/g
Phenols	361.4 mgGAE/g	708.5 mg/GAE/g
DPPH	56.2%	65%

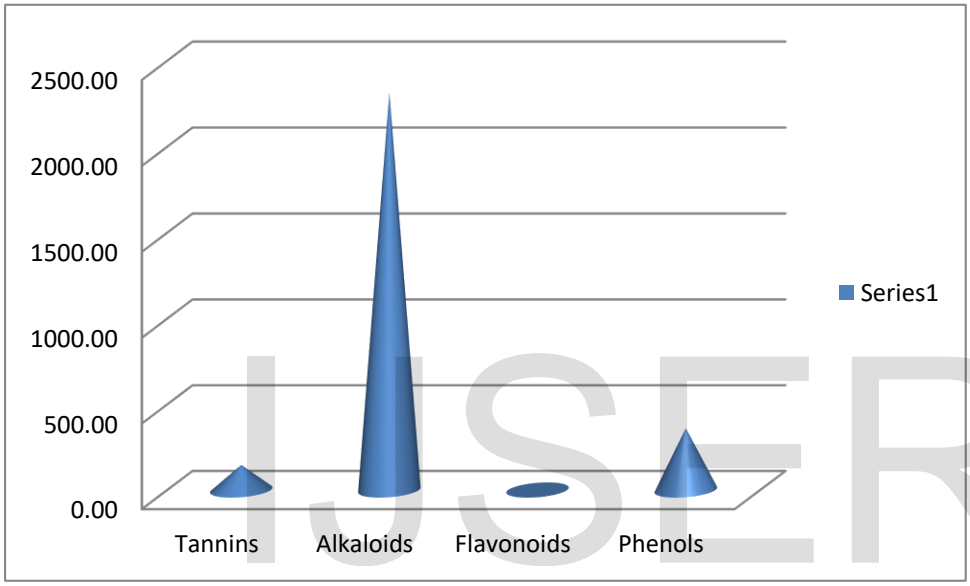

Figure 1: **Phytochemical analysis of *C. indica* (aqueous extract)**

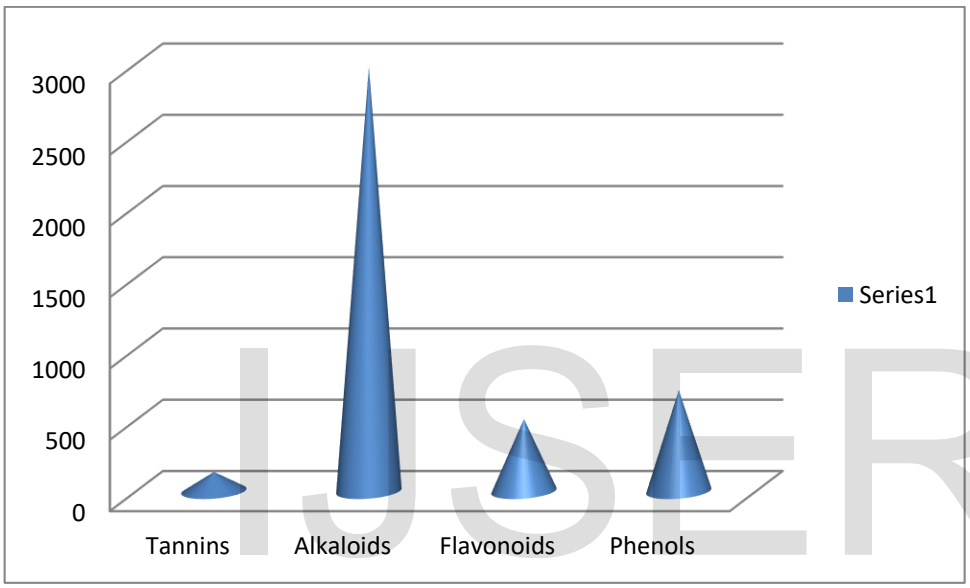

Figure 2: **Phytochemical analysis of *C. indica* (methanolic extracts)**

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