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Medicinal potential of *Morchella esculenta* oil inhibits cancer cell proliferation, metabolite characterization through GCMS, GC×GC–TOF–MS and UHPLC–QTOF–MS

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The current study investigated comprehensive phytochemical profile through high resolution techniques like GCMS, GC×GC–TOF–MS, UHPLC–QTOF–MS, total phenolic content, total flavonoid content, antioxidant, and anticancer activities of *Morchella esculenta* oil using *in vitro* approaches. The results showed the identification of 19 (GCMS) 20 (UHPLC–QTOF–MS) and 68 (GC×GC–TOF–MS) phytochemical compounds belonging to diverse classes of compounds. Hexadecenoic acid, oleic acid, palmitic acid, linoleic acid, octanoic acid, C16 sphinganine, allopumiliotoxin267A, 3′-Hydroxy-T2 Toxin were the main compounds identified. The *Morchella esculenta* oil showed moderate phenolic content (13.26 ± 2.52 mg GAE/g dw), flavonoid content (11.35 ± 1.58 mg RE/g dw) and antioxidant activities (DPPH-IC50 = 118.46 µg/mL; ABTS-IC50 = 125 µg/mL; and FRAP-IC50 = 117.90 µg/mL). Using human cervix (HeLa), human colon (HCT-116) and human breast cancer (MCF-7) cell lines, essential oil of *Morchella esculenta* showed a dose/time-dependent antiproliferative and cytotoxicity activity with GI50 values of 27.61 µg/mL, 19.52 µg/mL, and 31.34 µg/mL respectively. The obtained results confirmed that *Morchella esculenta* oil is a rich source of bioactive molecules with strong antiproliferative and cytotoxicity potential towards cancer cell lines.

Cancer is the genetic disease which can start anywhere in the body in which body's cells grow uncontrollably and can spread to other parts to form new tumors, through a process called metastasis[1]. Our cells are programmed by genes that monitor the way our cells must function, grow, and divide. Cancer abnormality arises due to errors in genes or damage to DNA which can be through chemical agents, ultra violet rays, lifestyle, or harmful substances in the environment[2]. In concern to Global cancer burden World Health Organization (WHO)'s cancer agency and the International Agency for Research on Cancer (IARC) depicted disproportionate impact on underserved populations and warned regarding cancer inequities worldwide.According to estimated data 20 million new cancer cases and 9.7 million deaths were reported in 2022 and a 77% increase from this is expected by 2050 with estimates of new 35 million cancer cases. Synthetic, natural, or biological agents are employed through chronic administration as a cancer chemoprevention approaches to delay or reduce malignancy occurrence in different cancers[3]. Conventional cancer treatments to cure or shrink a cancer from spreading encompasses radiation, surgery, medicines, and other therapies. Unpleasant side effects like fatigue, hair loss, nausea vomiting, loss of appetite, sex, and fertility issues more importantly cancer resistance and recurrence are associated with conventional cancer chemotherapy treatments[4,5]. Fungi have a variety of medical applications due to their capability of generating potent antimicrobial and anticancer products than recorded by bacteria[6]. Number of anticancer compounds have been reported from *Penicillium chrysogenum*, *Aspergillus niger*, *Bartalinia robillardoides*, *Penicillium citrinum*, *Aspergillus fumigatus*, *Aspergillus oryzae*,

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Aspergillus flavus, *Aspergillus versicolor*, *Aspergillus terreus*, and *Penicillium polonicum* and have been used for investigating the anticancer activity against the uterine cervix, pancreatic cancer, ovary, breast, colon, and colorectal cancers[7]. Morel mushrooms, *Morchella* species are well-known edible mushrooms belonging to the Morchellaceae family and has always been attractive for their delicate flavour, high nutritional value, biological effects like anticancer properties. *Morchella esculenta* is considered as herbal boon to pharmacology and is listed among the most highly priced mushrooms found in the world and labelled as"growing gold of mountains. *Morchella esculenta* is pharmaceutically and economically one of beneficial wild species of mushrooms and naturally grows in hilly altitude with cold environment usually found in the forests of Jammu and Kashmir and Himachal Pradesh (India) at a height of about 2500–3500 m above sea level[8,9]. Guchhi is the local name for *Morchella esculenta* in India and employed for medicinal applications by traditional hill societies. Studies have reported presence of polysaccharides, proteins, trace elements, dietary fibres and vitamins, bioactive substances which impart nutritional and medicinal values[10,11]. Antioxidant, antimicrobial, anti-allergenic, anti-inflammatory, immunostimulatory, neuroprotective and antitumor properties of *Morchella esculenta* are attributed to its active constituents that includea broad range of active constituents belonging to different classes like tocopherols (δ-to copherol, α-tocopherol and γ-tocopherol), carotenoids (β- carotene and Lycopene), phenolic compounds (Protocatechuic acid and p-Hydroxybenzoic acid) and organic acids which contain citric acid, oxalic acid, fumaric acid, quinic acid and malic acid etc[12]. Different extracts like methanol, ethanol, and chloroform from *Morchella esculenta* has been reported with potent antimicrobial properties against *Salmonella typhimurium*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Enterobacter cloacae* and *Escherichia coli*[13,14]. *Morchella esculenta* have been used in traditional Chinese medicine from 2000 years as well as in Malaysia and Japan for the treatment of many diseases like for the treatment of excessive phlegm, indigestion, to cure cardiac diseases, used an antiseptic and effective in wound healing etc[15–17]. Previous studies investigated and reported ethanol, methanol, chloroform extracts of *Morchella esculenta* showed potent antiproliferative activity against different cancer cell lines including breast cancer (MCF-7), colon cancer (HCT-116) and cervix cancer (HeLa) in a dose-dependent manner and are known to induce apoptosis through modulation of tumorigenesis at different stages. *Morchella esculenta* is edible and rich in proteins, carbohydrates, vitamins particularly vitamin B, contains minerals like calcium, iron, copper, zinc, magnesium, manganese, sodium, phosphorus, selenium, and potassium[18–20]. To the best of our knowledge this is the first scientific investigation undertaken to analyse the metabolite identification through high-resolution profiling using GCMS, GC×GC–TOF–MS and UHPLC–QTOF–MS, antioxidant capacity and anticancer properties of oil obtained from *Morchella esculenta* collected from Kashmir Himalayas. This study introduces the novelty as there was lack of oil-based extract analysis of *Morchella esculenta* and introduces this previously unreported oil from *Morchella esculenta* as a potential source of natural chemotherapeutic agent.

Materials and methods

Chemicals, enzymes, and dyes were of analytical grade supplied by sigma-Aldrich and Hi-media laboratories. Double distilled water followed by autoclave was used during experimentation and solution preparation.

Collection and identification of *Morchella esculenta*

The *Morchella esculenta* (Fig. 1 A) was manually collected in the month of march from village Lajoora, district Pulwama from Kashmir, India. The mushrooms were taken for identification to centre for Biodiversity and Taxonomy at University of Kashmir and were given the voucher specimen no as 9138-KASH.

Extraction of *Morchella esculenta* oil with hexane

The *Morchella esculenta* collected was allowed to shade dry and grinded into powder (Fig. 1B) and 100 g of powdered material was dissolved in 400 mL hexane in a conical flask and regularly shaken at intervals for 48 h[21]. The solvent was filtered through Watmann filter paper 1 and a light yellowish filtrate was obtained (Fig. 1 C). The filtrate was further allowed to evaporate in a rota evaporator under 40 ^{0}C temperature to keep volatile and thermolabile compounds safe and finally deep yellowish oil was obtained. The oil was stored in sterilized vials at 4 ^{0}C for further experiments and analysis.

Fig. 1. (**A**) Shade dried *Morchella esculenta* (**B**) Powdered *Morchella esculenta* (**C**) Filtered hexane solution of *Morchella esculenta* obtained after 48 h.

GCMS analysis

For the identification of non-polar and semipolar compounds in *Morchella esculenta* oil the Thermo Scientific TSQ 8000 Gas Chromatograph - Mass Spectrometer was employed by default method. The instrument consisted of MS part of Triple Quadrupole with MS/MS that allowed it for the lower detection limits and later connected with TRACE 1300 GC unit and Auto-sampler. The GCMS unit has Ion Source Type of EI source programmable to 350ºC with capability of Mass Range up to 2 to 1100 amu. The column temperature raised up to 400ºC.The Detectors incorporated are Flame Ionization Detector (FID) and Electron Capture Detector (ECD). For the analysis of *Morchella esculenta* oil the default method was set up with parameters of 35 min run time, injection volume (µL) of 1.00 and 9438 scans were acquired. Acquisition Low Mass(m/z) was 10 and High Mass(m/z) was 650. NIST Library equipped with instrument was used for compound identification. The sample for analysis was prepared in HPLC grade ethyl acetate with *Morchella esculenta* oil concentration of 0.5 mg/mL.

GC×GC–TOF–MS analysis

GC-GC-TOF-MS (Two-dimensional gas chromatography with time-of-flight mass spectrometer facility) was performed with the benefit for analysis of compounds from complex mixtures where identification is done after high end separation. GC-GC-TOF-MS involves two columns and have different attributes for separation. In First column a thermal modulator attached ensures cryo focusing of effluent before its release to the second column. The two-dimensional output aids specifically in resolving the peak of target analytes even interferences in matrix are present. The spectral harvesting rate of the TOF-MS detector together with the software capabilities can help in de-convolution of co-eluting compounds. For metabolite identification in *Morchella esculenta* oil: Leco, Pegasus 4D with Agilent 7890B GC instrument with liwiid/gas injection mode was used. The two columns employed were Primary Column: Rxi 5-MS (30 m) and Secondary Column: Rxi 17Sil MS (2 m). The GC column configuration involved GC oven temperature as 350 C, GC modulator at 360ºC and of capillary detector as 250ºC. The target flow was 1.50 (mL/min) and carrier gas used was helium. In the MS component start mass (u) acquisition was 40, end mass (u) as 500, Acquisition rate (Spectra/second) was 100, detectoracquisition voltage was 1600 and electron energy voltage was – 70. Mass detection mode was manual with mass detect (mu/100 u) as 66, ion source temperature was 250 C. The spectral acquisitions were used through Chromate software for data acquisition and processing. Contour plots were drawn for manual peak identification. Individual compound identification was performed using the in-house NIST library. The minimum similarity of 80% (800/1000) with the library was the criteria to identify the compounds.

UHPLC–QTOF–MS

The UHPLC–QTOF–MS analysis of *Morchella esculenta* oil was performed by default method. The device list in instrument consisted of HiP sampler, Binary pump, Column component, DAD, QTOF. TOF/Q-TOF Mass Spectrometer with component model G6550A had dual AJS ESI ion source with stop time of 30 min and in it MS Abs. threshold was kept 200, MS Rel. threshold (%) was 0.01, MS/MS Abs. threshold as 5, MS/MS Rel. threshold (%) as 0.01.The acquisition mode was set with MS Min Range (m/z) of 120, MS Max Range (m/z) of 1200, MS Scan Rate (spectra/sec) as 1.00, MS/MS Scan Rate (spectra/sec) as 1.00 and isolation width MS/MS was Medium (~ 4 amu).Precursor selection was maintained with maximum precursor per cycle of 10,Threshold (Abs) at 10,000, Threshold (Rel%) at 0.010, and Target counts/spectrum was 25,000. Source parameters include gas temperature ^{0}C (200), gas flow (L/min) was 13, Nebulizer (psig) was 35, Sheath Gas Temp as 300 and Sheath Gas Flow was 11.Scan source parameter values were 3500 in VCap, 1000 in nozzle voltage,175 in fragmentor,65 in Skimmer 1 and 750 in OctopoleRFPeak.TheHiP sampler with model G4226A in auxiliary mode had drawn speed of 100.0 µL/min, Eject Speed as 100.0 µL/min, wait time after drawing as 2.0 s, sample flush out factor as 5, injection volume was 5.00 µL, The Binary pump with model G4220B had flow of 0.300 mL/min, maintained at low pressure limit of 0.00 bar and high pressure limit of 1200 bar. The max flow rampsup in binary pump was 100.000 mL/min^2 and max flow ramp down was 100.000 mL/min^2 and stop time was set up to 35 min. Solvent composition was used through channels A and B with 0.1 formic acid in water used in channel A and 100% acetonitrile in channel B. The Column component with model G1316C was set in temperature control mode of up to 40.00 °C.

Total phenolic and flavonoid contents

The Folin-Ciocalteu method for phenolic content (TPC) determination and aluminium chloride method for flavonoid content (TFC)were employed for the *Morchella esculenta* oil, following the protocol adopted by Chandni et al. with slight changes[22]. Gallic acid and rutin served as the standard compoundswith TPC were expressed as milligrams of gallic acid equivalents per gram of dry extract weight (mg GAE/g DW) and TFC as milligrams of rutin equivalents per gram of dry extract weight (mg RE/g DW).

Antioxidant analysis

DPPH (2,2-diphenyl-1-picrylhydrazyl) assay

The different concentrations of *Morchella esculenta* oil were prepared ranging from 25 µg/mL to 800 µg/mL to observe its scavenging potential by neutralizing DPPH radical using the previous method with minor modifications[23]. The 300 µL from each concentration were allowed to incubate in test tubes covered with aluminium foils with 2 mL of 0.1mM DPPH radical solution for 30 min in dark at 37 ºC.The DPPH radical solution was prepared by dissolving 3.94 mg of DPPH in 100 mL of HPLC grade methanol. After the incubation period completed 280 µL from each test tube containing different concentrations was poured into 96 well plate with each concentration in triplicate and absorbance was recorded at 517 nm wavelength in an ELISA spectrophotometer plate reader. The free radical DPPH solution was used as control and rutin as standard antioxidant. The decrease in absorbance was used to calculate IC50 value through following given formula.

$$\%\text{Scavenging activity} = [(\text{Ab} - \text{As})] \div \text{Ab} \times 100 \quad (1)$$

where: Ab—Control reaction absorbance of Blank; As—Absorbance of sample.

ABTS (2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) assay

The *Morchella esculenta* oil was evaluated to check its potential to scavenge ABTS•+ radical cation solution at varying concentrations as used in DPPH[24]. First the generation of ABTS•+ radical cation was started by mixing 10 mL of 7mM aqueous ABTS and 10 mL of 2.45 mM of $K_2S_2O_8$ solution and this was kept up to 14 h till a dark blue colour occurs which confirms formation of ABTS radical cation solution. Secondly the absorbance of ABTS generated solution was allowed to maintained at 0.710 ± 2.1 at 734 nm with ethanol. Finally, 10 µL from each prepared concentration (25 µg/mL to 800 µg/mL) of *Morchella esculenta* oil was mixed with 195 µL ABTS cation solution in a 96 well plate and allowed to incubate at 30 ^{0}C for 10 min. The absorbance of 96 well plate was taken at a wavelength of 734 nm in an ELISA spectrophotometerplate reader. The absorbance recorded at various concentrations was used to calculate 50% inhibitory concentration (IC50 value) by the above given (1) formula.

Superoxide radical scavenging assay

Scavenging potential of *Morchella esculenta* oil for the superoxide (O2 •-) anion radical was conducted by adopted method of Chandni et al.[22]. The reaction was allowed to start by generating superoxide radicals in 100 mM of 9 mL of sodium phosphate buffer (pH 7.4) containing 3 mL of NBT (150 µM) followed by mixing of 3mL of NADH (468 µM), and later different concentrations (25 µg/mL to 800 µg/mL) of *Morchella esculenta* oil. The3mLPMS(60µM) was added to the mixture and incubated at 30 ^{0}C for 5 minutesand absorbance was recorded at 560 nm using an ELISA plate spectrophotometer with a blank and standard antioxidant rutin. Formula 1 was used to calculate the percentage of superoxide radical scavenging.

MTT (3-[4,5-dimethylthiazol-2-yl]−2,5 diphenyl tetrazolium bromide) assay

The MTT assay was utilized to check cytotoxicity and antiproliferativepotential of *Morchella esculenta* oil on cervix (HeLa), colon (HCT-116) breast cancer (MCF-7) cell lines and normal regular human mouse fibroblast cell line (L-929)[22]. Cell cultures of cancer cells and normal cell line were seeded in 96 well plate with a density of 1×10^4 per well. The cells were incubated for 24 h, allowed to adhere and obtain confluency. After that *Morchella esculenta* oil treatment using different concentrations ranging from 10 µg/mL to 320 µg/mL were given to each well and were left for next 24 h. After incubation completed with *Morchella esculenta* oil, the media containing dead cells and oil samplefrom each well was removed and serum fresh media was added again in each welland in it 20 µL MTT dye (5 mg/mL) was mixed in and allowed to incubate again for 4 hours.After 4 hours media containing MTT dye was removed and 100µL DMSO was added and absorbance was recorded at 570 nm using an ELISA spectrometer. The decrease in absorbance was used to calculate inhibitory concentration (GI50) at different concentrations and percent inhibition at for each concentration was calculated by the following given Eq. 2. The cell cultures were maintained in carbon dioxide (CO_2) incubator at 37 ^{0}C, The growth medium supplied contained FBS, DMEM media, streptomycin, penicillin G and gentamycin.

$$\%\text{Growth inhibition} = [(\text{ABC} - \text{ABS}) \div \text{AB}_\text{C} \times 100 \quad (2)$$

AB_C is the absorbance of Control and AB_S is the absorbance of sample.

DAPI and Rhodamine staining

HeLa, HCT-116 and MCF-7 cancer cells were seeded into a 24-well plate at an optimal density of approximately 1×10^5 cells/well and kept overnight to facilitate adhesion and proliferation. Each well was then exposed to GI50 concentration of the *Morchella esculenta* oil for 24 h. After completion of treatment period media containing unattached dead cells and oil sample was removed and each well was washed with 1XPBS (Phosphate Buffer saline). After washing, 0.300 mL of 1XPBS was added again in each well in which 10 µL DAPI (Stock solution-1 mg/mL) and 10 µL Rhodamine 123 (Stock solution-1 mg/mL) were mixed and were allowed to incubate in dark for 30 min inside CO_2 incubator. After incubation with respective dyes the excess dye was removed by washing twice with 1XPBS followed by visualizing through fluorescence microscopy (Nikon Eclipse T2, Japan) to observe nuclear alterations and loss of fluorescence for the assessment of mitochondrial membrane potential[25].

Statistical analysis

Experiment outcomes are presented as the mean ± standard error with readings carried in triplicate. GI50 and IC50 values were calculated using excel software (2019) by comparing data variables in triplicate.

Results

Total phenolic and flavonoid content

The oil of *Morchella esculenta* demonstrated weak, with a total phenolic content of 13.26 ± 2.52 mg of gallic acid equivalent (GAE) per gram of dry weight (dw) and a total flavonoid content of 11.35 ± 1.58 mg of rutin equivalent (RE) per gram of dry weight as presented in Table 1.

Antioxidant activity

The radical scavenging potential of *Morchella esculenta* oil was observed through DPPH, ABTS and Super oxide radical assays respectively. Moderate radical scavenging potential was observed in all three assays with percent inhibition of 86.42%, 88.33% and 88.57% respectively at higher concentration of 800 µg/mL. The radical inhibition was concentration dependent as shown in Fig. 2. The IC50 values recorded were 118.46 µg/mL,125 µg/

Sample	TPC (mg GAE/g dw)	TFC (mg RE/g dw)	DPPH IC_{50} (µg/mL)	ABTS IC_{50} (µg/mL)	SARS IC_{50} (µg/mL)
Morchella esculenta oil	13.26 ± 2.52	11.35 ± 1.58	118.46	125.42	117.90

Table 1. Total flavonoid, phenolic, free radical scavenging activity of *Morchella esculenta* oil. Values are presented as Mean ± S.E. Abbreviations: TPC-Total phenolic content, GAE-Gallic acid equivalent, IC_{50}–50% inhibitory concentration, RE- Rutin equivalent, TFC-Total flavonoid content, dw- dry weight. Values represent mean ± standard error of three repetitions.

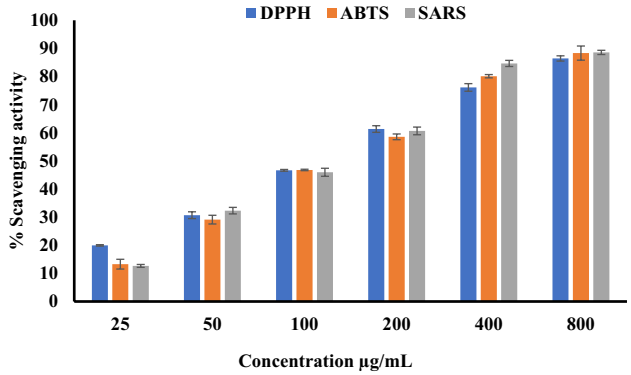

Fig. 2. Concentration dependent percent radical scavenging activity by *Morchella esculenta* oil in DPPH, ABTS and Superoxide assays which depicts that radical scavenging increased by increasing concentration of oil. Lowest scavenging activity recorded at 25 µg/mL and highest scavenging activity at 800 µg/mL.The data were represented as mean ± SE.

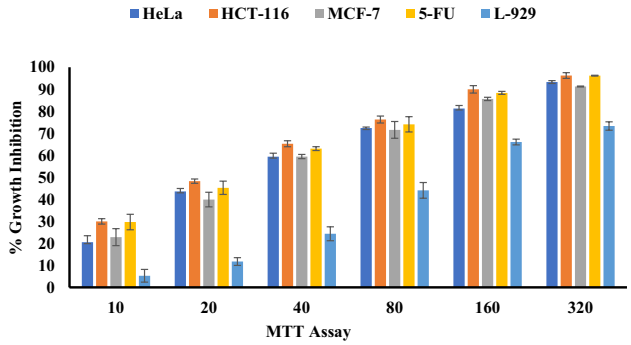

Fig. 3. Cytotoxicity of *Morchella esculenta* oil in HeLa, HCT-116 and MCF-7 cancer cell lines and L-929 normal cell lineafter 24 h treatment at concentrations ranging from 10 µg/mL to 320 µg/mL.The % growth inhibition was concentration dependent in all cell lines. The data is represented as Mean ± SE.

mL and 117.90 µg/mL in DPPH, ABTS and Superoxide radical assays as presented in Table 1. The IC50 values of standard antioxidant compound rutin was significantly very low with 28.25 µg/mL, 33.65 µg/mL and 29.21 µg/mL respectively.

Anticancer analysis

The *Morchella esculenta* oil showed strong inhibition towards proliferation of cervix (HeLa), colon (HCT-116) and breast cancer (MCF-7) cell lines with over 50% of HeLa, HCT-116 and MCF-7 cancer cellskilled at 27.61 µg/mL, 19.52 µg/mL, and 31.34 µg/mL respectively. The % inhibition was concentration dependent as presented in Fig. 3 and at higher concentration of 320 µg/mL it was 93.27%, 96.24% and 95.79% in HeLa, HCT-116 and MCF-7 cancer cells. The standard drug 5 -Fluro uracil used showed 50% inhibition at almost similar concentration with GI50 value of 24.89 µg/mL. The normal cell line L-929 evaluated showed less inhibition towards *Morchella esculenta* oil treatment with GI50 value of 101.23 which can be attributed to its antimetabolite activity.The selective index was calculated to evaluate the oils specificity toward cancer cells and was 3.66, 5.18 and 3.23 towards Hela, HCT-116 and MCF-7 respectively and demonstrate oils very desirable activity towards cancerous cells compared to the normal regular cell line.

Morphological characteristics by fluorescence microscopy

The observed cytotoxicity towards HeLa, HCT-116 and MCF-7 cancer cell lines was evaluated through DAPI and Rhodamine 123 staining to know insights regarding effect of *Morchella esculenta* oil on nuclear morphology and mitochondrial membrane potential to primally validate apoptotic effects of *Morchella esculenta* oil. Treatment of cancer cells with GI50 values of *Morchella esculenta* in HeLa, HCT-116 and MCF-7 cancer cells through DAPI staining showed nuclear deformity, fragmentation, blebbing and shrinkage which are characteristics of apoptotic nuclei as depicted in Fig. 4. Compared to treated cancer cells, normal shape and morphology was maintained by untreated cancer cells. Through rhodamine 123 staining for evaluation of mitochondrial membrane potentialcancer cells were observed with loss of fluorescence when treated with GI50 values of *Morchella esculenta* oil cells compared to untreated which showed high intensity of florescence due to uncompromised mitochondria as shown in Fig. 5. The loss of fluorescence in cancer cells depicts impaired mitochondrial membrane potential that demonstrates apoptotic inducing potential of *Morchella esculenta* oil.

GCMS, GC×GC–TOF–MS, UHPLC–QTOF–MS fingerprinting

A comprehensive approach was employed to detect metabolites in *Morchella esculenta* oil through GCMS, GC×GC–TOF–MS and UHPLC–QTOF–MS spectroscopic techniques. All techniques depicted that *Morchella esculenta* oil is a good reservoir of constituents belonging to different classes. As expected from the oil, the percentage of fatty acids and fatty acid derivatives was high followed by hydrocarbons, alcohols, esters, carboxylic acids etc. The GCMS analysis revealed 19 compounds with palmitic acid as dominant constituent with area percentage of 15.74% followed by linoleic acid (11.83%), nhexadecenoic acid (9.90%), oleic acid (7.13%) and 2-Undecanone (9.21%). The GC×GC–TOF–MS analysis showed presence of 128 identified compounds up to similarity level of 70% and 105 unknown fragmentations. The major compounds detected were 9,12-Octadecadienoic acid (Z, Z)- (9.19%), Tetracosane (5.81%), Octacosane (5.70), n-Hexadecenoic acid (4.20), Eicosane (4.17), Palmitoleic acid (5.15), Linoleic acid (2.66%), and Oleic Acid (4.14%). GC×GC–TOF–MS analysis revealed presence of other biologically active diverse compounds like Euric acid, 2,4-Di-tert-butylphenol, Nonanoic acid,1-Heptacosanol, n-Decanoic acid, n-Tridecan-1-ol, trans-13-Octadecenoic acid among others. In the Table 5 below we presented only 68 constituents due to their sound relevance. The UHPLC–QTOF–MS analysis revealed 5 compounds in negative ionization and 17 compound were detected in positive ionization mode. It was interesting to see that some compounds repeatedly detected at different retention times. We have presented tables of both positive and negative ionization modes obtained from spectral observations and data matching. The chromatograms of GCMS, GC×GC–TOF–MS and UHPLC–QTOF–MS are given in Figs. 6, 7 and 8 respectively. The identified compounds through above techniques are given in Tables 2, 3, 4 and 5 with representing retention time, area percentage, molecular formula, name of detected compounds and nature of compounds.

Discussion

Morchella esculenta is among the most highly prized mushroom commonly known as morel mushroom. *Morchella esculenta* is distributed throughout the world, and in India it is widely distributed in the Himalayan mountainous range. Traditionally *Morchella esculenta* is used for its special flavour and nutritional importance[18]. Scientific investigations studies on *Morchella esculenta* have revealed its antioxidant, anti-inflammatory,

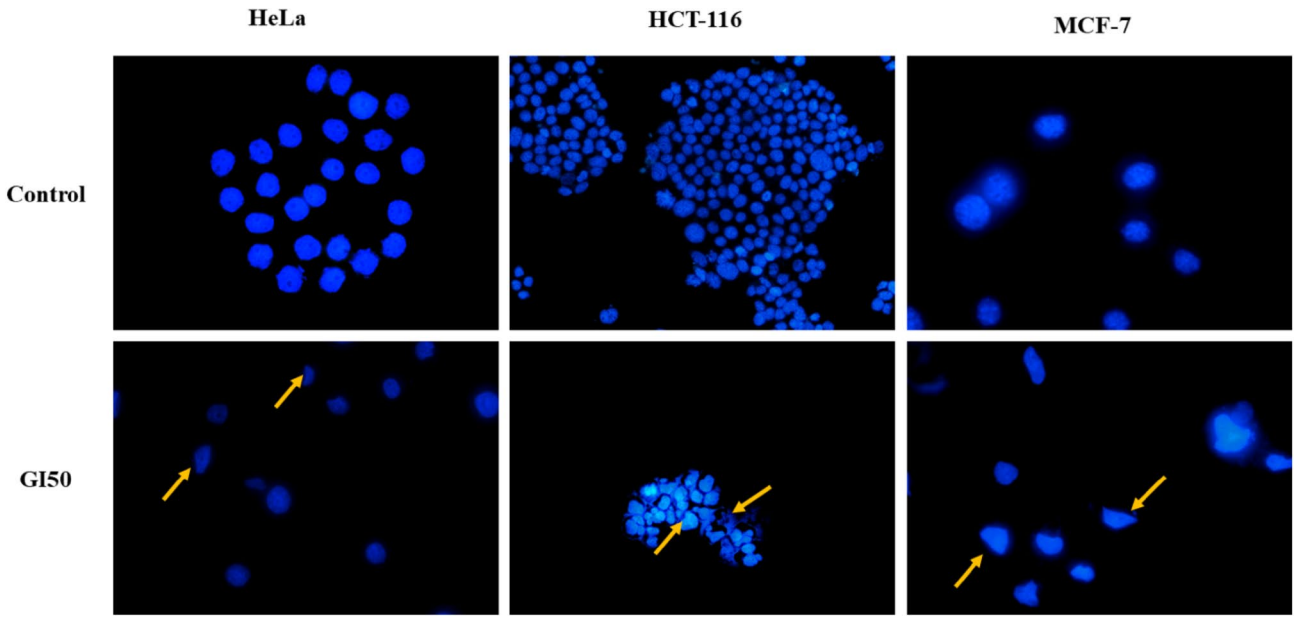

Fig. 4. DAPI staining after 24-hour treatment with GI50 concentration of *Morchella esculenta* oil in HeLa, HCT-116 and MCF-7 cancer cell lines, depicting apoptotic nature, fragmentation and blebbing of nuclei indicated with arrows.

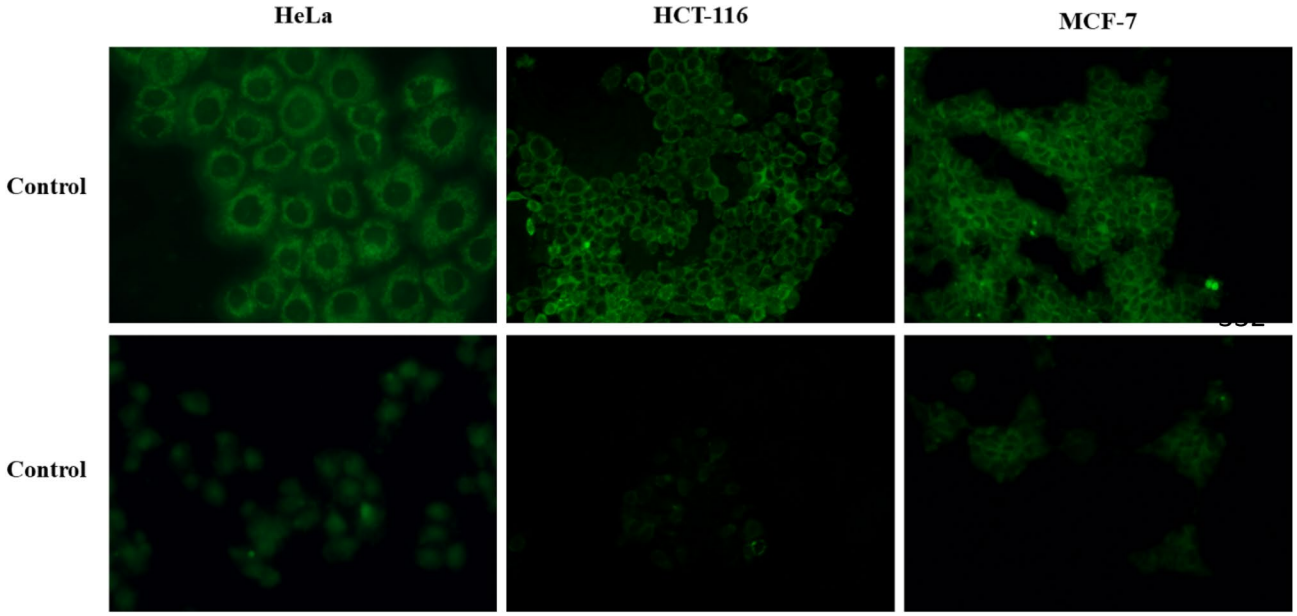

Fig. 5. Rhodamine 123 staining depicting loss of fluorescence and loss of mitochondrial membrane potential in HeLa, HCT-116 and MCF-7 cancer cell lines upon treatment with GI50 concentration of *Morchella esculenta* oil for 24 h.

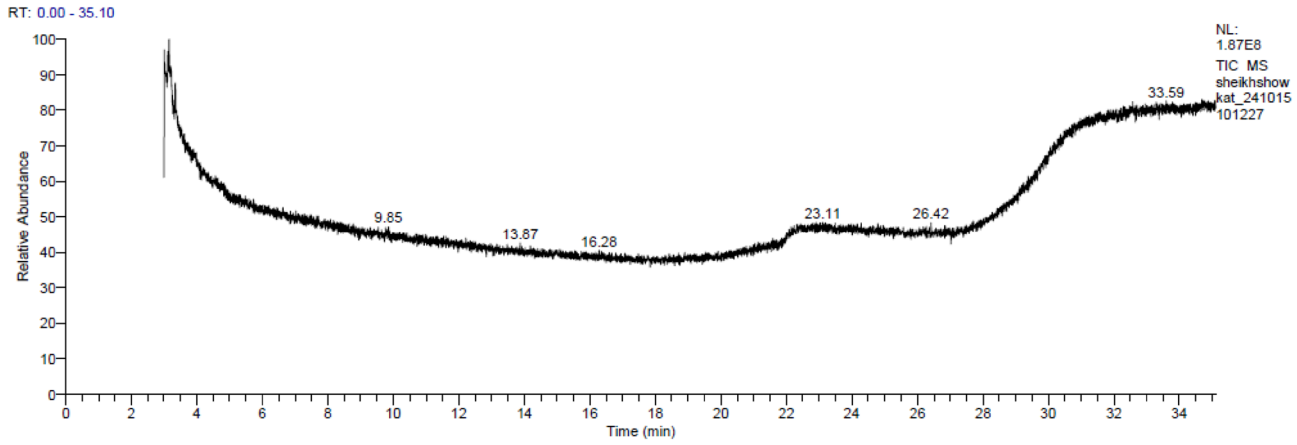

Fig. 6. GCMS chromatogram of *Morchella esculenta* oildepicting different peaks at different intervals when run over a time period of 35 min.

antimicrobial, immunomodulatory, nephroprotective, anticancer and hepatoprotective activities.Presence of phenolic and flavonoid compounds are considered vital in defence responses, such as anti-aging, anti-inflammatory, antioxidant, and anti-proliferative activities and cut down the incidence of numerous chronic diseases, for instance diabetes, cardiovascular diseases, through the management of oxidative stress[26,27]. The phenolic and flavonoid contents observed in *Morchella esculenta* oil in our study were low (TPC = 13.26 ± 2.52 mg GAE/g dw, TFC = 11.35 ± 1.58 mg RE/g dw) compared to *Morchella dunalii*, *Morchella purpurascens*, *Morchella deliciosa*, *Morchella mediterraneensis* with phenolic content as high as 281.96 mg gallic acid equivalent (GAE)/g dry weight and flavonoid content as high as (846.07 ± 1.87 mgEQ/gE),and in six Morchella species studied from turkey our results were in accordance with phenolic and flavonoid contents reported[28–30]. The observed results validate that extraction methods, geographical location affect phenolic and flavonoid contents[31]. The antioxidant activity for scavenging free radicals by *Morchella esculenta* oil was moderate with IC50 as high 125.42 µg/mL in DPPH assay. According to the previous studies, antioxid antactivity of different extracts from *Morchella esculenta* have previously been reported to exhibit excellent activity patterns in different antioxidant assays with % scavenging reported from 78.8–94.6%. Our results are parallel with them, except with some minor differences. According to literature search, there are numbers of reports on that antioxidant activity in some Morchella species that has been attributed to linoleic and oleic acids detected also in GCMS and GC×GC–TOF–MS analysis in our study[28,30,32]. The anticancer effect of *Morchella esculenta* oil in our study demonstrated strong

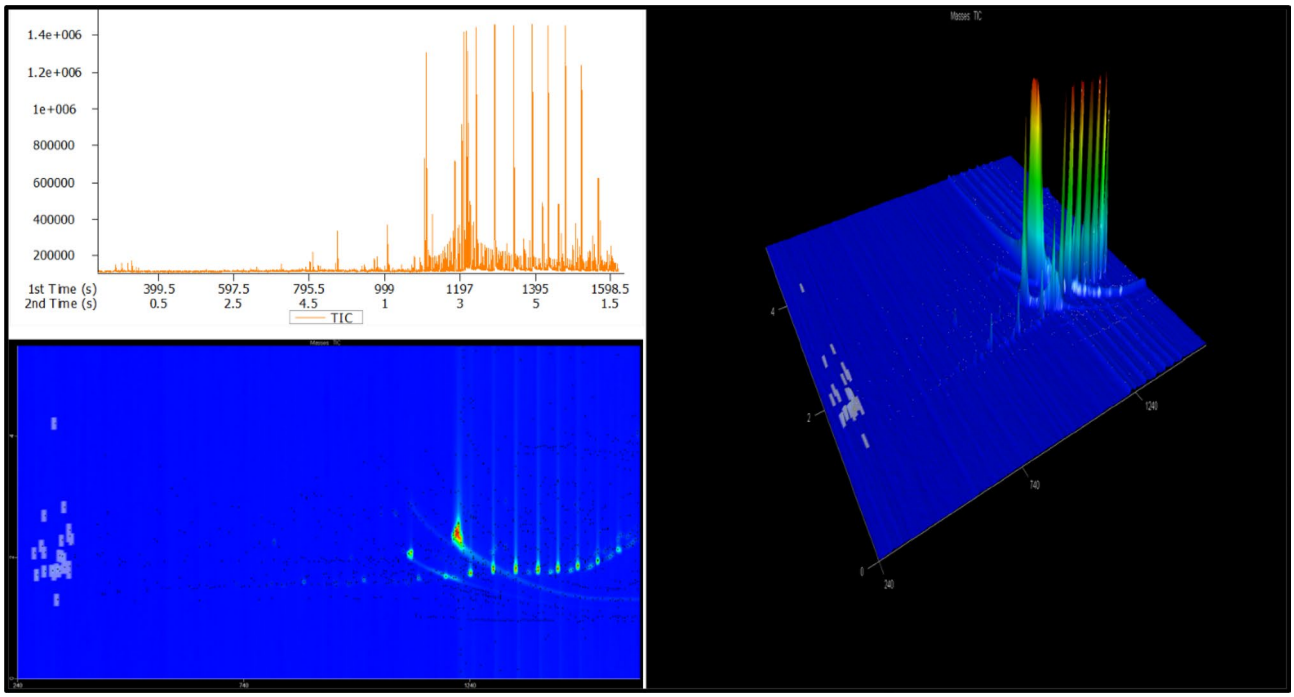

Fig. 7. (**A**) GC×GC–TOF–MS chromatogram of *Morchella esculenta* oil (**B**) Contour plot of oil and (**C**) Dimensional plot representing major compounds.

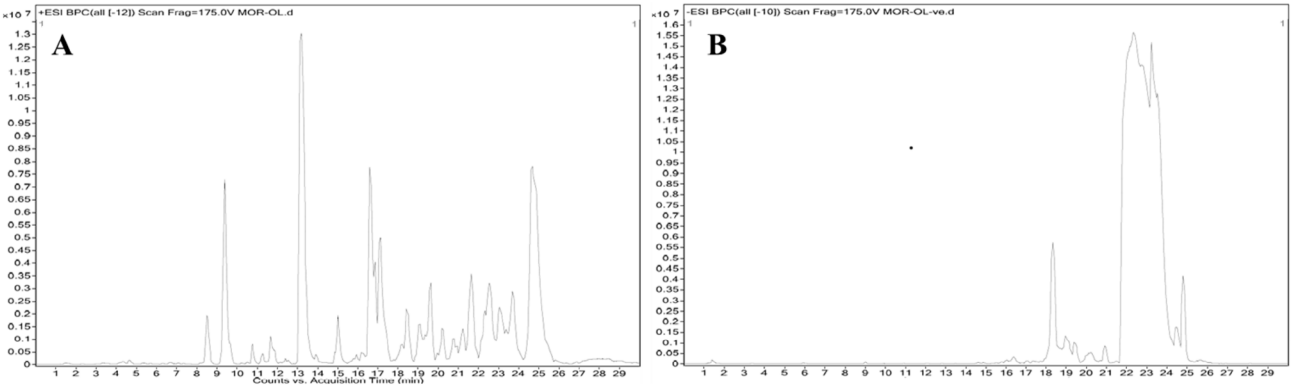

Fig. 8. UHPLC–QTOF–MS chromatograms (**A**) Positive Ionization mode (**B**) Negative Ionization mode.

antiproliferative potential towards cervix (HeLa), colon (HCT-116) and MCF-7 cancer cell lines with GI50 value as low as 19.52 µg/mL in HCT-116, supporting previous investigations that reported strong anticancer, antiproliferative and apoptotic inducing potential of different extracts of *Morchella esculenta.* Methanol extract of *Morchella esculenta* inhibited growth of different human lung adenocarcinoma cell lines (A549, H1264, H1299, and Calu-6) with GI_{50} values ranging from 0.49 to 1.35 mg/mL. *Morchella conica*, *Morchella esculenta* and *Morchella delicosa* extracts (Methanol and ethanol) reduced breast (MCF-7) and colon (SW-480) cancer cells proliferation at half inhibitory concentration (IC_{50}) of 0.02 ± 0.01 to 0.68 ± 0.30 mg/mL and significantly increased gene expressions of apoptotic genes like Bax, caspase-3, caspase-7, and caspase-9 and downregulated the gene expression of Bcl-2 in MCF-7 and SW-480 cell lines[33–37]. Octanoic acid, hexadecenoic acid, oleic acid, trans-cinnamic acid, palmitic acid, lineolic acid and stearic acid detected in GCMS and GC×GC–TOF–MS are reported to possess insecticidal, antimicrobial, antitumor, antibacterial, anti-inflammatory, antifungal, antiviral and antimalarial properties[38–43]. C16 Sphinganine, phytosphingosine, palmitic amide, 16-Hydroxy hexadecenoic acid, ergosterol peroxide, 3'-Hydroxy-T2 Toxin metabolites identified in HRLCMS have been reported to possess anticancer properties[44–48] and supports our results that strong anticancer effect could be observed due to synergistic effect of these constituents.Previous findings carried with ovarian cancer models and patient-derived xenografts (PDXs) depicted that stearic acid can directly inhibit tumour growth through mechanisms involving DNA damage and apoptosis mediated by the unfolded protein response (UPR) pathway[49]. Studies

R. Time	Compound	Area %	Formula	M.W	Nature
4.57	Heneicosane	2.41	$C_{21}H_{44}$	296.57	Hydrocarbon
22.08	2-Undecanone	9.21	$C_{11}H_{22}O$	170.29	Ketone
22.19	(*E*)−2-Octena	6.92	$C_8H_{14}O$	126.29	Acetals
22.23	Octanoic acid	3.06	$C_8H_{16}O_2$	144.21	Fatty acid
22.32	n-Hexadecanoic acid	9.90	$C_{16}H_{32}O$	256.42	Fatty acid
22.41	Pentadecanoic acid	4.25	$C_{15}H_{30}O_2$	242.39	Fatty acid
22.55	Silicic acid, diethyl bis(trimethylsilyl) ester	1.90	$C_{15}H_{36}O_5Si_3$	380.70	Aliphatic ester
22.66	Decanal	1.76	$C_{10}H_{20}O$	156.29	Fatty aldehyde
30.06	8,14-Seco-3,19-epoxyandrostane-8,14-dione	1.33	$C_{24}H_{36}0_6$	420.05	Steroid derivative
30.17	Eicosane	1.49	$C_{20}H_{42}$	282.54	Hydrocarbon
30.28	Oleic acid	7.13	$C_{18}H_{34}O$	282.46	Fatty acid
30.36	trans-Cinnamic acid	0.95	$C_9H_8O_2$	148.16	Monocarboxylic acid
30.52	Hexanal	3.65	$C_6H_{12}O$	100.16	Alkyl aldehyde
30.62	Benzoic acid	1.72	$C_7H_6O_2$	122.12	Carboxylic acid
30.72	1-Butanol, 3-methyl-	2.01	$C_5H_{12}O$	88.14	Alcohol
30.97	Acetic acid, methyl ester	1.95	$C_3H_6O_2$	74.07	Carboxylate ester
31.09	Palmitic acid	15.74	$C_{16}H_{32}O_2$	256.43	Fatty acid
32.58	Linoleic acid	11.83	$C_{18}H_{32}O_2$	280.44	Fatty acid
33.56	Stearic acid	2.62	$C_{18}H_{36}O_2$	284.48	Fatty acid

Table 2. Phytochemicals in *Morchella esculenta* oil identified by GCMS analysis.

RT	Mass	Name	Formula	DB Diff (ppm)	Nature
10.944	267.2183	Allopumiliotoxin 267A	$C_{16}H_{29}NO_2$	5.69	Alkaloid
13.14	273.2663	C16 Sphinganine	$C_{16}H_{35}NO_2$	1.59	Sphingolipids
13.196	554.5141	Helianyl octanoate	$C_{38}H_{66}O_2$	14.11	Triterpenoid.
13.934	255.2537	Palmitic amide	$C_{16}H_{33}NO$	10.01	Fatty acid amides
15.118	317.2899	Phytosphingosine	$C_{18}H_{39}NO_3$	9.85	Sphingoid
16.745	354.258	1,1'-[1,12-Dodecanediylbis(oxy)] bisbenzene	$C_{24}H_{34}O_2$	−5.93	Aromatic ether
16.746	349.3027	Dihomo-γ-linolenoyl-EA	$C_{22}H_{39}NO_2$	−13.22	Fatty acid
16.953	354.2578	1,1'-[1,12-Dodecanediylbis(oxy)] bisbenzene	$C_{24}H_{34}O_2$	−5.4	Aromatic ether
17.021	355.3416	Guazatine	$C_{18}H_{41}N_7$	2.01	Dioctylamine
19.023	580.3526	alpha, alpha'-Trehalose 6- palmitate	$C_{28}H_{52}O_{12}$	−11.58	Lipopolysaccharide
19.079	272.2339	16-Hydroxy hexadecenoic acid	$C_{16}H_{32}O_3$	4.7	Fatty acid
19.08	550.39	1-O-(cis-9-Octadecenyl)−2-O- acetyl-sn-glycero-3- phosphocholine	$C_{28}H_{57}NO_7P$	−4.98	Phospholipid
19.133	488.3294	7',8'-Dihydro-8'- hydroxycitraniaxanthin	$C_{33}H_{44}O_3$	−0.7	Triterpenoid
19.207	428.3301	Ergosterol peroxide	$C_{28}H_{44}O_3$	−2.42	Phytosterol
19.465	272.2346	16-Hydroxy hexadecenoic acid	$C_{16}H_{32}O_3$	1.93	Fatty acid
19.705	254.2241	Citronellyl hexanoate	$C_{16}H_{30}O_2$	1.98	Carboxylic ester
19.785	272.2344	16-Hydroxy hexadecenoic acid	$C_{16}H_{32}O_3$	2.56	Fatty acid

Table 3. UHPLC–QTOF–MS analysis of *Morchella esculenta* oil in positive ionization mode.

RT	Mass	Name	Formula	DB Diff (ppm)	Nature
18.441	294.2222	9-HOTE	$C_{18}H_{30}O_3$	−9.21	Fatty acid
22.192	912.5698	PI (20:4(8Z,11Z,14Z,17Z)/20:1 (11Z))	$C_{49}H_{85}O_{13}P$	3.23	Phosphatidylinositol
22.763	486.298	Hydrocortisone cypionate	$C_{29}H_{42}O_6$	0.26	Steroid
23.291	280.2443	Ethyl 2E,4Z-hexadecadienoate	$C_{18}H_{32}O_2$	−14.4	Fatty acid ester
23.782	482.2191	3'-Hydroxy-T2 Toxin	$C_{24}H_{34}O_{10}$	−8.18	Trichothecenes

Table 4. UHPLC–QTOF–MS analysis of *Morchella esculenta* oil in negative ionization mode.

S. no.	Peak	Name	R.T. (s)	Area %	Formula	Nature
1.	654	Tetracosane	1340, 1.830	5.8159	$C_{24}H_{50}$	Alkane
2.	798	Heneicosane	1433.5, 1.820	3.4411	$C_{21}H_{44}$	Alkane
3.	300	n-Hexadecenoic acid	1103.5, 2.070	4.2053	$C_{16}H_{32}O_2$	Fatty acid
4.	486	Eicosane	1241, 1.750	4.1758	$C_{20}H_{42}$	Alkane
5.	860	Octacosane	1477.5, 1.870	5.7011	$C_{28}H_{58}$	Alkane
6.	121	2,4-Di-tert-butylphenol	806.5, 2.260	0.40215	$C_{14}H_{22}O$	Alkylbenzene
7.	52	Octanoic acid	526, 1.920	0.032	$C_8H_{16}O_2$	Fatty acid
8.	403	9,12-Octadecadienoic acid (Z, Z)-	1202.5, 2.380	9.1958	$C_{18}H_{32}O_2$	Fatty acid
9.	840	Eicosane, 2-methyl-	1466.5, 1.800	0.70331	$C_{21}H_{44}$	Alkane
10.	80	2,4-Decadienal	641.5, 2.180	0.08372	$C_{10}H_{16}O$	Fatty aldehyde
11.	291	Palmitoleic acid	1092.5, 2.110	0.1543	$C_{16}H_{30}O_2$	Fatty acid
12.	942	Linoelaidic acid	1532.5, 1.330	0.66047	$C_{18}H_{32}O_2$	Fatty acid
13.	726	Tridecane, 3-methyl-	1389.5, 1.790	0.15578	$C_{14}H_{30}$	Alkane
14.	249	Pentadecanoic acid	1043, 1.990	0.04527	$C_{15}H_{30}O_2$	Fatty acid
15.	111	9-Oxononanoic acid	779, 2.670	0.02902	$C_9H_{16}O_3$	Fatty acid
16.	853	(Z)6,(Z)9-Pentadecadien-1-ol	1472, 2.890	0.10326	$C_{15}H_{28}O$	Fatty alcohol
17.	231	Isopropyl myristate	1021, 1.830	0.04679	$C_{17}H_{34}O_2$	Fatty acid ester
18.	640	9-Octadecenamide, (Z)-	1323.5, 2.780	0.03915	$C_{18}H_{35}NO$	Fatty acid amide
19.	41	Undecane	465.5, 1.440	0.02374	$C_{11}H_{24}$	Alkane
20.	131	1-Nonene, 4,6,8-trimethyl-	834, 1.570	0.04099	$C_{12}H_{24}$	Alkene
21.	1029	Oxalic acid, cyclohexylmethyl nonyl ester	1593, 2.400	0.18586	$C_{18}H_{32}O_4$	Ester
22.	1058	Hexadecane, 3-methyl-	1609.5, 2.300	0.01632	$C_{17}H_{36}$	Alkane
23.	304	Sulfurous acid, 2-ethylhexyl undecyl ester	1109, 1.640	0.10727	$C_{19}H_{40}O_3S$	Ester
24.	816	Dodecane, 2-cyclohexyl-	1450, 1.870	0.03222	$C_{18}H_{36}$	Cycloalkane
25.	325	Cyclotetradecane	1125.5, 1.690	0.11295	$C_{14}H_{28}$	Cycloalkane
26.	357	Octadecanoic acid	1164, 2.030	0.07493	$C_{18}H_{36}O_2$	Fatty acid
27.	107	Hexadecane	773.5, 1.530	0.04306	$C_{16}H_{34}$	Alkane
28.	343	1-Tridecyne	1147.5, 2.220	0.02317	$C_{13}H_{24}$	Hydrocarbon
29.	944	1-Iodo-2-methylnonane	1532.5, 1.950	0.0233	$C_{10}H_{21}I$	Organoiodides
30.	890	Cyclohexane, nonadecyl-	1494, 1.910	0.07155	$C_{25}H_{50}$	Cycloalkane
31.	467	Nonanamide	1230, 2.600	0.08903	$C_9H_{19}NO$	Glycol ester
32.	1041	Oxalic acid, cyclohexylmethyl decyl ester	1598.5, 2.450	0.03479	$C_{19}H_{34}O_4$	Ester
33.	187	Oxalic acid, isobutyl nonyl ester	944, 1.600	0.15583	$C_{15}H_{28}O_4$	Ester
34.	939	6,11-Dimethyl-2,6,10-dodecatrien-1-ol	1527, 2.420	0.19281	$C_{14}H_{24}O$	Sesquiterpenoids
35.	861	(E)-Hex-3-enyl (E)–2-methylbut-2-enoate	1477.5, 2.030	0.09168	$C_{11}H_{18}O_2$	Fatty acid ester
36.	186	Hexadecane, 2,6,10,14-tetramethyl-	944, 1.540	0.01195	$C_{20}H_{42}$	Alkane
37.	407	1-Decanol, 2-hexyl-	1208, 1.670	0.00335	$C_{16}H_{34}O$	Aliphatic alcohol
38.	436	trans-13-Octadecenoic acid	1219, 2.250	0.59808	$C_{18}H_{34}O_2$	Fatty acid
39.	1068	Erucic acid	1615, 1.300	0.01045	$C_{22}H_{42}O_2$	Fatty acid
40.	550	1-Undecanol	1268.5, 1.910	0.08872	$C_{11}H_{24}O$	Fatty alcohol
41.	134	Dodecanoic acid	839.5, 1.990	0.01375	$C_{12}H_{24}O_2$	Fatty acid
42.	243	Hexadecanal, 2-methyl-	1032, 1.880	0.01516	$C_{17}H_{34}O$	Aldehyde
43.	79	Tridecane	641.5, 1.550	0.01377	$C_{13}H_{28}$	Alkane
44.	322	5,7-Dodecadiene, (E, Z)-	1120, 2.890	0.05439	$C_{12}H_{22}$	Alka diene
45.	337	9,12-Tetradecadien-1-ol, (Z, E)-	1142, 2.680	0.01869	$C_{14}H_{26}O$	Fatty alcohol
46.	44	Nonanal	471, 1.940	0.0147	$C_9H_{18}O$	Fatty aldehyde
47.	999	Oleic Acid	1571, 1.320	0.14289	$C_{18}H_{34}O_2$	Fatty acid
48.	201	5-Undecene, 9-methyl-, (Z)-	971.5, 1.590	0.00501	$C_{12}H_{24}$	Alkene
49.	564	n-Decanoic acid	1274, 1.880	0.21863	$C_{10}H_{20}O_2$	Fatty acid
50.	382	1-Dodecene	1186, 1.950	0.00946	$C_{12}H_{24}$	Alkene
51.	970	1,1'-Bicycloheptyl	1549, 2.180	0.03713	$C_{14}H_{26}$	Hydrocarbon
52.	13	Ethanone, 1-(2-methylcyclopropyl)-	339, 2.030	0.3767	$C_6H_{10}O$	Ketone
53.	151	Asarone	878, 3.000	0.01227	$C_{12}H_{16}O_3$	Phenylpropanoid
54.	273	Phthalic acid, cyclobutyl isobutyl ester	1070.5, 2.780	0.02875	$C_{16}H_{20}O_4$	Ester
55.	281	Pentadecanoic acid, 14-methyl-, methyl ester	1081.5, 1.930	0.02496	$C_{17}H_{34}O_2$	Ester
56.	741	1-Heptacosanol	1400.5, 1.870	0.00705	$C_{27}H_{56}O$	Fatty alcohol
Continued						

S. no.	Peak	Name	R.T. (s)	Area %	Formula	Nature
57.	288	Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)–4-hydroxy-, methyl ester	1087, 2.560	0.01399	$C_{18}H_{28}O_3$	Ester
58.	718	Hexane nitrile, 5-methyl-	1384, 1.540	0.15641	$C_7H_{13}N$	Alkane
59.	384	Cyclooctane, ethenyl-	1186, 2.380	0.00628	$C_{10}H_{18}$	Cycloalkane
60.	4	Ether, 3-butenyl propyl	300.5, 1.780	0.59504	$C_7H_{14}O$	Ether
61.	664	Undecanoic acid	1351, 1.590	0.00567	$C_{11}H_{22}O_2$	Fatty acid
62.	818	Propanoic acid, 3-mercapto-, dodecyl ester	1450, 2.330	0.00226	$C_{15}H_{30}O_2S$	Ester
63.	637	9-Octadecenoic acid	1323.5, 1.730	0.64866	$C_{18}H_{34}O_2$	Fatty acid
64.	15	Hexanoic acid	344.5, 1.840	0.01591	$C_6H_{12}O_2$	Fatty acid
65.	358	1, E-11, Z-13-Octadecatriene	1164, 2.520	0.0403	$C_{18}H_{32}$	Hydrocarbon
66.	902	Cyclohexane, 1-isopropyl-1-methyl-	1499.5, 1.970	0.00662	$C_{10}H_{20}$	Cycloalkane
67.	275	Cyclohexane, 1-methyl-3-propyl-	1076, 1.750	0.00994	$C_{10}H_{20}$	Cycloalkane
68.	69	Nonanoic acid	608.5, 1.950	0.01394	$C_9H_{18}O_2$	Fatty acid

Table 5. Compounds identified in GC×GC–TOF–MS analysis.

showed linoleic acid demonstrated anti-proliferative and anti-invasive potential in endometrial cancer cell lines validated also through Lkb1fl/flp53fl/fl mouse model of endometrial cancer[50]. Palmitic acid detected as one of the major constituents in *Morchella esculenta* oil has been reported to be involved in induction of cell apoptosis through the disruption of mitochondrial pathway, interference with the cancer cell cycle, induces programmed cell autophagy death, inhibits angiogenesis, and synergistically promotes the activity of chemotherapy drugs while neutralizing the adverse reactions[51]. Oleic acid has been shown to acts on different intracellular and extracellular targets like modulating tumor cell signalling pathways, P53 etc[40]. C16 Sphinganine reported to mediate antiproliferative responses via inhibiting cancer cell growth, migration and parallelly inducing autophagy and apoptosis and downregulated Cdk4 expression and phosphorylation of phospho-Rb has been observed in Intestinal adenoma RIE cells[52]. Phyto sphingosine inhibits the growth of lung adenocarcinoma cells both in vitro and in vivo validated through induction of G2/M-phase arrest, apoptosis, and mitochondria-dependent pathway cell death and increase ROS levels in cancer cell line[54]. Previous findings reported that Trans-cinnamic acid exerted strong inhibitory effect against invasion of A549 cells and the inhibitory result was drawn from observing reducing matrix metalloproteinase (MMP-2 and − 9) activities[54]. Similarly, cytotoxicity of benzoic acid on ten different cancer cell lines showed strong anticancer activity and benzoic acid inhibits tumor growth and metastasis in murine models of bladder cancer through inhibition of TNFα/NFKB and iNOS/NO pathways[55]. The current and previous investigations are supporting the anticancer potential of *Morchella esculenta* and further in-depth studies for targeted isolation, toxicity profiles and molecular mechanisms should be widely and soundly explored.

Limitations of study

The detailed mechanisms by which *Morchella esculenta* oil exert apoptotic effects remain unclear which could be explored through different gene expression molecular mechanisms and studying apoptotic and cell cycle pathways and in vivo studies using animal cancer models.The lack of in vivo animal studies restricts the ability to draw potential conclusions about the pharmacokinetics, bioavailability, metabolism, and systemic toxicity of the active constituents. Though *Morchella esculenta* oil showed detection of rich metabolites but targeted isolation for characterization of individual constituent is lacking. As the oil demonstrated strong cytotoxicity towards cancer cell lines this warrants its further exploration with limitations provided.

Conclusion

Morchella esculenta is an edible mushroom widely used for its nutritional values and parallelly have high economic values. In present study hexane extract of *Morchella esculenta* collected from Himalayan regions of Kashmir depicted moderate antioxidant activity but demonstrated strong anticancer potential against cervix (Hela), colon cancer (HCT-116) and breast cancer (MCF-7) cell lines. The comprehensive phytochemical analysis through GCMS, GC×GC–TOF–MS and UHPLC–QTOF–MS revealed diverse classes of metabolites like fatty acids, esters, alcohols, alkanes etc.

Considering the strong anticancer potential the future directions of *Morchella esculenta* oil studies can be extended for in-depth in-vivo, toxicological studies exploring the chemo preventive potential of *Morchella esculenta* oil into the development of possible novel functional cancer preventive source. Besides, further research on the molecular mechanisms of action in *Morchella esculenta*oil will broaden its applications in understanding its target sites with physiological impacts.

Data availability

Data related to manuscript included in this article.

Received: 1 April 2025; Accepted: 17 June 2025
Published online: 26 September 2025

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Acknowledgements

The author expresses gratitude to IIT Bombay and SAIF Punjab University Chandigarh for providing necessary facilities. College of Science, King Saud University Saudi Arabia for funding and sources.

Author contributions

All authors contributed significantly.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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