Full Length Research Article

© 2024, Centre for Advanced Research in Agricultural Sciences
Research Journal of Agricultural Sciences
CARAS Volume 15; Issue 04 (Jul–Aug 2024); pp 1055–1059

ISSN: 0976-1675 (P)
ISSN: 2249-4538 (E)

# Phytochemical Analysis of Wild Mushroom *Calocybe indica* Associated with Roots of Date Palm (*Phoenix sylvestris* L Roxb)

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Received: 18 Jun 2024; Revised accepted: 12 Aug 2024

## Abstract

Mushrooms have been valued as traditional sources of natural bioactive compounds for many centuries and targeted as promising therapeutic agents. The bioactive mycomolecules of mushrooms are reported to have antioxidant, antitumor, antidiabetic, anti-inflammatory, and antimicrobial activity, which are the important medicinal targets in terms of drug discovery today. 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 *Calocybe indica* was collected from the roots of the date palm tree *Phoenix sylvestris.* To know about their medicinal values, therapeutic uses the phytochemicals analysis by TLC and HPTCL were done. From the methanolic extracts of mushroom the phytochemicals components identified are Tannin, Flavonoids, Glycosides, Alkaloids, Coumarin and Cardiac Glycoside.

***Key words*:** Phytochemical analysis, TCL, HPTCL, *Calocybe indica, Phoenix sylvestris*

*Calocybe indica* also possesses various medicinal and therapeutic applications. *Calocybe* indica, commonly known as the milky mushroom, is recognized for its medicinal and therapeutic properties. It is known to possess antioxidant, antimicrobial, anti-inflammatory, and immunomodulatory activities. These attributes make it valuable in promoting health and potentially preventing or managing various diseases. A detailed review on the account of nutritional and medicinal properties of *Calocybe indica* was presented by Shashikant [1] in which its potential anti-oxidant, anti-cancer, anti-obesity, hepatoprotective and anti-aging activities were reported. Several extraction techniques have been used to prepare the mushroom extract including the hot aqueous extract of fresh fruit bodies [2], methanolic extract [3], ethanolic extraction, and modified solvent evaporation extraction [4], and hot water extraction [5]. Bioactive compounds found in edible mushrooms include phytochemicals (alkaloids, phenolic acids, flavonoids, carotenoids), fiber, polysaccharides, selenium, vitamins (e.g., niacin, thiamin, riboflavin, ascorbic acid, and vitamins B and D), and the significant antioxidants ergothioneine and glutathione, which may play a role in the prevention of cancer [6]. Researchers recently identified and documented the chemical composition of several substances with biological activity and secondary metabolites from *Calocybe indica* [7]. Records available in this shows that these phytochemicals have some beneficial health effects. Some of these phytochemicals have been shown to have antioxidant properties [8].

Therefore, keeping in view the importance of milky mushroom in India, phytochemical characterization of a collected wild *Calocybesp* on roots of date palm *Phoenix sylvestris* were carried out.

## MATERIALS AND METHODS

The *Calocybe indica* was collected from the roots of the date palm tree *Phoenix sylvestris.*

*Preparation of extract*

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

*Phytochemical tests*

All the phytochemical parameters were carried out as per the standard test procedures [9].

Qualitative analysis of phytochemicals was carried out according to the methods described by Wandati [10] with some modifications.

a) *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.

b) *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

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**Citation:** Ebency CIL, Jenefa BA, Rani BU. 2024. Phytochemical analysis of wild mushroom *Calocybe indica* associated with roots of date palm (*Phoenix sylvestris* L Roxb). *Res. Jr. Agril. Sci*. 15(4): 1055-1059.

shaking vigorously. The presence of persistent foam lasting for at least 5minutes indicated the presence of saponins.

c) *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.

d) *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.

e) *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.

f) *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.

g) *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.

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

i) *Test for steroids*: One milliliter of the mushroom extract was put into a test tube in which 0.5 ml of sulphuric acid, acetic anhydride, and chloroform in similar amount was added. A red coloration indicates presence of sterols while a green colour indicated the presence of steroids.

j) *Test for proteins*: To 2 ml of the extract, a few drops of concentrated nitric acid were added. Formation of yellow colour indicated the presence of proteins.

k) *Test for cardiac glycosides*: This was done following Bromine water test. The plant extract was taken in a beaker and a few drops of bromine water is added. A yellow colour precipitate indicates the presence of cardiac glycosides.

l) *Test for reducing sugars*: This was done following Benedicts test. Take 0.5ml of filtrate in a beaker and add 0.5ml of Benedicts reagent. Mix thoroughly and boil it for 2minutes. A formation of green/yellow/red colour shows the presence of reducing sugars.

m) *Test for quinones*: This was done following Alcoholic KOH test. Take One millilitre (1ml) of plant extract and a few ml of alcoholic potassium hydroxide is added. Mix thoroughly, the change in red to blue colour indicates the presence of quinines.

n) *Test for coumarins*: This was done following NaOH test. Take few grams of plant extract and add 10% NaOH with it. Mix well and Chloroform is added. The formation of yellow colour shows the presence of coumarins.

*Sample preparation for TLC*

Sample (1g) was sonicated with 10 ml of Methanol for 15 minutes and filtered. This solution was used for TLC/HPTLC.

*Thin-layer chromatography methodology*

Applied 10 µl Methanol extract of Mushroom sample on TLC plate using Camag's ATS4 applicator and developed by the mobile phase, Toluene: Ethyl acetate: Formic acid (5:2.5:0.5 *v/v/v*) up to 9 cm distance. After development, the plate was photo documented using Camag's TLC Visualizer under UV 254 nm and UV 366 nm and then scanned using Camag's Scanner 4 at ($D_2$ lamp/Absorption mode, Hg lamp/Fluorescent mode) finger print profiles of the extract were documented. Then the plate was dipped in 5% vanillin-sulphuric acid reagent followed by heating at 105°C till development of coloured spots. The plate was then photo documented in white light and scanned at 520 nm for finger print profile.

*HPTLC analysis*

One hundred milligrams of the methanol extract were dissolved in 1 ml of HPTLC grade methanol and centrifuged at 3000 rpm for 5 min. This solution was used as test solution for HPTLC analysis. The HPTLC was performed on 7.0 × 10.0 cm precoated silica gel 60 F 254 HPTLC plate (E. MERCK KGaA). No pre-washing and modification of the plate were done. The sample solution was applied as bands to the plate by CAMAG Linomat applicator fitted with 100 µl syringe. The stable application rate was 150 nl/s. The sample loaded plate was kept in automatic development chamber with mobile phase chloroform: ethylacetate: formic acid (5:4:1 v/v/v). Densitometric scanning was performed with CAMAG TLC scanner-4 equipped with winCATS software. The bands were visualized using CAMAG visualizer, and the images were captured in white light and 254 nm (short UV), 366 nm (long UV) wavelengths and 560 nm. When exposed to short-wave UV light of 254 nm, UV-active compounds will undergo fluorescence quenching and appear as dark spots on a bright background. Conversely, compounds that absorb 366 nm UV light will appear as bright spots on a dark background.

Table 1 Phytochemical details

| Phytochemical parameters | Inference |
|---|---|
| Phenol | - |
| Tannin | + |
| Flavonoids | + |
| Tri terpenoids | |
| Steroids | - |
| Glycosides | + |
| Reducing sugar | - |
| Anthraquinone | - |
| Quinones | - |
| Alkaloids | + |
| Saponins | - |

| | |
|---|---|
| Coumarin | + |
| Cardiac glycoside | + |
| Acids | - |
| Proteins | - |

+ = presence, - = absence

These are the compounds present in the mushroom sample

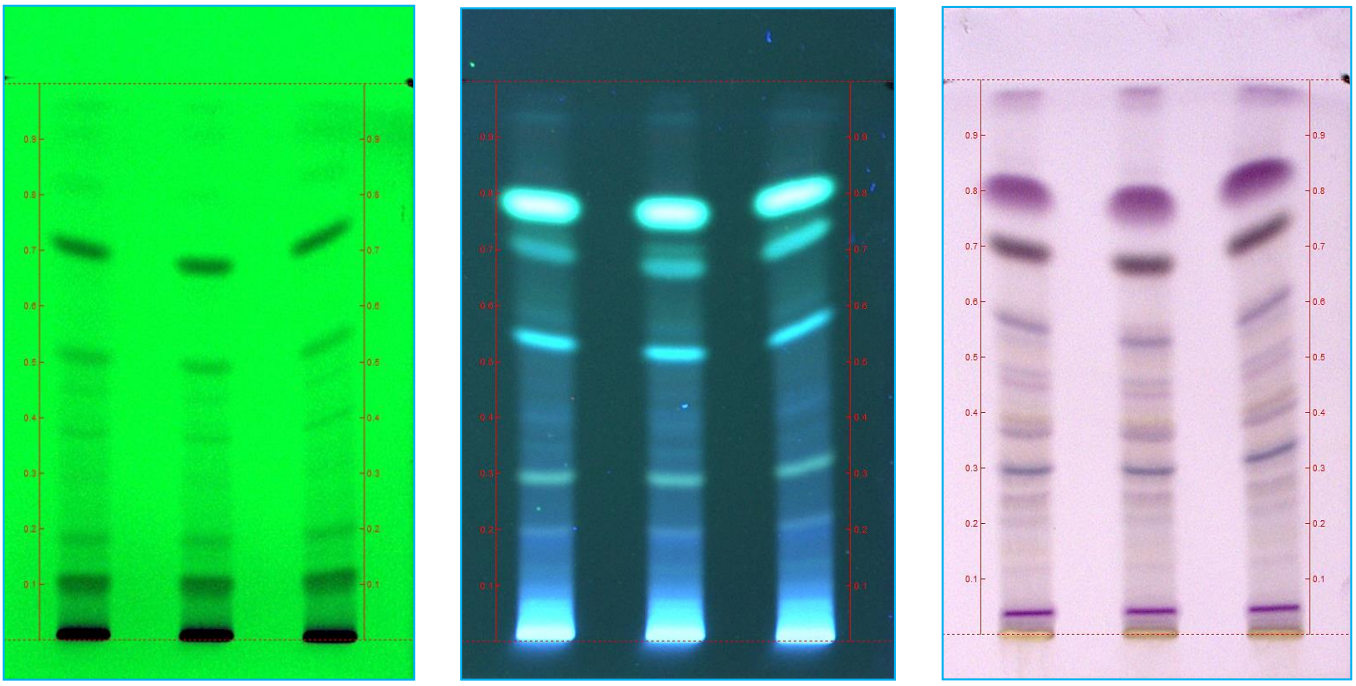
## RESULTS AND DISCUSSION

*Phytochemical tests*

The methanolic extracts revealed the presence of tannin, flavonoids, glycosides, alkaloids, coumarin and cardiac glycoside. The methanol extract is subjected to TLC and HPTLC to confirm the further presence of these compounds.

*TLC results*

Thin Layer Chromatography (TLC) profiling of the mushroom extract indicated the presence of diverse types of phytochemicals. Different Rf values of the compound also reflect an idea about their polarity. This information will help in selection of appropriate solvent system for further separation of compound from these extracts.



Under Short UV λ=254 nm

Under Long UV λ=366 nm

Under White light after derivatization

Fig 1 TLC photo document

*$R_f$ value and color of spots*

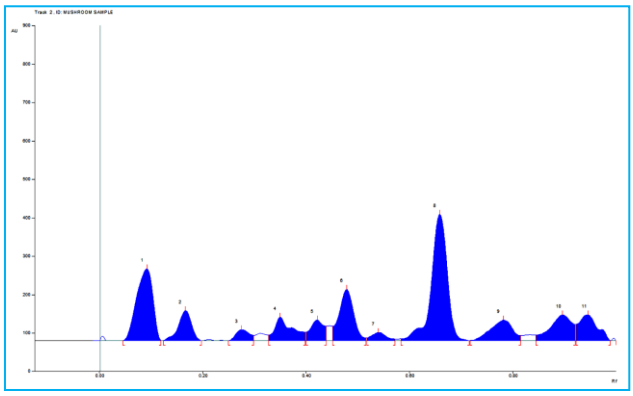
Table 2 $R_f$ value and color spot

| λ = 254 nm | | λ = 366 nm | | Under white light after derivatization with vanillin sulphuric acid reagent (Derivatized) | |
|---|---|---|---|---|---|
| Color | $R_f$ value(s) | Color | $R_f$ value(s) | Color | $R_f$ value(s) |
| Green | 0.10 | Fluorescent sky blue | 0.04 | Ash | 0.03 |
| Green | 0.18 | Fluorescent sky blue | 0.08 | Purple | 0.05 |
| Green | 0.36 | Blue | 0.09 | Ash | 0.12 |
| Green | 0.43 | Ash | 0.14 | Ash | 0.21 |
| Green | 0.49 | Blue | 0.20 | Ash | 0.25 |
| Green | 0.68 | Green | 0.29 | Ash | 0.28 |
| | | Blue | 0.34 | Blue | 0.31 |
| | | Blue | 0.39 | Blue | 0.36 |
| | | Fluorescent green | 0.52 | Yellow | 0.39 |
| | | Fluorescent green | 0.67 | Purple | 0.44 |
| | | Fluorescent green | 0.70 | Blue | 0.46 |
| | | Fluorescent green | 0.76 | Blue | 0.53 |
| | | Ash | 0.94 | Black | 0.66 |
| | | | | Purple | 0.77 |
| | | | | Purple | 0.96 |

*HPTLC analysis results*

The High-Performance Thin Layer Chromatography (HPTLC) densitometric analysis of the methanolic extract of *Calcocybe indica* was carried out using HPTLC system, and the results were obtained in the form of chromatograms (scanned at the wavelength of 254 nm, 366 nm and 520 nm) representing several peaks. The phytochemical profile of the mushroom was determined and presented in the tables showing the total number of peaks, peak heights, peak area, percent area, and Rf values.

*HPTLC Chromatogram @ 254 nm*

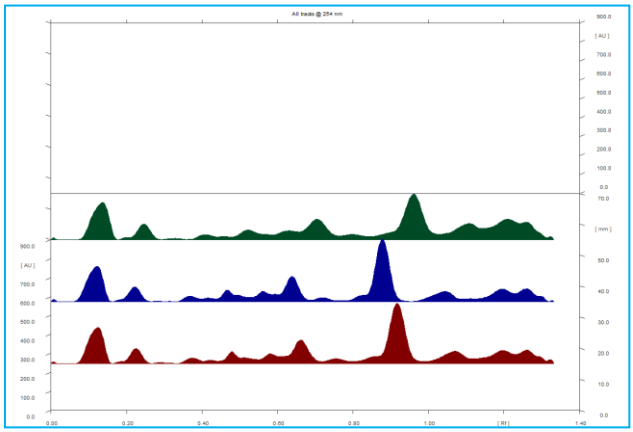


*Peak table @ 254 nm*

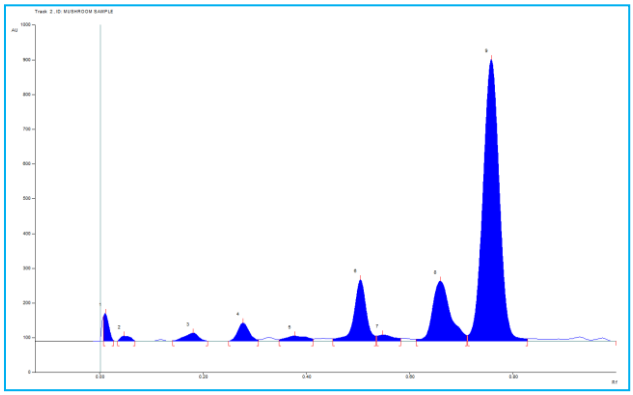
Track 2, ID: MUSHROOM SAMPLE

| Peak | Start Position | Start Height | Max Position | Max Height | Max % | End Position | End Height | Area | Area % |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 0.06 Rf | 0.1 AU | 0.12 Rf | 187.0 AU | 17.35 % | 0.16 Rf | 1.0 AU | 5423.5 AU | 17.25 % |
| 2 | 0.17 Rf | 0.2 AU | 0.22 Rf | 78.1 AU | 7.24 % | 0.26 Rf | 0.2 AU | 1823.2 AU | 5.80 % |
| 3 | 0.33 Rf | 0.0 AU | 0.37 Rf | 28.3 AU | 2.62 % | 0.40 Rf | 13.3 AU | 687.7 AU | 2.19 % |
| 4 | 0.44 Rf | 14.8 AU | 0.47 Rf | 61.0 AU | 5.66 % | 0.53 Rf | 21.9 AU | 1913.8 AU | 6.09 % |
| 5 | 0.53 Rf | 22.1 AU | 0.56 Rf | 53.6 AU | 4.97 % | 0.58 Rf | 37.5 AU | 1272.8 AU | 4.05 % |
| 6 | 0.60 Rf | 37.4 AU | 0.64 Rf | 133.1 AU | 12.35 % | 0.69 Rf | 6.7 AU | 3453.0 AU | 10.99 % |
| 7 | 0.69 Rf | 6.9 AU | 0.72 Rf | 20.9 AU | 1.94 % | 0.76 Rf | 3.8 AU | 557.6 AU | 1.77 % |
| 8 | 0.78 Rf | 4.5 AU | 0.88 Rf | 329.0 AU | 30.52 % | 0.95 Rf | 0.3 AU | 9432.8 AU | 30.01 % |
| 9 | 0.96 Rf | 0.5 AU | 1.04 Rf | 53.1 AU | 4.93 % | 1.09 Rf | 12.8 AU | 2098.0 AU | 6.67 % |
| 10 | 1.13 Rf | 14.8 AU | 1.20 Rf | 66.7 AU | 6.19 % | 1.23 Rf | 41.8 AU | 2561.7 AU | 8.15 % |
| 11 | 1.23 Rf | 42.1 AU | 1.26 Rf | 67.2 AU | 6.23 % | 1.32 Rf | 0.4 AU | 2209.3 AU | 7.03 % |

*3D chromatogram @ 254 nm*



*HPTLC chromatogram @ 366 nm*

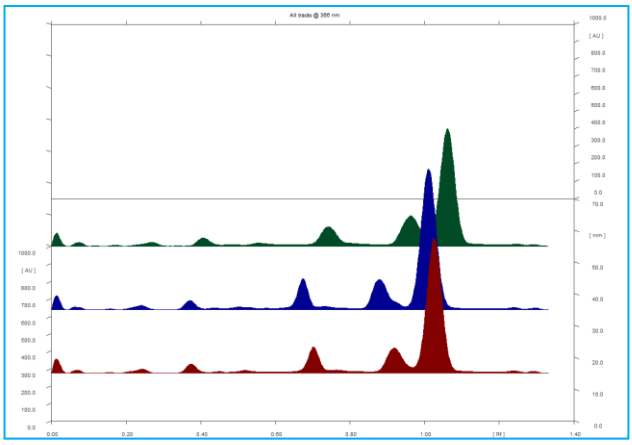


*Peak table @ 366 nm*

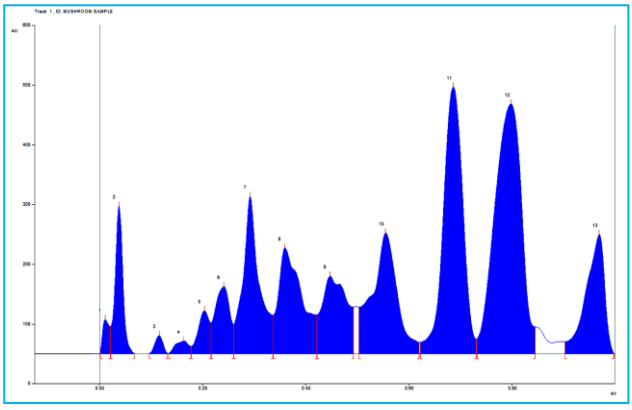
Track 2, ID: MUSHROOM SAMPLE

| Peak | Start Position | Start Height | Max Position | Max Height | Max % | End Position | End Height | Area | Area % |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 0.01 Rf | 71.9 AU | 0.01 Rf | 79.6 AU | 5.85 % | 0.04 Rf | 0.8 AU | 742.6 AU | 2.02 % |
| 2 | 0.05 Rf | 0.5 AU | 0.06 Rf | 14.4 AU | 1.06 % | 0.09 Rf | 0.2 AU | 254.4 AU | 0.69 % |
| 3 | 0.19 Rf | 0.7 AU | 0.24 Rf | 23.4 AU | 1.72 % | 0.28 Rf | 0.6 AU | 582.2 AU | 1.59 % |
| 4 | 0.33 Rf | 0.0 AU | 0.37 Rf | 52.1 AU | 3.83 % | 0.41 Rf | 3.8 AU | 1064.9 AU | 2.90 % |
| 5 | 0.46 Rf | 4.4 AU | 0.50 Rf | 14.6 AU | 1.07 % | 0.55 Rf | 6.0 AU | 563.4 AU | 1.54 % |
| 6 | 0.60 Rf | 6.2 AU | 0.67 Rf | 177.0 AU | 13.01 % | 0.71 Rf | 14.3 AU | 3851.8 AU | 10.50 % |
| 7 | 0.71 Rf | 14.4 AU | 0.73 Rf | 17.5 AU | 1.28 % | 0.78 Rf | 7.6 AU | 505.2 AU | 1.38 % |
| 8 | 0.82 Rf | 4.8 AU | 0.88 Rf | 172.3 AU | 12.66 % | 0.95 Rf | 16.3 AU | 5501.8 AU | 15.00 % |
| 9 | 0.95 Rf | 16.3 AU | 1.01 Rf | 809.9 AU | 59.52 % | 1.11 Rf | 6.9 AU | 23616.0 AU | 64.38 % |

*3D chromatogram @ 366 nm*



*HPTLC chromatogram @ 520 nm*

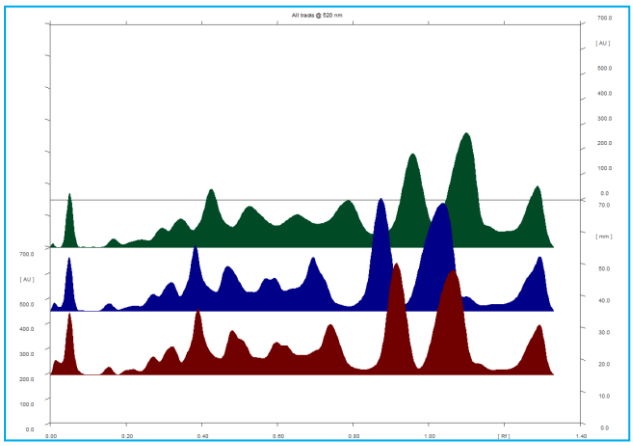


*Peak table @ 520 nm*

Track 1, ID: MUSHROOM SAMPLE

| Peak | Start Position | Start Height | Max Position | Max Height | Max % | End Position | End Height | Area | Area % |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 0.00 Rf | 6.5 AU | 0.01 Rf | 57.4 AU | 2.41 % | 0.03 Rf | 45.4 AU | 692.2 AU | 0.84 % |
| 2 | 0.03 Rf | 45.8 AU | 0.05 Rf | 247.6 AU | 10.41 % | 0.09 Rf | 0.1 AU | 3381.4 AU | 4.12 % |
| 3 | 0.13 Rf | 0.2 AU | 0.16 Rf | 30.5 AU | 1.28 % | 0.18 Rf | 0.5 AU | 433.2 AU | 0.53 % |
| 4 | 0.18 Rf | 0.2 AU | 0.22 Rf | 21.4 AU | 0.90 % | 0.24 Rf | 12.3 AU | 508.8 AU | 0.62 % |
| 5 | 0.24 Rf | 12.3 AU | 0.27 Rf | 71.8 AU | 3.02 % | 0.29 Rf | 51.6 AU | 1453.7 AU | 1.77 % |
| 6 | 0.29 Rf | 52.0 AU | 0.32 Rf | 112.4 AU | 4.73 % | 0.35 Rf | 49.1 AU | 2967.3 AU | 3.62 % |
| 7 | 0.35 Rf | 49.6 AU | 0.39 Rf | 263.7 AU | 11.09 % | 0.45 Rf | 64.7 AU | 7704.4 AU | 9.39 % |
| 8 | 0.45 Rf | 64.9 AU | 0.48 Rf | 177.3 AU | 7.46 % | 0.56 Rf | 65.0 AU | 7491.9 AU | 9.13 % |
| 9 | 0.56 Rf | 65.2 AU | 0.60 Rf | 130.0 AU | 5.47 % | 0.66 Rf | 78.5 AU | 5744.8 AU | 7.00 % |
| 10 | 0.67 Rf | 78.5 AU | 0.74 Rf | 201.9 AU | 8.49 % | 0.83 Rf | 19.1 AU | 9124.4 AU | 11.12 % |
| 11 | 0.83 Rf | 19.3 AU | 0.92 Rf | 446.5 AU | 18.78 % | 0.98 Rf | 24.3 AU | 15688.9 AU | 19.13 % |
| 12 | 0.98 Rf | 24.8 AU | 1.07 Rf | 418.3 AU | 17.59 % | 1.13 Rf | 44.9 AU | 20190.7 AU | 24.61 % |
| 13 | 1.20 Rf | 19.8 AU | 1.29 Rf | 199.4 AU | 8.38 % | 1.33 Rf | 1.4 AU | 6645.6 AU | 8.10 % |

*3D chromatogram @ 520 nm*



The investigation into the phytochemical composition of the methanolic extracts of *Calocybe indica* revealed the presence of significant bioactive compounds, including tannins, flavonoids, glycosides, alkaloids, coumarins, and cardiac glycosides. The subsequent application of TLC and HPTLC analyses provided further confirmation and detailed profiling of these compounds [11].

TLC profiling successfully identified the diverse range of phytochemicals within the extract, with various Rf values indicating differences in polarity and aiding in the selection of an appropriate solvent system for future separation efforts. The visualization under short UV, long UV, and white light after derivatization highlighted the complex nature of the extract, showing distinct spots with varying colors and Rf values [12].

The HPTLC densitometric analysis provided a more refined and quantitative assessment of the extract. Chromatograms scanned at 254 nm, 366 nm, and 520 nm revealed multiple peaks, representing the phytochemicals' profiles. The detailed peak tables and 3D chromatograms presented comprehensive data on the number of peaks, peak heights, areas, and percent areas, offering a deeper insight into the concentration and distribution of these compounds within the extract.

Overall, the combined use of TLC and HPTLC techniques in this investigation effectively characterized the phytochemical composition of *Calocybe indica*. The findings underscore the potential medicinal and therapeutic applications of this mushroom, paving the way for further research into its bioactive properties and possible uses in pharmaceutical formulations [13].

## CONCLUSION

The study concluded that methanolic extract of the *Calocybe indica* whole contains a rich variety of phytochemicals which might be accountable for its therapeutic value. This study has further elaborated the knowledge of medicinal and health benefits of mushrooms. The presence of the phytochemicals in the tested materials could possibly account for these benefits.

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