Isolation and Characterization and Antimicrobial Activity of *Calocybe Indica* Apk-2

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ABSTRACT

The present study was aimed to Isolate and characterize the active constituents from higher Basidiomycetes *Calocybe Indica* Apk-2, an indigenious variety developed by Tamil Nadu Agricultural University, Coimbatore. Extraction was done with various solvents like petroleum ether, ethyl acetate, chloroform, methanol and water. Methanolic extract was found to contain Alkaloids, Carbohydrates, Glycosides, Phytosterols, Terpenoids and Flavonoids. Aqueous extract was found to contain Protein, Amino acids, Carbohydrates and Glycosides. Methanolic extract was found to have significant inhibitory activity against *Bacillus cereus and Bacillus lentus* and moderate effect against other micro organisms. Aqueous extract was found to have significant activity against *pseudomonas and azotogensis and bacillus cereus.* Methanolic extract was subjected to column chromatography to isolate its components and the active compound was characterised by using spectral analysis.

INTRODUCTION

Mushrooms are one of the major constituents of fungi family and commonly used as a food supplements. Medicinal mushrooms have been used since ancient times in folk medicine throughout the world. Mushrooms are considered to be natural nutraceuticals and are cultivated for both edible and medicinal purposes[1, 2]. Mushrooms are often used as adaptogen and immunostimulant[3]. Cellular components and secondary metabolites of many mushrooms have been shown to affect host immune systems and might be used to treat immunodeficiency diseases, immuno suppression after drug treatment and a variety of diseases including cancer[4].

The major drawback of such medicinal products from plant and fungi sources is lack of chemical characterization and standardization. Identification still remains unclear for the buyer, manufacturer, distributor and consumer.

The research scenario on medicinal properties of mushrooms in India is on a promising note with lot of opportunities for young scientists. The National center for mushroom research and training and chambaghat, solan, H.P was set up with the objective to promote research on mushrooms in India.

We had made an attempt to isolate and characterize the constituents from Indian Milky white mushroom *Calocybe Indica* (Apk 2) an indigenous variety developed by Tamilnadu Agricultural University, Coimbatore. Medicinal property of this variety had not been reported, we worked on the identifying the components and antimicrobial activity of *Calocybe Indica* (Apk2).

MATERIALS AND METHODS

Plant extraction

Calocybe Indica (Apk 2) was obtained from Tamilnadu agriculture university, Coimbatore. Extraction was done by Soxhlet extraction and Percolation method. 200 gm of *Calocybe Indica* (Apk2) was extracted with various solvents like petroleum ether, ethyl acetate, chloroform, methanol and water.

Phytochemical Analysis

Identification of various phytoconstituents such as alkaloids, phenolic compounds and tannins, flavonoids, carbohydrates and sugar were performed using standard protocols[5, 6].

Antimicrobial Screening

Methanolic and Aqueous extracts of *Calocybe Indica* (Apk 2) was subjected to antimicrobial screening by disc diffusion method on different test organisms. Antimicrobial activity of the extract was quantitatively estimated by measuring the diameter of zone of inhibition[7].

Isolation and characterization of components

The components present in methonolic extract of *calocybe Indica* (APK-2) was isolated by column chromatography. Isolation was carried out with different solvents of increasing polarity in different ratios[8]. The isolated components were then characterized by using UV, IR, NMR & Mass spectras. The purity of isolated compound was established by using HPTLC finger print analysis.

RESULTS AND DISCUSSION

The percentage yields of the extracts with various solvents were calculated and the data is given in Table 1. The percentage yield of Methanol was highest with 35% followed by Aqueous and Ethyl acetate extracts. The phytochemical analysis for qualitative determination of the phytoconstituents present in various extracts is given in Table - 2. The data shows that methonolic extract was found to contain Alkaloids, Carbohydrates, Glycosides, Phytosterols, Terpenoids and Flavonoids. Aqueous extract was found to contain Protein, Amino acids, Carbohydrates and Glycosides.

Table -1: Percentage yields of extracts

S.NO	Extract	% Yield
1	Petroleum ether	5%
2	Ethyl acetate	17.15%
3	Chloroform	10%
4	Methanol	35%
5	Aqueous	28%

Table - 2 Preliminary phytochemical analysis of *Calocybe Indica* (APK-2) Mushroom extract

Extracts	Alkaloids	Protein & Amino acid	Carbohydrates	Tannins	Glycosides	Phyto sterols	Terpenoids	Flavonoids
Pet ether	-	+	-	-	-	+	-	-
Ethyl acetate	-	-	+	-	-	-	+	+
Chloroform	+	-	-	+	-	-	-	-
Methanol	+	-	+	-	+	+	+	+
Aqueous	-	+	+	-	+	-	-	-

(+) – Present (-) – Absent

Antimicrobial Screening of Methanolic and aqueous extracts

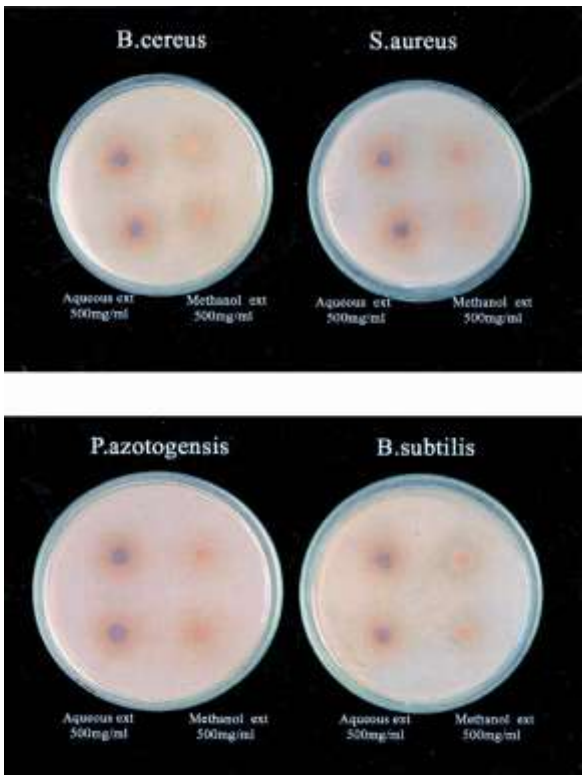
Methanolic and Aqueous extracts of *Calocybe Indica* (Apk 2) was subjected to antimicrobial screening by disc diffusion method on different test organisms. Antimicrobial activity of the extract was quantitatively estimated by measuring the diameter of zone of inhibition. Results were shown in Table - 3.

Table – 3: Antibacterial activity of Methanolic aqueous extracts of *calocybe indica* (APK-2) by disc diffusion method

S.No	Micro Organism	Diameter of Zone of Inhibition in mm		
		Methanolic extract 500 mg / ml	Aqueous extract 500 mg/ml	Standard Oflaxacin 5 mcg/disc
1	*Escherichia coli* (NCIM 2065)	15	19	21
2	*Pseudomonas aeruginosa* (NCIM 2200)	14	19	23
3	*Pseudomonas azotogensis* (NCIM 2075)	15	23	25
4	*Klebsiella aeruginosa* (NCIM 2239)	17	20	19
5	*Bacillus subtilis* (NCIM 2063)	15	18	33
6	*Bacillus cereus* (NCIM 2155)	24	22	27
7	*Bacillus lentus* (NCIM 2018)	18	21	18
8	*Staphylococcus aureus* (NCIM 2079)	18	20	30

From the data it was observed that methanolic extract was found to have significant inhibitory activity against *Bacillus cereus and Bacillus lentus* and moderate effect against other micro organisms. Aqueous extract was found to have significant activity against *pseudomonas and azotogensis and bacillus cereus* (Fig. 1).

Fig 1: Antibacterial activity of Methanolic aqueous extracts of *Calocybe Indica* (APK-2) by disc diffusion method



Isolation of components

Based on the antimicrobial screening data, we chose to isolate the components present in methonolic extract *calocybe Indica* (APK-2) by column chromatography. Isolation was carried out with different solvents of increasing polarity in different ratios. Elution was started with pure petroleum ether and polarity was increased slowly by gradual addition of other solvents. When extract was eluted with petroleum ether, ethyl acetate (99:1) solvent system compound I was obtained. It was found to be less in quantity. When methanolic extract was eluted with petroleum ether, ethyl acetate (96:4) solvent system compound II was obtained and the yield was good. It was further subjected to characterization. Phytochemical analysis data of the eluted components were given in Table–4. From the data it was found that compound eluted with 4% petroleum ether, ethyl acetate (96:4) gave positive results for phytosterols and triterpenoids.

Table – 4: Preliminary Phytochemical analysis of eluted components from the Colum Chromatography

S.No	Tests	Compounds		
		1%	2%	3&4%
1	Alkaloids	-	-	-
2	Carbohydrates	-	-	-
3	Protein & Amino Acids	-	-	-
4	Flavonoids	-	-	-
5	Phytosterols & Triterpinoids	+	-	+
6	Glycosides	-	-	-
7	Tannins	-	-	-
8	Resins	-	+	-
9	Saponins	-	-	-

Table – 5 gives the description of eluted compounds. Compound II isolated with 3% & 4% petroleum ether and ethyl acetate was found to be white crystals in nature with a melting point of 140^{o}C and Rf value of 0.083. Compound I isolated with 1% was white powder in nature with a melting point of 110 ^{o}C and Rf value of 0.461

Table – 5: Description of eluted compounds

Compound	Eluted compound	Nature	Solubility	Melting point	Rf value
I	1%	White Powder	Soluble in petroleum ether Insoluble in water Insoluble in ethanol	110°C	0.461
II	3&4%	White crystals	Slightly soluble in water Soluble in ethyl acetate Insoluble in petroleum ether	140°C	0.083

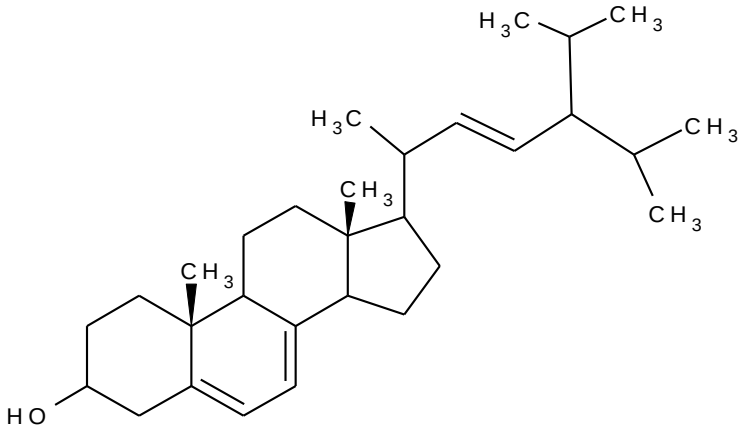
Characterisation of compound II

Its molecular formula was established as $C_{30}H_{48}O$. Elemental analysis shows that C=84.90 and H=11.32. From the UV spectrum λ max of the compound II was found to be at 330.0 and 306.5nm. Its IR spectrum exhibited absorptions at 3450 (br), 2955, L 1374 cm-1, showed the presence of –OH, alkyl and isopropyl groups, respectively.

The 1H NMR spectrum showed a doublet at δ 5.39 and a multiplet at δ 3.82 which are the characteristic pattern of the Δ5-3β - hydroxyl steroids. The signals in the region δ 0.54–2.49 suggested the presence of CH_2 and CH_3 entity of the steroid nucleus and as the side chain. A quartet at δ 5.21 was assigned for the protons at C22 and C23 denoting a double bond between C22 and C23. A doublet at δ 5.57 was assigned for C7 proton. From its IR and 1H NMR spectra, it was inferred that the compound might be a steroid.

The mass spectrum showed the peaks at m/z 271, 255, 231 and 213 which demonstrated the presence of Δ2 – 3 β - hydroxyl steroid nucleus with one degree of unsaturation in the side chain. The other fragment ions were appeared at m/z 397 [M+ - CH3], 394 [M+ - H2O], 379 [M+ - (CH3+H2O)], 314 [M+ - part of side chain GH14)], 273 [M+ - (side chain)] and 271 [M+ - (side chain + 2H)]. The loss due to the part of the side chain (m/z 314) is the characteristic pattern suggested to arise by a McLafferty rearrangement involving the C20-H. From the above spectral data, the structure of the compound was assigned as in fig 2

Fig: 2 Structure of Compound II



HPTLC Finger Print Analysis of methanolic extract

From the area of normalization percentage the purity of the isolated compound was found to be 73.52% and the purity tends to decrease as the concentration of the sample incorporated increases. Fig. 3 gives the HPTLC data.

Fig 3: HPTLC of methanolic extract Before Derivitization

Image at 254nm

Image at 366nm

After Derivitization

Image at 366nm

Image at visible light

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