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In-vitro antimicrobial and anti-inflammatory activity of modified solvent evaporated ethanolic extract of *Calocybe indica*: GCMS and HPLC characterization

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ABSTRACT

Recent interest in the utilization of mushroom-based bioactive compounds has increased due to their potential bioactivities and as alternatives in the reduction of high concentrations of chemical utilization. Therefore, we evaluated the physicochemical, functional, antimicrobial, and anti-inflammatory activity of the *Calocybe indica*. The nutritional composition of the mushroom was found to be a good source of proteins (12.48%) and fiber (6.87%). Polysaccharide and protein moiety showed both hydrophilic and hydrophobic domains and the sample showed higher water (3.01 g/g), oil binding (2.45 g/g) emulsifying (68.94), and foaming properties (59.39%). Structural characterization revealed the porous and small crystalline structure of the mushroom powder. Ethanolic extract was quantified for total phenolics and flavonoids and revealed 11.1534 ppm caffeic acid, 0.057 ppm syringic acid, 1.6385 ppm p-coumaric acid, and 0.3495 ppm rutin, respectively. Presence of ethyl tridecanoate, hexadecanoic acid ethyl ester, pentadecanoic acid ethyl ester, undecanoic acid ethyl ester, N, α, α′-trimethyl diphenethylamine, nicotinonitriles, phosphonic acid decyl-, 1-hexyl-2-nitrocyclohexane, diallyl divinylsilane, 3-phenyl-pyrrolo(2,3-β) pyrazine was confirmed during GC–MS analysis. Furthermore, the mushroom extract showed effective antimicrobial against Gram-positive (23.67 mm) and negative bacteria (20.33 mm) in terms of zone of inhibition. Significantly comparable anti-inflammatory activity was observed for mushroom extract during protein denaturation (43.72–85.69%) and membrane stabilization. In conclusion, the mushroom extract has shown good functional properties and potential bioactivity, therefore, it can be scaled up as an effective food preservative, potential anti-inflammatory, and antimicrobial agent at the industrial level.

1. Introduction

Mushrooms have a reproductive phase called the fruiting body and the vegetative state of mushrooms is called mycelia (Bains and Chawla, 2020). The edible mushroom has been widely known for its unique aroma and taste and has been proven to contain several health properties (Chelladurai et al., 2021). Furthermore, they have a wide range of bioactive compounds such as polyphenolic components (terpenes, terpenoids, steroids) proteoglycans, lectins, polysaccharides, and antioxidants that act as therapeutic agents and can be used as raw materials for the development of various functional foods (Sánchez, 2017). Apart from the nutritional aspects, mushrooms possess active compounds having potential medicinal value with an antimicrobial activity that can be isolated and used for the benefit of humans. These compounds

Abbreviations: AOAC, Association of Official Analytical Collaboration; DMSO, Dimethyl sulfoxide; DPPH, (2,2-diphenyl-1-picrylhydrazyl); FTIR, Fourier transform infrared spectroscopy; GC–MS, Gas chromatography - mass spectroscopy; UPLC, Ultra-pressure liquid chromatography; DSC, Differential scanning calorimetry; MSE, Modified solvent evaporation; MTCC, Microbial Type Culture Collection; SEM, Scanning electron microscopy; XRD, Xray diffraction.

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possess anticarcinogenic, immunomodulatory, antioxidant, antimicrobial, anti-inflammatory, antiviral, antiaging, hepatic protective, hypoglycaemic, hypocholesterolaemia, and various other biological activities (Gupta et al., 2018). Therefore, edible mushrooms can be explored broadly for their effective bioactivity and nutritional value.

Calocybe indica commonly known as milky mushrooms is one of the most extensively cultivated mushrooms in the world. They are the third most important commercially grown mushroom in India following button and oyster mushrooms (Patel and Trivedi, 2016). According to the taxonomical classification, *Calocybe indica* falls under Phylum: Basidiomycota, Class: Agaricomycetes, Order: Agaricales, Family: Tricholomataceae (Singh et al., 2022). In appearance, they are a fleshy, milky white, and compositionally, rich source of proteins, lipids, fibers, minerals, and carbohydrates. According to various studies, the pileus and gills are found to be higher in protein (40–60%), lipid (30–60%), and ash content (5–10%) than that of the stipe, whereas, the stipe containing higher fiber (40–50%) and carbohydrate (10–15%) content, respectively (Alam et al., 2022). Furthermore, the fruiting body is comprised of several amino acids (alanine, aspartic acid, glutamine, glutamic acid, proline, glycine, histidine, lysine, threonine, tyrosine, valine, and arginine), and vitamin (thiamine, riboflavin, ascorbic acid, nicotinic acid). In addition, convenient method of cultivation, comparatively lower investments, appealing fruiting body, desirable milky white color, extended shelf life, thermo-tolerant, wholesome and the growth period is of lesser extent are major benefits of the strain *Calocybe indica* over other strains (Subbiah and Balan, 2015). Milky white mushrooms are a good source of dietary fiber and are considered an alternative economical protein food for people with low income who cannot afford other expensive protein sources such as meat, and it can be used for potential applications such as formulation of functional and nutraceutical foods (Cheung, 2013). According to the literature, several solvents have been used for the extraction of bioactive compounds from milky mushrooms which possessed versatile bioactivities and it clearly justifies the theorem of one drug with multiple targets (Ghosh and Acharya, 2022). Over the past few years, several reports have been published on different extraction techniques of bioactive compounds including ultrasonication, heat treatment, and microwave-assisted. However, it has been proven that these methods might lead to auto-oxidation of the bioactive compounds (Bains and Chawla, 2020). Hence, to overcome this issue, the modified solvent evaporation (MSE) technique could be a better approach. In this technique, with a condition of refrigerated temperature, the polyphenolic components which are distributed in ethanol and the molecules of ethanol or any other organic solvent congregate enough kinetic energy from their exchange with neighboring molecules to escape from the bonds with another molecule. Therefore, these molecules leave the mass of liquids to join the air as vapors (Chawla et al., 2019 & (Bains and Chawla, 2020)). To characterize and quantify the ethanolic extract of *Calocybe indica*, certain analytical techniques have been performed. In addition, it has been believed that mushroom extracts have excellent antimicrobial activity. For instance, in a study, Bains et al., 2021 compared the antimicrobial activity of *Pleurotus floridanus* extract between vacuum oven drying and the modified solvent evaporated techniques. They concluded that the modified solvent evaporated technique can be considered one of the most effective techniques for the extraction of bioactive compounds from mushrooms. Therefore, we characterized the extracted bioactive compounds from *Calocybe indica* using chromatographic techniques with a modified solvent evaporated extraction method and intended to investigate the polyphenolic profiling. Based on the fragmentation pattern of mass spectra and their direct comparison with mass spectra as well as with the chemical profiles revealed the structure of the obtained compound. (Najda et al., 2021). High-Performance Liquid Chromatography is a precise analytical technique for the separation and identification of biological substances in a mixed solution used for the quantification of individual compounds and accurately determines each compound based on peak area and concentrations (Kuppusamy et al., 2018). Inflammation is the natural and necessary reaction by the body to signals caused by tissue damage or any pathogenic infestation primarily arises as a series of events, beginning with a quick induction phase that results in a pro-inflammatory response, followed by a resolution phase (Najda et al., 2021). As a result, inflammation is a fundamental key to wound healing, however, if this well-coordinated response is disrupted, inflammation can become uncontrolled or chronic, leading to the development and progression of a variety of inflammatory disorders such as cancer, obesity, sepsis, cardiovascular, neurological and autoimmune diseases (Bains et al., 2021). Therefore, the present study was carried out based on the following objectives: i) Formulation and characterization of the *Calocybe indica* powder, ii) evaluation of antimicrobial efficacy of *Calocybe indica*, and iii) determination of anti-inflammatory of *Calocybe indica* extract.

2. Materials and methods

2.1. Materials

Fresh milky mushrooms were obtained from The Natura Agro Farms, Chennai, Tamil Nadu, India. Identification of mushrooms was done by the Department of Botany, Lovely Professional University, Phagwara, Punjab, India.

2.1.1. Chemicals

HPLC grade standards include caffeic acid, syringic acid, p-coumaric acid, rutin, L-ascorbic acid, BSA (bovine serum albumin), DPPH (2-2–diphenyl-1-picrylhydrazyl), and phosphate buffer saline (PBS) were procured from Sigma Aldrich Co. St. Louis, USA. Other 'Analytical Reagent grade chemicals including sodium hydroxide, ethanol, citric acid, hydrochloric acid, nitric acid, phosphate buffer, and diclofenac sodium were procured from Loba Chemie Pvt Ltd. Navi Mumbai, India. For microbiological experiments, standard antibiotics (ciprofloxacin), Mueller Hinton Agar (MHA), nutrient agar, and nutrient broth were purchased from Hi-Media Pvt. Ltd., Mumbai, India. Pathogenic microbial strains *Staphylococcus aureus* (MTCC 3160) and *Escherichia coli* (MTCC 443), were procured from the Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, India. For the accomplishment of the research, deionized water and aqua-regia washed class 'A' glassware was used.

2.2. Methods

2.2.1. Nutritional composition of Calocybe indica

Fruiting bodies of fresh *Calocybe indica* were subjected to assess the nutritional value in terms of moisture (Subbiah and Balan, 2015), fat (Prameela et al., 2020), fiber (Chelladurai et al., 2021), protein, ash and carbohydrate) content. The moisture content of *Calocybe indica* was determined by using the method of a hot air oven. In addition, protein content in terms of total nitrogen content was estimated using the Kjeldahl apparatus and the obtained values were multiplied by a factor of 6.25. Soxhlet apparatus was used for fat content estimation and the difference method was used to estimate the carbohydrate content of the sample.

2.2.2. Preparation of Calocybe indica powder

Fresh and healthy milky mushrooms were selected to formulate the mushroom powder. Briefly, selected mushrooms were gently wiped with a cotton cloth to remove any dust particles present on the outer layer. These mushrooms were then sliced into smaller uniform pieces and consequently placed in a tray dryer (Labfit India Pvt. Ltd., Mumbai, India) at 40 °C for 24 h to remove the free form of water. The dried slices of mushroom were then grounded in fine powder using a mechanical grinder (Rex 900, Bajaj Electricals Ltd., India). The obtained mushroom powder was then kept in glass containers at −20 °C.

Table 1
Proximate analysis and functional properties of *Calocybe indica*.

	Observation
Proximate components	
Moisture (%)	68.38 ± 1.13
Fat (%)	1.84 ± 0.63
Ash (%)	6.44 ± 0.58
Fiber (%)	6.87 ± 0.88
Protein (%)	12.48 ± 0.68
Carbohydrate	3.99 ± 0.43
Functional properties	
Bulk density	0.74 g/cm^3
Oil binding capacity	2.45 ± 0.05 g/g
Water binding capacity	3.01 ± 0.24 g/g
Foaming capacity	63.67 ± 1.54%
Foaming stability	59.28 ± 0.98%
Emulsifying activity	62.68 ± 1.78%
Emulsifying stability	68.94 ± 1.92%

Data are presented as means ± SD ($n = 3$).

Table 2
Quantification of polyphenolic and flavonoid components using high-pressure liquid chromatography.

Compounds	Type	RT (min)	Quantity (ppm)
Caffeic acid	Polyphenol	3.063	11.1534
Syringic acid	Polyphenol	3.287	0.057
p-Coumaric acid	Polyphenol	4.200	1.6385
Rutin	Flavonoid	8.780	0.3495

RT - retention time, ppm - parts per million*.

2.2.3. Functional properties of Calocybe indica powder

2.2.3.1. Bulk density. The powdered mushroom sample was subjected to the estimation of the bulk density by following the proposed method of (Sadh, et al., 2018). Samples were gently filled in a 5 ml graduated glass measuring cylinder that comprises a minimum count of 0.5 ml. The bottom of the measuring cylinder was mildly and gently tapped ten times till further reduction of the volume of powdered samples after complete filling up to the 5 ml mark. The bulk density of powdered *Calocybe indica* was determined as the weight of the powdered mushroom sample occupied per unit volume of the powder and it was expressed as g/cm^3.

2.2.3.2. Oil and Water binding properties of Calocybe indica. The water and oil binding efficacy of powdered *Calocybe indica* was examined by following the proposed method of Chawla et al. (2020). To assess these properties, pre-weighed centrifuge tubes with a capacity of 15 ml were taken and a 1 g powdered sample was dispersed with 10 ml triple distilled water. Alternatively, another 1 g of sample was dispersed with soyabean oil, and both the samples were kept undisturbed for 30 min. Centrifugation was done at 5000 rpm for 5 min at room temperature (27 °C) and the supernatant of both oil and water was discarded. The weight of tubes with pellets was then measured and water and oil absorption capacity were expressed as g of water and g of oil reserved per g of the powdered mushroom samples.

2.2.3.3. Emulsifying properties of Calocybe indica. To evaluate the emulsion properties, the emulsion was formulated by following the

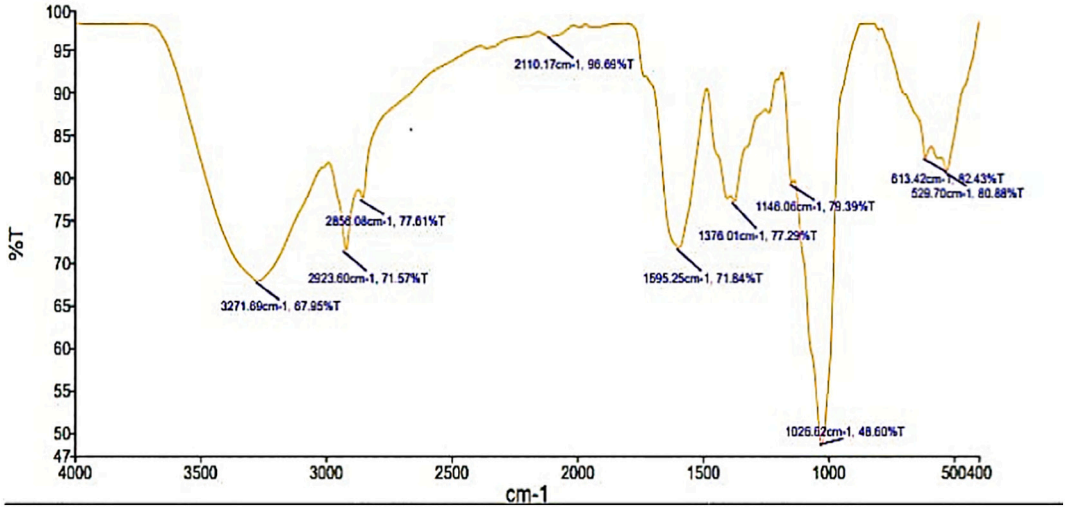
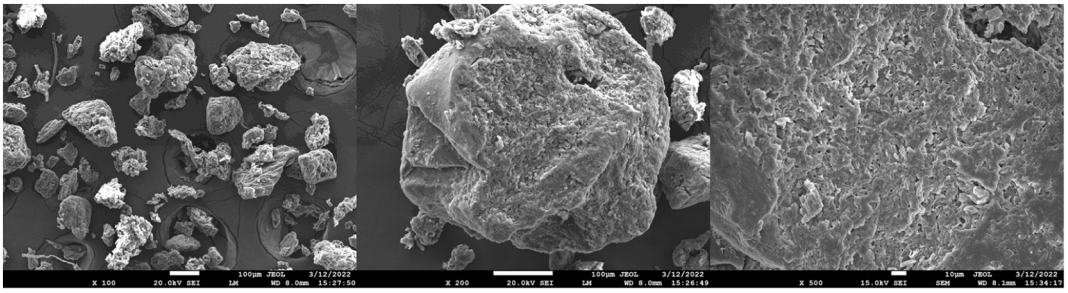


Fig. 1. Fourier transform infrared spectrum of *Calocybe indica* powder.



(a) **(b)** **(c)**

Fig. 2. Morphology of *Calocybe indica* powder with scanning electron micrographs of (2a), 100×, (b)200×, (2c), and 500×.

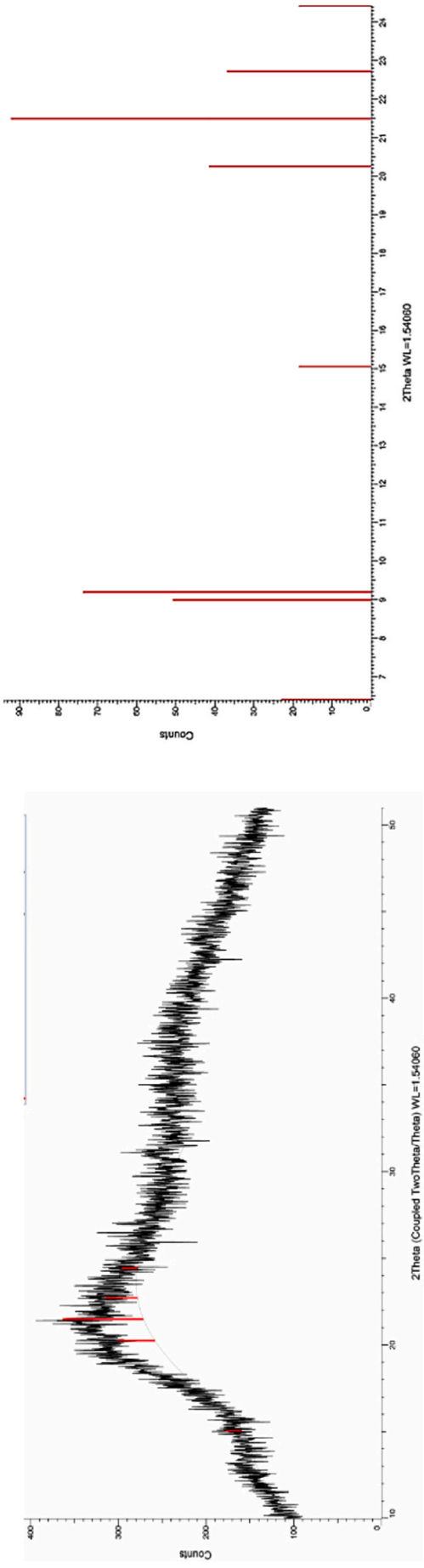


Fig. 3. Crystalline structure evaluation of *Calocybe indica* powder using X-ray diffraction pattern.

method of Chawla et al. (2017). Herein, a 2% (*w/v*) solution of mushroom powder was prepared and the sample was subjected to a centrifuge at 10000 ×*g* to collect the soluble fractions of the mushroom. Low energy method was used to formulate the emulsion and 2 ml of soyabean oil was added in the obtained aqueous phase (soluble fraction, 100 ml). The sample was further mixed using a magnetic stirrer (SPINOT MC 02, Tarsons, Kolkata, India) at 1500 rpm for 2 h. The milky white appearance of the sample confirmed the formulation of emulsion. To assess the emulsifying properties of the mushroom powder the prepared emulsion samples were centrifuged at 500 rpm for 5 min. The height of the emulsified sample was then measured using a graduated measuring cylinder for the calculation of emulsifying activity.

$$Emulsifying\ Activity\ (\%) = \frac{Emulsified\ layer\ height}{Height\ of\ the\ total\ content} \times 100$$

For the evaluation of emulsion stability, the emulsion was subjected to heat at 80 °C for 30 min and further centrifuged at 1500 rpm for 5 min and calculated as follows:

$$Emulsifying\ Stability\ (\%) = \frac{Emulsified\ layer\ after\ heating}{Emulsified\ layer\ before\ heating} \times 100$$

2.2.3.4. Foaming capacity and stability. The method proposed by Chawla et al. (2020) was employed to evaluate the foam capacity and foam stability of the powdered mushroom samples. Briefly, a 2% (*w/v*) solution was prepared using a magnetic stirrer at 500 rpm for 30 min. Herein, soluble fraction was collected to assess the foaming properties of the mushroom, and samples were centrifuged at 10000 ×*g* for 15 min. The obtained sample was then whipped in an auto-mix blender at its maximum speed for about 10 min. Immediately, the sample was transferred to a 250 ml graduated glass measuring cylinder and the volume was measured both before and after stirring.

$$Foaming\ Capacity\ (\%) = \frac{Whipped\ volume - the\ volume\ before\ whipping}{The\ volume\ before\ whipping} \times 100$$

The change in foam volume after 20 min (30 °C) was measured as foam stability.

$$Foaming\ Stability\ (FS\%) = \frac{Volume\ after\ resting - Volume\ before\ whipping}{Volume\ before\ whipping} \times 100$$

2.2.4. Characterization of Calocybe indica powder

2.2.4.1. Fourier transform infrared spectroscopy (FTIR). Functional groups present in the powdered mushroom were evaluated and spectra were recorded using FTIR with Diamond Attenuated total reflectance and Pellet accessories (Perkin Elmer, Spectrum Two). To obtain the results, a sample (5 mg) was placed on the clean surface of the mirror of the machine, and the lens was confined over the sample. Spectra were recorded at a wavenumber of the mid-infra-red region (4000–400 cm^{-1}) using air as the background and data were consequently obtained in terms of transmittance using inbuilt Spectrum 10 software.

2.2.4.2. Scanning electron microscopy. Morphological characteristics of powdered milky mushrooms were evaluated using Field Emission-Scanning Electron Microscope coupled with an Energy-dispersive detector and gold sputter coater (FE-SEM: JEOLJSM-7610F Plus EDS: OXFORD EDS LN2 free, gold coater: JEOL Smart Coater). Micrographs were recorded and the images were taken at 100×, 200×, and 500× with an accelerating voltage of 0.1 kV to 30 kV at a working distance of 1.0–40 mm.

2.2.4.3. X-ray diffraction (XRD). XRD reveals vital characteristics of the 3D crystalline structure, the chemical bonding, and the affinity of particles (Carducci et al., 2019). Herein, the Powder XRD (Bruker D8

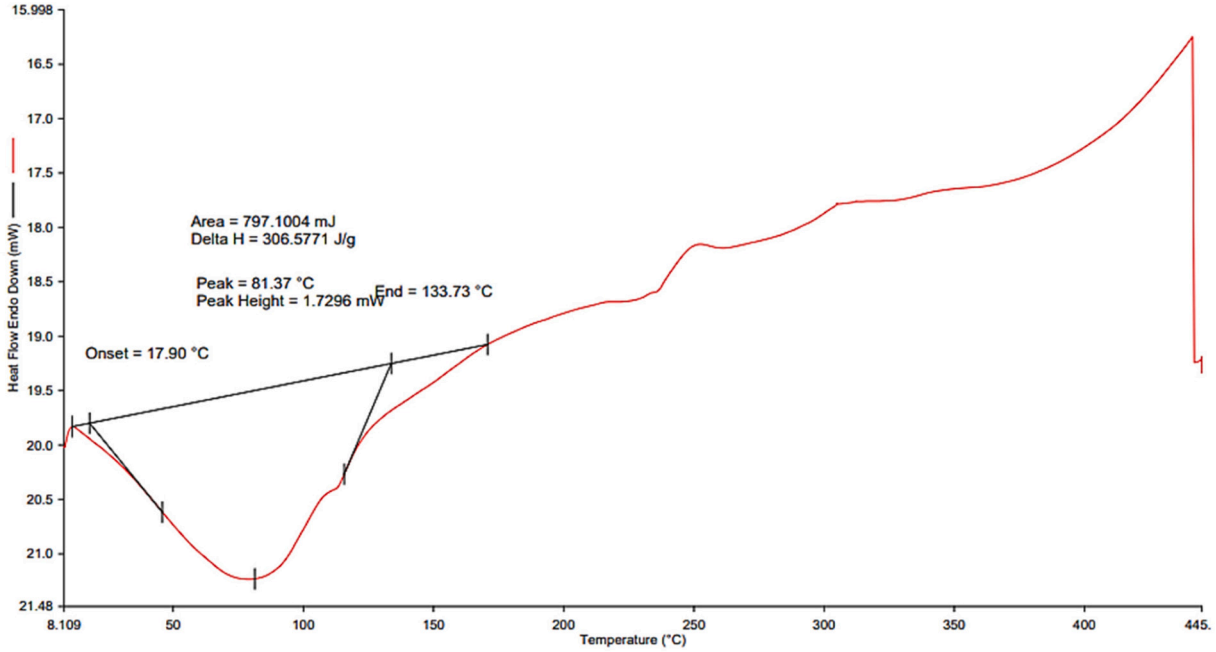


Fig. 4. Differential scanning calorimetry spectra of *Calocybe indica* powder.

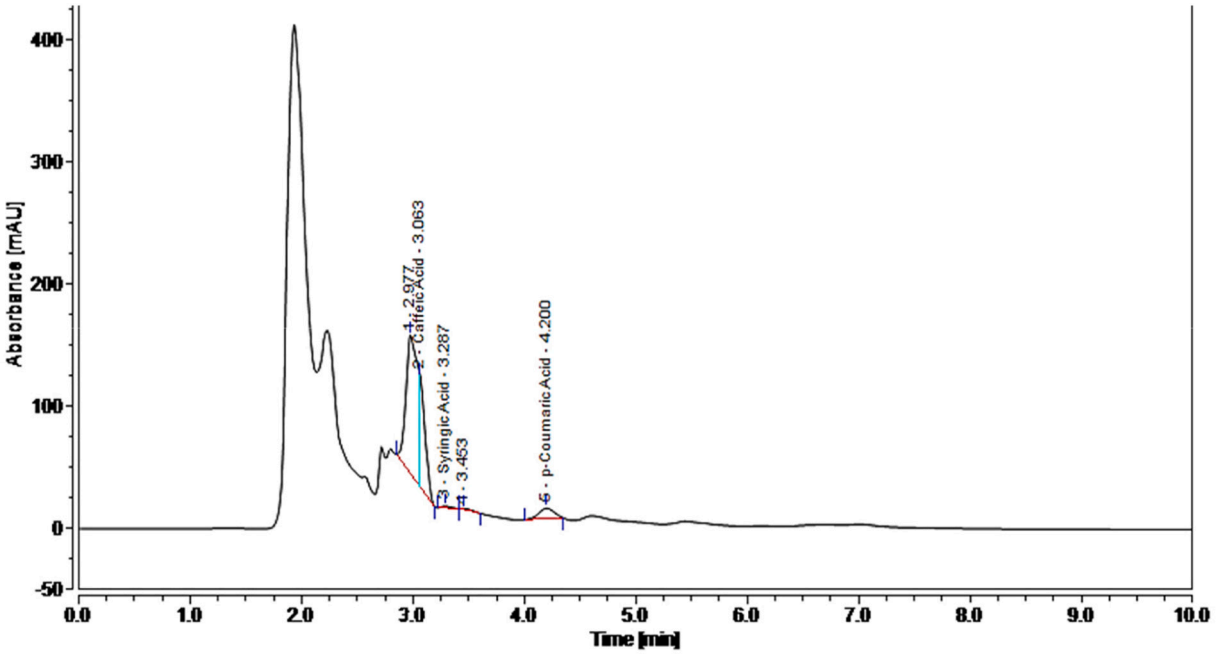


Fig. 5. Chromatogram of polyphenolic components of ethanolic extract of *Calocybe indica.*

Advance) equipment was used to determine the diffraction pattern of the powdered mushroom sample (5 mg). The sample was kept in a sample holder within the chamber of analytical X-ray having a wavelength of 1.5406 Å of Cu-Kα radiation and the spectra were scanned in the 2θ range from 10° to 50° at a 10°/min scan rate.

2.2.4.4. Differential scanning calorimetry. Differential scanning calorimetry (Perkin Elmer DSC 6000) was performed in a 99.999% nitrogen gas atmosphere with the rate of increase in temperature being 0.01 °C/min to 100 °C/min. The measurements were recorded using the thermal protocol initiating at 10–450 °C at a 10°/min scan rate. Thermocouple-based temperature sensors were used to detect and plot the results.

2.2.5. Extraction of Calocybe indica by modified solvent evaporation technique

A method proposed by (Bains and Chawla, 2020) was employed for the extraction of bioactive compounds from powdered *Calocybe indica.* Briefly, in a 250 ml conical flask, powdered mushroom (10 g) was carefully dispersed in absolute ethanol (100 ml) and the flask was then placed in an orbital shaker (Max Q 4000 series, Thermofisher Scientific private limited, Mumbai, India) at 100 rpm for 72 h. The supernatant was collected by sample filtration using Whatman no. 1 filter paper. To obtain the ethanol-free extract, evaporation of ethanol was carried out using a modified solvent evaporation technique (refrigerated temperature, 4–7 °C) for 72 h. The dried extract was then stored in glass vials at

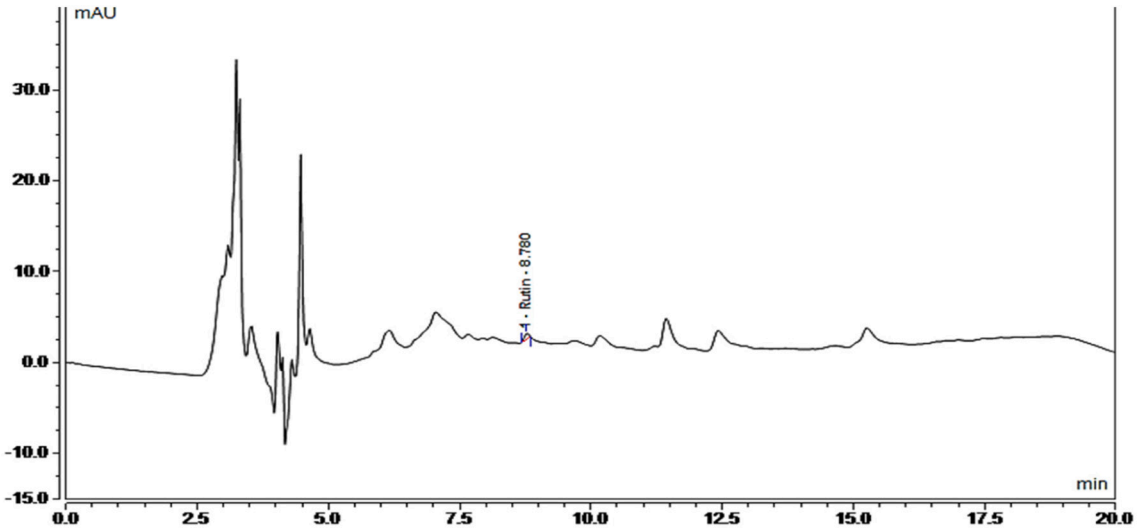


Fig. 6. Chromatogram of flavonoid components of ethanolic extract of *Calocybe indica.*

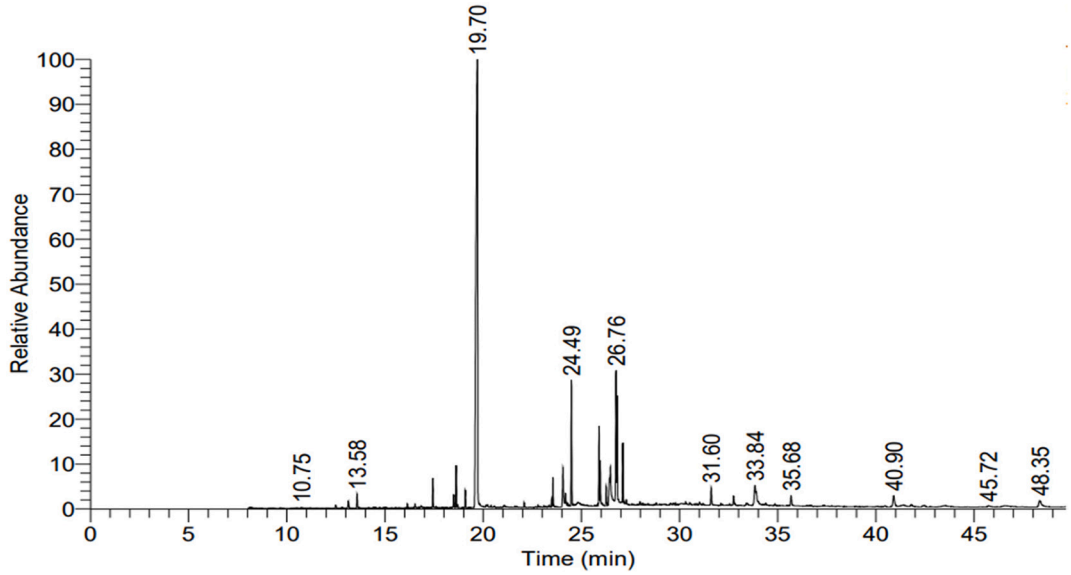


Fig. 7. GC-MC chromatogram of ethanolic extract of *Calocybe indica* showing bioactive constituents.

−20 °C for further analysis.

2.2.6. Quantification of polyphenolic and flavonoid compounds

Bioactive polyphenolics and flavonoids of ethanolic extract of mushroom were determined using Ultra-pressure liquid chromatography (Thermo Fisher Scientific Model: Dionex Ultimate 3000). The system was equipped with column 20RBAX eclipse XDB-C18 (4.6 × 250 mm, 5 µm, Agilent), and 1% acetic acid, acetonitrile (70:30) were used as mobile phase. A 20 µl sample volume was injected manually in the injector and an isocratic run with a flow rate of 1 ml/min for 10 min was employed. For the polyphenol detection, Diode Array Detector (280 nm) with 30 °C column oven temperature was used. In addition to this, the quantification of flavonoids was carried out and water (solvent A) and acetonitrile (Solvent B) was used as a mobile phase. Briefly, the gradient system (solvent A: 15, 30, 50, and 15% and solvent B: 85, 70, 50, 85%) with a flow rate of 0.7 ml/min for 0–20 min, respectively were used. The Chromeleon 7.0 software was used to quantify the bioactive compounds (Bains and Chawla, 2020).

2.2.7. GC–MS analysis of Calocybe indica ethanolic extract

For bioactive compound profiling, ethanolic extract of *Calocybe indica* was subjected to GC–MS analysis (Thermo Fisher Scientific, USA). The system was equipped with an autosampler (TriPlus RSH), gas chromatography (GC 1300), and a Mass Selective quadrupole detector. For the stationary phase, a column (40 m, 0.15 mm ID, and 0.15 µm film thickness) was used and the sample was diluted with GC grade hexane (1:99) and then injected with an autosampler in the splitless mode process. The temperature of the injector was kept at 250 °C, and the GC program was started from 60 °C (1 min) to 180 °C (3 min), and the final temperature was achieved at 240 °C (12 min). The ramping of temperature was maintained at 10 °C/min until the complete run. In addition, a helium gas flow of 0.7 ml/min was maintained and the ion source temperature was maintained at 230 °C along with the temperature of the transfer line (250 °C). Ionization and fragmentation of the components were attained by 70 eV electron impact. The mass filter was adapted to a scan range of *m/z* 45 and 450. The obtained results were administered using inbuilt Xcalliber Software.

Table 3
Bioactive compounds identified in modified solvent evaporated ethanol extract of *Calocybe indica*.

Retention time (min)	Compound name	Molecular formula
10.75	1,2,4-Trioxolane, 3,5-diphenyl-	$C_{14}H_{12}O_3$
13.58	2,4-Decadienal	$C_{10}H_{16}O$
13.58	2,4-Nonadienal	$C_9H_{14}O$
19.70	1H-Pyrido(4,3-b) indole, 2,3,4,5-tetrahydro-2,8-dimethyl	$C_{13}H_{16}N_2$
19.70	Quinoxaline-6-carboxylic acid, (1-oxo-1,3-dihydroisobenzofuran-5-yl) amide	$C_{17}H_{11}N_3O_3$
24.49	Hexadecanoic acid, ethyl ester	$C_{18}H_{36}O_2$
26.76	9,12-Octadecadienoic acid, ethyl ester	$C_{20}H_{36}O_2$
31.60	4-Cyanophenol, TBDMS derivative	$C_{13}H_{19}NOSi$
31.60	N, α, α'-Trimethyldiphenethylamine	$C_{19}H_{25}N$
33.84	1,1,1,5,7,7,7-Heptamethyl-3,3-bis (trimethylsiloxy) tetrasiloxane	$C_{13}H_{40}O_5Si_6$
35.68	1-Hexyl-1-nitrocyclohexane	$C_{12}H_{23}NO_2$
40.90	Z-6-Phenyldec-4-en-2-yne	$C_{16}H_{20}$
45.72	2-(1-Hydroxycyclopentyl)-furan	$C_9H_{12}O_2$
48.34	3(2H)-Benzofuranone, 6-methoxy-2- (phenyl methylene)-	$C_{16}H_{12}O_3$

2.2.8. Antimicrobial activity of Calocybe indica ethanolic extract

The *Calocybe indica* ethanolic extract was examined for its antimicrobial susceptibility against *S. aureus* and *E. coli by employing the agar well diffusion assay (*Bains et al., 2021*). Briefly, MHA plates were prepared and the inoculum of each bacterial strain (1* × 10^8 cells/ml) was spread uniformly on it by the spread plate technique method, and wells were formulated with the help of a cork borer (6 mm). A stock solution containing 10 mg extract dispersed in Dimethyl sulfoxide (10 ml) was prepared and 0.1 ml of it was pipetted into the agar well. The plates were kept at 37 °C for 24 h for incubation and results were obtained by measuring the zone inhibition diameter in mm.

2.2.9. Kinetic time-kill study

For the kinetic study, 0.1 ml ethanolic extract of *Calocybe indica* was added to the test tubes containing microbial strains, and each sample was then observed during the interval of 0, 24, and 48 h. Results were obtained by calculating log CFU/ml for each sample.

2.2.10. Anti-inflammatory activity of ethanolic extract of Calocybe indica

The method proposed by Bains et al. (Bains et al., 2021) was used to evaluate the anti-inflammatory of the extract. For negative control, deionized water was used, whereas diclofenac sodium was used as a positive control. The percentage of HRBC membrane stabilization was calculated using the following equation:

$$Protection\ (\%) = 100 - \frac{optical\ density\ of\ test\ sample}{optical\ density\ of\ control} \times 100$$

In the albumin denaturation assay percentage of inhibition of protein denaturation was calculated using the following equation:

$$Inhibition\ (\%) = 100 \times \frac{Oa}{Oc} - 1$$

Herein, Oa is the absorbance of the test sample and Oc is the absorbance of the control.

2.2.11. Statistical analysis

For the statistical analysis, the method proposed by Chawla et al. (2019) was employed. The significant difference among the samples was calculated by one-way analysis of variance (ANOVA) and the comparison between means was calculated by critical difference (CD value). Microsoft Excel, 2019 (Microsoft Corp., Redmond, WA) was used for the calculation of means and standard deviation.

3. Results and discussion

3.1. Proximate analysis

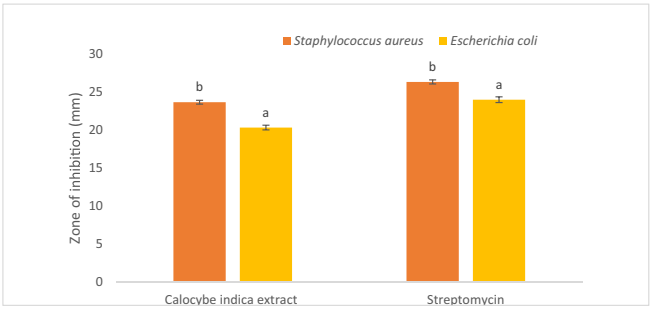
The results of proximate analysis of *Calocybe indica* are presented in (Table 1). Determination of the moisture content is important for the shelf–life of food products and the moisture content of milky mushrooms were found to be 68.38%. In addition, fat content was observed to be 1.84% and protein content was found to be 12.48%. Ash content in mushrooms was found to be 6.44% and fiber content was found to be 6.87%. Our results are well supported by the finding of (Shams et al., 2022) who determined the crystalline structure of freeze and cabinet dried button mushrooms powder.

3.2. Functional properties

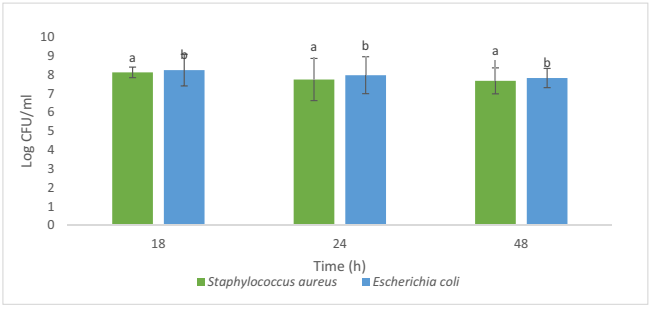
Industrial imported functional properties of powdered *Calocybe indica* were evaluated and the results are presented in (Table. 1). The bulk density of the powdered sample was obtained to be 0.74 g/cm^3 which indicates the porosity of mushroom powder. The obtained result revealed the impact of bulk density on the design of the package and it can be used in determining the type of the required packaging material (Awuchi et al., 2019). According to the nutritional aspects, a loose bulk density of porous mushroom powder increases the digestibility of food and enhances nutrient and energy value, which is greatly helpful in the formulation of functional foods. As well, a suitable reduction in the size of mushroom powder can improve the bulk density. The results of water and oil binding capacity are presented in Table 2. This property of the porous mushroom powder significantly depends on the compositional aspects including protein structure, amino acid profiling, and existing interaction sites on the surface (Tosif et al., 2021). Furthermore, good oil binding value reflects good textural properties of food products and a significant amount of oil can be entrapped which prevents the loss of oil and aromatic compounds from food products. Hydrophobic sites exiting on proteinaceous part of mushroom powder absorbed 2.45 g/g of oil droplets (Ishara et al., 2018). In addition to this water-binding capacity is the capacity of a sample to hold water molecules when it is exposed to a peripheral centrifugal force. It always comprises the total linked water, physically entrapped water, and hydrodynamic water (Alpizar-Reyes et al., 2017). In *Calocybe indica*, the water-binding capacity (3.01 g/g) was dependent on the hydrophilic sites of biopolymeric components including high amounts of polysaccharides (mannose and galactose 1,4 linked Glc*p*, t-Glc*p*, 1,2,3,6-Glc*p*, 1,2,4,6-Glc*p*, 1,3,6-Glc*p*, 1,4,6-Glc*p*, 1,2-Man*p*, 1,3-Gal*p*, and (1–3)-β-D-glucan, β-D-glucans (Tu et al., 2021) and proteins (one α-helix between cysteine (Wu et al., 2017), which are related to diffusion phenomena and affinity for water. As well, *Calocybe indica* powder was subjected to emulsifying properties and it revealed 62.68% emulsifying activity and 68.94% emulsion stability. The emulsion properties of the powder are associated with hydrophobic and hydrophilic sites of the biopolymeric moiety. The foaming capacity of the mushroom powder depends upon compositional factors including protein and polysaccharides structures, which uphold the interruption of air bubbles and slow down the rate of coalescence. *Calocybe indica* powder showed 63.67% foaming capacity and 59.28% foaming stability. From the above results, *Calocybe indica* powder could be used as an important ingredient for the fabrication of foam and emulsion-based functional foods.

3.3. FTIR spectroscopy of Calocybe indica

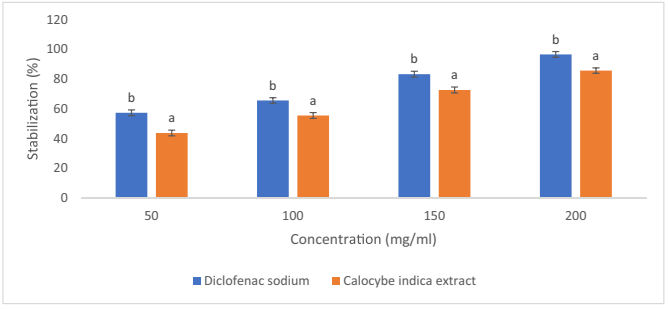
Based on functional properties, the sample was subjected to infrared spectroscopy ranging from 4000 to 400 $cm^{-1,}$ and the results are represented in Fig. 1. According to the FTIR spectra, *Calocybe indica* revealed 10 peaks detected at 3271.69 cm^{-1}, 2923.60 cm^{-1}, 2856.08 cm^{-1}, 2110.17 cm^{-1}, 1595.25 cm^{-1}, 1376.01 cm^{-1}, 1146.06 cm^{-1}, 1026.62 cm^{-1}, 613.42 cm^{-1} and 529.70 cm^{-1}. The FTIR vibrational frequencies



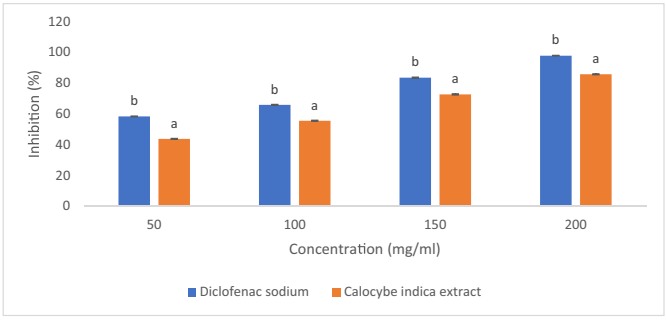
(a)



(b)



(c)



d

Fig. 8. (a) the antibacterial activity of ethanolic extract of *Calocybe indica* against Gram-positive and Gram-negative microorganisms (b), Time-kill study of ethanolic extract of *Calocybe indica* against Gram-positive and Gram-negative microorganisms (c), the anti-inflammatory activity of *Calocybe indica* ethanolic extract in comparison with standard diclofenac sodium during the HRBC membrane stabilization assay (d), the anti-inflammatory activity of *Calocybe indica* ethanolic extract in comparison with standard diclofenac sodium during the albumin denaturation assay of *Calocybe indica*
Data are presented as means ± SD ($n = 3$).
[a-b]Means within the column with different lowercase superscripts are significantly different ($p < 0.05$) from each other.

obtained at 3271.69 cm^{-1} inferred the O − H stretching (hydrogen-bonded intermolecular), 2923.60 cm^{-1} indicated the existence of C − H, 2856.08 cm^{-1} corresponded to C − H, 2110.17 cm^{-1} indicated C≡C, symmetric carboxylate stretching of C═O at 1595.25 cm^{-1} was observed which can confirm the presence of amino acids (Oliveira et al., 2016) and 1376.01 cm^{-1} can be attributed to N═O stretching. The C − O stretching appeared at 1146.06 cm^{-1}, 1026.62 cm^{-1} was associated with the presence of C − O, 613.42 cm^{-1} indicated C − Cl, and 529.70 cm^{-1} corresponded to C − H revealing α- and β- pyranose compounds, both glycosidic and non-glycosidic (Bekiaris et al., 2020). Our results are comparable with the finding of Chelladurai et al. (2014) who performed the *in vitro* cultivation technology and nutritional status of *Calocybe indica* and revealed similar peaks.

3.4. Scanning electron microscopy (SEM)

The structural characteristic of the *Calocybe indica* provided a three-dimensional view and the results were represented in (Fig. 2a, b, and c) at 100× 200× 500×, respectively. The higher magnifications of the surface of the mushrooms appeared to have a rough morphology with evident craters. Herein, the surface morphology is studied which is indefinite in shape due to rough handling during grinding. Due to excellent rehydration ability, the powdered samples consisting of highly porous surfaces could be an exceptional component for the food industry (Deshpande et al., 2013; Her et al., 2015). Our micrographs of the powdered mushrooms were comparable with the finding of (Her et al., 2015) who developed a spray freeze-drying process for the development of volatile shiitake mushrooms (*Lentinus edodes*) powder.

3.5. X-ray diffraction

The crystalline characteristics of powdered *Calocybe indica* were determined using XRD analysis and the results are depicted in (Fig. 3). The XRD patterns revealed potential peaks, which revealed the crystalline nature of the powdered mushrooms. The distinctive diffraction patterns of powdered mushrooms revealed a substantial peak at 2θ values of 20.258°, 21.499°, 22.724°, and 24.435°, respectively. Moreover, broad peaks were observed for the mushroom powder which revealed the small crystalline structure of the samples.

3.6. Differential scanning calorimetry

The thermal characteristics including denaturation temperature (T_d) and enthalpy change (ΔH) and identification of changes in phase transitions of *Calocybe indica* powder were examined using differential scanning calorimetry. The results obtained from (Fig. 4) summarized the DSC parameters obtained at different temperatures ranging from 10 to 445 °C for endothermic reaction at on-set temperature (T_{onset}) = 17.90 °C, end temperature (T_{endset}) = 133.73 °C, denaturation peak temperature (T_{peak}) = 81.37 °C, and ΔH = 306.5771 J/g. The first (T_{onset}) peak at 17.90 °C was due to the release of water molecules.

3.7. HPLC of bioactive compounds

Different polyphenols and flavonoids including caffeic acid, syringic acid, p-coumaric acid, and rutin were determined using HPLC and the results are represented in (Table 2) and (Figs. 5 and 6). According to the results, among the four phenolic acids and flavonoids, moderate amounts of caffeic acid (11.1534 ppm) were present followed by p-coumaric acid (1.6385 ppm) and syringic acid (0.057 ppm). Current results showed a moderate amount of flavonoid content majorly comprising of rutin (0.3495 ppm). Herein, caffeic acid possesses various biological and pharmacological activities including anti-inflammatory, antioxidant, anticancer, and neuroprotective effects. They are linked with the existence of two hydroxyl groups at its aromatic ring, the structure has a phenol ring with OH at positions 3 and 4 of the ring and a hydrocarbon chain at position 1 with an acid group (Alam et al., 2022). Syringic acid is used as a therapeutic agent in various diseases (diabetes, cardiovascular diseases, cancer, neuro, and liver damage) and exhibits anti-oxidant, antimicrobial, anti-inflammatory, and antiendotoxic activities (Srinivasulu et al., 2018). However, p-coumaric acid was found to have antimicrobial properties which inhibited most of the organisms (Stojković et al., 2013). Rutin is reported to improve the stability of color during storage, antioxidant *in vitro* properties which have many therapeutic properties, mainly attributed to its potent antioxidant and anti-inflammatory activities (Frutos et al., 2019). All results were in accordance with the findings of Bains et al. (Bains et al., 2021) who revealed polyphenolic and flavonoid components from modified solvent evaporated ethanolic extract of mushrooms.

3.8. GC–MS analysis

The analysis of GC–MS results revealed different types of bioactive compounds from the extract and the results are represented in (Fig. 7). The structure of the obtained compounds was analyzed. The chemical profile accompanied by the retention time and molecular formula was presented in the following (Table. 3b) Herein, the peaks were recorded at 10.75, 13.58, 19.70, 24.49, 26.76, 31.60, 33.84, 35.68, 40.90, 45.72 and 48.35. They revealed the presence of ethyl tridecanoate, hexadecanoic acid ethyl ester, pentadecanoic acid ethyl ester, undecanoic acid ethyl ester, N, α, α′-trimethyl diphenethylamine, nicotinonitriles, phosphonic acid decyl-, 1-hexyl-2-nitrocyclohexane, diallyl divinylsilane, 3-phenyl-pyrrolo (2,3-β) pyrazine and many other compounds possessing potential health benefits to the human body. Herein, 1,2,4-Trioxolane, 3-phenyl- had antimicrobial, food preservative, a neuroprotective agent, and an antifungal agent. 2,4-Decadienal and 2,4-Nonadienal were used as flavoring agents. 1H-Pyrido(4,3-b) indole, 2,3,4,5-tetrahydro-2,8-dimethyl reduces anesthetic and opioid requirements; and causes sedation and analgesia. Quinoxaline-6-carboxylic acid, (1-oxo-1,3- dihydroisobenzofuran-5-yl) amide showed inhibition properties and hexadecanoic acid is the fatty acid used as a food additive. Therefore, this characterization revealed the occurrence of secondary metabolic components that show distinctive therapeutic importance including antimicrobial, antioxidant, anti-inflammatory, and anticancer properties

3.9. Antimicrobial analysis

The antimicrobial properties of ethanolic extract of *Calocybe indica* were evaluated against both Gram-positive and Gram-negative bacterial strains. The result of antimicrobial activity is presented in (Fig. 8a). Herein, in the case of Gram-positive bacteria, extract of *Calocybe indica* nanoemulsion showed a significantly ($p < 0.05$) higher zone of inhibition (23.67 mm) whereas, in the case of Gram-negative bacteria, *Calocybe indica* extract showed a significantly ($p < 0.05$) lower zone of inhibition (20.33 mm). In the case of Gram-negative bacteria, the outer cell membrane consists of two layers one is the phospholipid protective layer bound to the inner leaflets and the other is the lipopolysaccharide layer bonded to the outer leaflets. The modification in these structures includes porin mutation, variation in hydrophobic properties, and other factors that may result in resistance of *E. coli* towards the ethanolic extract of *Calocybe indica* (Bains and Chawla, 2020; Bains et al., 2021). Furthermore, the outer cell wall of Gram-positive bacteria is composed of a thick porous hydrophobic cell wall to which the number of proteins and lipids can bind and could be the reason for the susceptibility of *S. aureus* towards ethanol extract of *Calocybe indica* (Bains and Chawla, 2020). In addition to this in the present study, several phytocompounds that include ethyl tridecanoate, undecanoic acid ethyl ester, diallyl divinylsilane, 3-phenyl-pyrrolo(2,3-β) pyrazine are identified by GCMS technique and these compounds reported to have antimicrobial properties.

3.10. Time kill study

Time-kill study of ethanolic extract of *Calocybe indica* extract was done and the results were presented in (Fig. 8b). Against Gram-negative bacteria *E. coli*, extract showed a significantly ($p < 0.05$) higher (8.24 Log CFU/ml) value as compared to *S. aureus* (8.12 Log CFU/ml). With increasing time, *Calocybe indica* extract showed a significantly ($p < 0.05$) lower value against *S. aureus* in comparison with *E. coli*. The secondary metabolites present in extract result in inhibition of the growth of *S. aureus* by inhibiting the biochemical pathway, synthesis of proteins, and causing outer membrane disintegration. *E. coli* showed a higher log CFU value as the external cell membrane of the bacteria is composed of irregular bilayer phospholipids and lipopolysaccharides layers that are fixed with the β-barrel channels and proteins. These channels and proteins prevent the entry of extract inside the cell (Najda et al., 2021). The present results are well supported by the finding of (Bains and Chawla, 2020; Bains et al., 2021) who revealed a similar trend against *Escherichia coli* and *Staphylococcus aureus*.

3.11. Anti-inflammatory activity

Protein (BSA) denaturation assay and HRBC membrane stabilization assay were used for the *in-vitro* anti-inflammatory assay and the results are represented in (Fig. 8c and d). During membrane stabilization and protein, denaturation assay significant ($p < 0.05$) difference was observed in the anti-inflammatory activity of *Calocybe indica* extract as compared to a standard anti-inflammatory drug. As well, *Calocybe indica* extract showed 43.72–85.69% membrane stabilization as compared to diclofenac sodium (57.32–96.58%) in a concentration-dependent manner (20–100 mg/ml). A similar trend was observed in the case of BSA denaturation for *Calocybe indica*. In the present study, HRBC membrane stabilization and BSA denaturation assay were performed to evaluate the anti-inflammatory effect of extract as the HRBC membrane is similar to the lysosomal membrane, and denaturation of albumin proteins is also correlated with the inflammatory response thereby resulting in numerous inflammatory diseases. The denaturation of proteins results due to the action of the intracellular substances that cause injuries to tissues. The synthesis of major inflammatory mediators and cell membrane stabilization was supposed to be prevented by the ethanolic extract of *C. indica* (Bains and Chawla, 2020). The anti-

inflammatory effect of the extract in the present study can be due to the phytochemical's compounds identified in the extract namely ethyl tridecanoate, diallyl divinylsilane, 3-phenyl-pyrrolo (2,3-β) pyrazine, and N, α, α′-trimethyl diphenethylamine by GC–MS analysis. 1,2,4-Trioxolane, 3,5-diphenyl- ($C_{14}H_{12}O_3$) with potential chemopreventive activities, mediates anti-inflammatory effects and inhibits cyclooxygenase and hydroperoxidase functions.

4. Conclusion

Calocybe indica is a macro fungus and it is well-known for its nutritional and therapeutic aspects. They are among the easiest and most cost-effective cultivatable mushrooms in tropical areas. The nutritional composition of the mushroom was evaluated and found to be a good source of proteins and carbohydrates. From the results of functional properties, it can be concluded that *Calocybe indica* powder could be used as an important ingredient for the fabrication of foam and emulsion-based functional foods. Furthermore, mushroom powder revealed a porous structure and a small crystal structure. Polysaccharide and protein moiety showed both hydrophilic and hydrophobic domains and the sample showed higher techno-functional properties. A modified solvent evaporation technique was used to prepare ethanolic extract and it was quantified for total phenolics and flavonoids by spectrophotometric and chromatographic techniques. Furthermore, the mushroom extract showed effective antimicrobial against Gram-positive and negative bacteria. Significantly comparable anti-inflammatory activity was observed for mushroom extract. Caffeic acid, syringic acid, p-coumaric acid, and rutin present in *Calocybe indica* have been found to possess adequate anti-oxidant, antimicrobial, anti-inflammatory, anti-endotoxic activities, and other therapeutic properties. Furthermore, through GC–MS many compounds that possess antimicrobial activity, neuroprotective agents, antifungal agents, and used as food preservatives, and flavoring agents were detected. In conclusion, the mushroom extract has shown great potential for antioxidant and anti-inflammatory activity, therefore, it could be used as an effective food preservative and potential anti-inflammatory and antimicrobial agent.

Declaration of competing interest

Authors are declaring no conflict of interest.

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