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A Parveen Nisha
Department of Biology,
Gandhigram Rural Institute – Deemed University,
Gandhigram- 624302.

R Kumuthakalavalli
Department of Biology,
Gandhigram Rural Institute – Deemed University,
Gandhigram- 624302.

Correspondence
A Parveen Nisha
Department of Biology,
Gandhigram Rural Institute – Deemed University,
Gandhigram- 624302.

Fractionation, radical scavenging activity of bioactive compounds and FT-IR spectroscopy study of milky mushroom, *Calocybe indica* Var. APK2

A Parveen Nisha, R Kumuthakalavalli

Abstract
Antioxidant activity of solvent fractions of *Calocybe indica* Var.APK2 was assessed by methanolic extraction. The chloroform and ethanol fractions of *C. indica* were evaluated via hydroxyl radical scavenging assay and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. Six fractions (F1 to F6) were obtained from chloroform-ethanol partition using silica gel chromatography. Among the six fractions, fraction -3 and fraction -4 recorded high antioxidant properties. In DPPH radical scavenging activity, 1ml concentration of fraction 3 expressed 50.19% of EC50 value, where as in Hydroxyl radical scavenging activity, 0.8ml concentration of fraction 4 expressed 50% of EC50 value, which was compared with the standards namely L- Ascorbic acid and Butylated Hydroxy Anisole (63.33 and 69.19 respectively). The FTIR analysis of methanol extracts of *Calocybe indica* var APK.2, confirmed the presence of alcohols, phenols, alkanes, aldehydes, saturated aliphatic, unsaturated ketones, primary amines, alkanes, aromatics, alkyl halides and aliphatic amines, with their major peaks.

Keywords: Antioxidant, silica gel chromatography, FT-IR spectroscopy, *Calocybe indica* Var.APK2, phytochemicals.

1. Introduction
From time immemorial, mushrooms have been valued by humankind as a culinary wonder and folk medicine in Oriental practice. The last decade has witnessed the overwhelming interest of Western research fraternity in pharmaceutical potential of mushrooms. Many mushroom species are reported to be the miniature pharmaceutical factories producing hundreds of novel constituents with wild array of biological properties. As a result, mushrooms have been emerged as wonderful source of nutraceuticals, anti-oxidants, prebiotic, anti-cancerous, immune-modulating, anti-inflammatory, cardiovascular, anti-microbial, and anti-diabetic agents. They are also valued as highly nutritive and low-calorie food, with good quality proteins, vitamins and minerals (Aina, 2012) [2].
In recent time the ongoing research projects are aimed to promote mushrooms as new generation "bio-therapeutics" for various dreadful diseases like tumors and cancer through in-vitro assays and FT-IR studies. Identification and chemical nature of the phytochemical compounds present in the medicinal mushrooms will provide information on the different functional groups responsible for their medicinal properties. By using chromatography separation of the potential bioactive fractions can be obtained to carry out *in vitro* assays (Yanjun Zhang, 2002) [15]. Antioxidants are chemicals with the property to neutralize or scavenge free radicals or reactive oxygen species (ROS) Aina (2012) [3]. Free radicals are unstable, highly reactive, chemically incomplete substances that remove electrons from other molecules, consequently causing damage to chemicals in the body such as enzymes, making them less effective (Acworth and Bailey, 2000) [4]. Free radicals are engendered by normal metabolic activity as well as lifestyle factors such as smoking, exercise, and diet; when present in the body, free radicals can damage tissues and delicate cell membranes. They can also damage DNA, which may lead to the initiation of certain cancers (Barros, 2008) [6].
The FT-IR analysis is being used to study the medicated compounds from Indian medicinal plants (Ashok Kumar, 2014) [1]. The present study seeks to assess the potential antioxidant fractions of the fruiting bodies of *Calocybe indica* by using various polar and non-polar solvents such as of methanol, chloroform and ethanol.

2. Materials and Methods

2.1 Solvent extraction method

Fruiting bodies of *Calocybe indica* var. APK2 were obtained from the RPJ Mushroom farm, Usilanakottai, Ramanathapuram, Tamilnadu, India and were authenticated by Dr. A.S. Krishnamoorthy, Professor, Department of Plant Pathology, Tamilnadu Agricultural University, Coimbatore. The fruiting bodies were shade dried, ground into fine powder (20 mesh size) and subjected for methanolic extraction using soxhlet apparatus for 6 hrs at 50-60 ^{0}C (Feyzaoke, 2011) [7]. The crude extract was concentrated under vaccum by using rotary evaporator and stored under 4 ^{0}C.

2.2 Fractionation of the extract using Chromatography

5gm of crude methanolic extract was subjected to silica gel column (200 to 300 mesh). For this purpose a glass column was packed with silica gel upto 25 cm height and washed with 200 mL of hexane. Then, the extract-silica gel slurry was loaded onto the column followed by different ratios of chloroform ⁄ ethanol mixture by increasing polarity of 8:2 to 2:8 (v⁄v) (Mi ja Chung, 2010 and Bokyung Sung, 2015) [10, 5]. About 100 mL of each solvent system was used for elution and 5 ml of each fraction was collected.

2.3 DPPH Radicals scavenging activity

The DPPH free radical scavenging activity was performed for six fractions (F1 to F6) and the active fractions were used to determined DPPH assay. 0.1mM solution of DPPH was prepared in 100% methanol, and 1 ml of this solution was added to 4 ml of sample in 40% methanol at various concentrations (10-160 µg/ml). This mixture was shaken vigorously and incubated for 15 min at 30 °C in the dark. The reduction of the DPPH radical was measured by continuous monitoring of the decrease of absorption at 517 nm. The L-ascorbic acid and BHA (Butylated Hydroxy Anisole) were used as standards (Vaddadi Sridevi, 2015) [14].

DPPH Scavenging effect (%) = [(A0-A1)/A0] x 100

where A0 was the absorbance of the control reaction and A1 was the absorbance in the presence of the standard sample or extract.

2.4 Hydroxyl radical-scavenging assay

The reaction mixture containing sample (0.1–3.2 mg/ml) was incubated with deoxyribose (3.75 mM); H_2O_2 (1 mM); $FeCl_3$ (100 mM); EDTA (100 mM) and ascorbic acid (100 mM) in potassium phosphate buffer (20 mM, pH 7.4) for 60 minutes at 37 ^{0}C (Ranju Pal *et al.,* 2011) [12]. The reaction was terminated by adding 1ml of TBA (1%, w/v) and 1ml of TCA (2%, w/v) and the mixture was heated in a boiling water bath for 15 minutes. The contents were cooled and the absorbance of the mixture was measured at 535 nm against reagent using blank. Decreased absorbance of the reaction mixture indicated decreased oxidation of deoxyribose.

2.5 Fourier Transform Infrared Spectrophotometer (FTIR)

Fourier Transform Infrared Spectrophotometer (FTIR) is perhaps the most powerful tool for identifying the types of chemical bonds (functional groups) present in compounds. The wavelength of light absorbed is characteristic of the chemical bond as can be seen in the annotated spectrum. By interpreting the infrared absorption spectrum, the chemical bonds in a molecule can be determined.

The potential antioxidant fractions which revealed from silica gel chromatography were used for FTIR analysis. 10 mg of the dried extract powder was encapsulated in 100 mg of KBr pellet, in order to prepare translucent sample discs. The powdered sample of each fraction specimen was loaded in FTIR spectroscope with a scan range from 400 to 4000 [cm -1] with a resolution of 4 [cm -1] (Kishore Kumar, 2014) [9].

3 Results

By using methanolic extract 10.5 g of brown sticky crude phyto compound was obtained from 200 g of dried fruiting bodies of *Calocybe indica* Var APK2. Under fractionation of solvent-solvent partition using silica gel chromatography, six fractions were obtained. DPPH scavenging assay and hydroxyl radical assays were done for all these six fractions and fraction 3 and fraction 4 were evolved as potent fractions. In DPPH scavenging activity, EC50 value for fraction 3 at 1ml concentration was 51.19% while the standards namely L-ascorbic acid and BHA it was recorded as 72.90% and 72.50% respectively (Table 1). For fraction 4, it was 62% where as the standard recorded as 72.9%and 76.50% respectively. In Hydroxyl radical scavenging assay, EC50 value for fraction 3 at 1 ml concentration was 52% and for fraction 4 was 60% while in the standards namely ascorbic acid and BHA, it was 71.86% and 82.79% respectively (Table 2).

The structural analysis of FT-IR for fraction 3 and fraction 4 consisted of those specific compounds which are responsible for the anti-oxidant property in *Calocybe indica* VarAPK.2

Table: 1 DPPH radical scavenging assay for Fraction 3 and 4

DPPH Radical assay in ml	Fraction 3%	Fraction 4%	L-Ascorbic Acid %	BHA %
0.1	28.22	24.12	51.41	54.11
0.5	36.80	40.00	63.44	65.89
1	51.19	62.00	72.90	76.50

Table 2: Hydroxyl radical assays for fraction 3 and 4

Hydroxyl radical assay in ml	Fraction 3%	Fraction 4%	L-Ascorbic Acid %	BHA %
0.2	26	28	39.92	44.25
0.4	35	38	47.02	51.22
0.6	40	45	55.40	60.45
0.8	45	50	63.33	69.19
1.0	52	60	71.86	82.79

Table 3: FTIR frequency and functional group of *Calocybe indica* Fraction 3

S. No	Peak values	Functional groups
1.	3500-3200	Alcohols, Phenols
2.	3000-2850	Alkanes
3.	3000-2800	Alkanes
4.	1760-1665	Carbonyl and carboxylic acid
5.	1680-1640	Alkenes
6.	1370-1350	Alkenes
7.	1370-1000	Alcohol, carboxylic acid, esters and ethers
8.	1320-1000	Alcohol, carboxylic acid, esters and ethers
9.	1000-650	Alkenes
10.	910-665	Primary and secondary amines
11.	900-675	Alkyl halides and aromatics

Table 4: FTIR frequency and functional group of *Calocybe indica* Fraction 4

S. No	Peak values	Functional groups
1.	3500-3200	Alcohols, Phenols
2.	3000-2850	Alkanes
3.	3000-2800	Alkanes
4.	1740-1710	Aldehyde and aliphatic
5.	1470-1450	Alkanes
6.	1380-1350	Alkanes
7.	1335-1250	Aromatic amines
8.	1300-1150	Esters, ethers aliphatic amines
9.	1200-1020	Aliphatic amines
10.	680-1000	Alkenes
11.	900-680	Aromatics

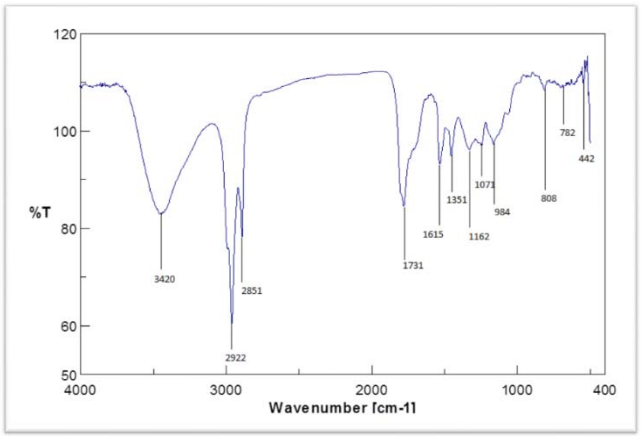

Fig 1: FTIR frequency of *Calocybe indica* Fraction 3

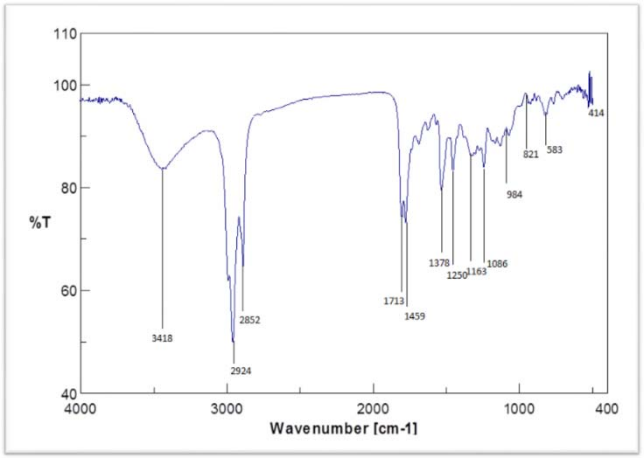

Fig 2: FTIR frequency of *Calocybe indica* Fraction 4

4. Discussion

Solvent extraction and phytochemical screening serve as the initial step in predicting the types of potential bioactive compounds. In the current study methanolic extract of *C. indica* VarAPK2 recorded higher yield than other non-polar and polar solvents, such as chloroform, ethyl acetate, methanol and aqueous extraction. Feyzaoke (2011) [7] reported that the methanolic extraction of *Auricularia auricular*-judae and *Pleurotus eryngii* showed the highest degree of phytochemical compounds.

Many bioactive compounds produce positive healthful changes in human body. These compounds exhibit antioxidant properties, and also protect the body against cardiovascular and various immune disorders Packer *et al.*, (1999) [11]. The free radical and other reactive oxygen species (ROS) are mostly considered to be associated with pathogenesis and responsible to inhibit the diseases. The antioxidants are biologically synthesized as defensive mechanism having an important role in preventing the disease by reducing the oxidative damage to cellular components Kishor Kumar (2014) [9]. In the potential fractions of *C. indica* for DPPH activity to fraction 3 and fraction 4 were expressed as 51.19% and 62% respectively. Similarly hydroxyl radical scavenging activity for fraction 3 and fraction 4 were expressed as 51% and 60% respectively. Tsun Thai -Chai (2015) [13] reported that the methanolic extraction and solvent fractions of *Avrainvillea erecta* possessed 27.6% of DPPH radical scavenging activity.

The FTIR spectroscopy has proven to be a valuable tool for characterization and identification of compounds and functional groups present in the unknown mixture of samples. Moreover FT-IR spectroscopic analysis of the two fractions of *C. indica* revealed the presence of phenols, flavonoids, alcohols, amines, aldo and keto groups. Similarly Israilides (2008) [8] reported the phenolic compounds such as flavonoids and tannins present the mushroom fruiting bodies of *Lentinula edodes*

The present study clearly dictates the potential antioxidant activity and free radical scavenging properties of *C. indica* VarAPK2 through their functional compounds. However studies on fractionation and characterization of these compounds validating their therapeutic potential against several diseases should be extended.

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