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ORIGINAL ARTICLE

# Expanding knowledge on *Russula alatoreticula*, a novel mushroom from tribal cuisine, with chemical and pharmaceutical relevance

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**Abstract** Since antiquity, numerous macrofungi are being worshiped as food and natural medicine especially in Asian tribal communities. Recent investigation has correlated these medicinal properties with bioactive components including phenols and flavonoids. However, research on mushrooms is not satisfactory; as several traditionally prized members remain undiscovered or poorly explored yet. This backdrop tempted us to unveil secondary metabolites empowered with therapeutics from an ethnic delicacy, *Russula alatoreticula* that was justified as a novel macrofungus in our previous publication. Accordingly, methanol extract was prepared from dried basidiocarps that was found to be enriched with phenolic compounds (pyrogallol > cinnamic acid > *p*-coumaric acid) and ascorbic acid. As a result, the fraction exhibited strong antioxidant activity evident by the ability of quenching free radicals, chelating $Fe^{2+}$ ion and reducing components with $EC_{50}$ of 263–2382 µg/ml. Besides, effective antibacterial potential against six investigated microbes was also noticed where MIC value ranged from 99 to 2673.74 µg/ml. Furthermore, the extract revealed promising anticancer property as it induced apoptosis of Hep3B cell ($IC_{50}$ 358.57 µg/ml) by imparting morphological changes, interfering cell cycle, depleting MMP and alleviating ROS through Bax, Bcl2, caspases 9 and 3 intrinsic mitochondrial pathway. Overall study implied that the immense bioactive potential of *R. alatoreticula* could possibly be utilized as a good source of natural supplement to combat against free radicals, pathogenic bacteria and hepatocellular carcinoma as well as in food safety industry.

**Keywords** Antibacterial potential · HPLC · Hep3B liver cancer · Novel species · Phenolics · Antioxidant activity

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## Introduction

From ancient times, mushrooms have been collected as food by humans for their taste, flavour, nutritional and culinary values. Subsequently, they have also been viewed as tonic and used in home remedies to treat various diseases across the globe especially in Asia (Khatua et al. 2013). Such extensive knowledge has historically been accumulated by indigenous people and transmitted to the next generation to make use of these bio-resources. As a consequence of the lifestyle, macrofungi still play an important part of rural alimentary strategies, cultural issues and economic activities (Ruan-Soto et al. 2017); although, their empirical application is deteriorating in civilized

societies due to lack of bio-availability, awareness and propaganda. Besides, documentary evidence of ethno-mycological information has been preserved only in certain countries like China and Japan, but not in other areas including India (Panda and Tayung 2015). Thus, it can easily be assumed that such traditional practice is till now restricted to several Indian mycophagy communities that need to be exposed for human welfare and conservation of historical culture.

In that note, our research team is being working since past 10 years with an aim to unveil Indian myco-diversity mainly confining to West Bengal (21°38′–27°10′N latitude and 85°50′–89°50′E longitude, area of 88,752 $km^2$). This phyto-geographically unique state is extended from Himalayas in northern to Bay of Bengal in southern side with different geological regions that helps in flourishing natural growth of mushrooms (Khatua et al. 2015). Consequently during recent inspection, a morphologically unique macro-fungus was collected with the help of native people during their hunting for food. After thorough characterization based on macro and micro morphological features accompanied by phylogenetic study the taxon was confirmed as novel and designated *Russula alatoreticula* K. Acharya, S. Khatua, A.K. Dutta and S. Paloi. Further, polysaccharidic fraction was prepared from the ethnic myco-food for evaluation of putative bioactivities as described in our previous publication (Khatua et al. 2017a).

Besides carbohydrate, medicinal mushrooms consist another class of therapeutic compounds derived from secondary metabolism. These constituents like phenols and flavonoids are synthesized for protection against insects, viruses and bacteria; as a result many of them are ideal for inhibition of microbial growth. At present, these natural substances are in high demand due to worldwide emergence of antibiotic resistance that is creating an urgent health crisis (Tiong et al. 2016). Simultaneously, phenolic components are also ascribed for ability to diminish free radical in biological systems providing antioxidative protection. They have the capacity to scavenge free radicals, quench lipid peroxidation, chelate redox active metals and prevent DNA oxidative damage hindering initiation or progression of several degenerative diseases like cancer (Kozarski et al. 2015). Therefore, these bio-resources have also proved to possess the capacity of regulating cancer cell proliferation, through different mechanisms including growth inhibition and apoptosis induction (Ivanova et al. 2014). Consequently, hepatocellular carcinoma represents one of main health problems across the globe characterized by its high incidence and mortality rates (Shiraha et al. 2013). Treatment dealing with the disease is extremely limited due to non-responsiveness to chemotherapy. At the same time, sorafenib, only approved therapy for patients in advanced stage, alone or in combination with oxaliplatin remain unsatisfactory as they cause adverse side effect. Hence, quest for novel agent with more safety and higher selectivity against the ailment is one of the cornerstone of modern medicine (Wang et al. 2017). In this context, Hep3B cell line has been widely used as a cellular reference model to search for unique drug (Qiu et al. 2015).

Therefore, the present study was designed for determination of phenolic composition reflecting therapeutic activities of the folk mushroom, *R. alatoreticula* and for that methanol extract was prepared. The fraction was characterized by spectrophotometry and high performance liquid chromatography (HPLC) to identify bioactive constituents. Alongside, it was also subjected for evaluation of different medicinal properties like antibacterial, antioxidant and anticancer effects.

## Materials and methods

### Collection and identification

Basidiocarps of the specimen were collected during subsequent field trips (2011–2013) at West Bengal and identified following standard protocols (Khatua et al. 2017a). The representative voucher specimen (Accession no: CUH AM 114) was deposited in Calcutta University Herbarium (CUH).

### Preparation of methanol extract

Lyophilized powder was extracted by stirring with 50 ml of methanol overnight and subsequently separated by Whatman filter paper. Residue was then re-extracted with 30 ml of methanol and the combined methanolic formulation was evaporated at 40 °C to reduce volume. The fraction was stored at − 20 °C in dark bottle until analysis, for no more than 1 month.

Determination of major bioactive compounds

To estimate total phenolic compounds 1 ml of methanol extract was mixed with equal volume of Folin–Ciocalteu reagent and 35% sodium carbonate solution. Volume was adjusted to 10 ml by water, incubated for 90 min and absorbance was measured at 725 nm. Gallic acid was considered as standard and result was expressed as µg of gallic acid equivalent per mg of extract. For quantification of flavonoid content, 1 ml methanol fraction was mixed with 0.1 ml of 1 M potassium acetate and 0.1 ml of 10% aluminium nitrate. Further, 4.1 ml of 80% aqueous ethanol was added followed by measurement of absorbance at 415 nm. Quercetin was used to calculate standard curve and result was expressed as µg of quercetin equivalent per mg of extract. To determine carotenoid content, 100 mg methanol fraction was mixed with 10 ml acetone:hexane (4:6) solution, filtered through Whatman filter paper and absorbance was noted at 453, 505 as well as 663 nm. Finally, presence of ascorbic acid was detected by titration method using 2,6-dichlorophenol indophenol dye (Khatua et al. 2017c).

HPLC profiling

Extract dissolved in 1 ml of HPLC grade methanol was filtered and 20 µl of the filtrate was analysed by HPLC system equipped with an Agilent Eclipse Plus C18 column (100 mm × 4.6 mm, 3.5 µm). Elution was carried out by using eluent A (acetonitrile) and eluent B (0.1% v/v aqueous phosphoric acid) through a gradient procedure: 0–5 min, 5% A; 5–10 min, 15% A; 10–15 min, 40% A; 15–20 min, 60% A; 20–22 min, 90% A. The compounds were identified by comparing UV spectra and retention time with authenticate standards namely gallic acid, *p*-hydroxybenzoic acid, chlorogenic acid, vanillic acid, *p*-coumaric acid, ferulic acid, myricetin, salicylic acid, quercetin, cinnamic acid and pyrogallol (Chatterjee et al. 2016).

Estimation of antibacterial action

Antibacterial effect was estimated by determining minimum inhibitory concentration (MIC) values according to microdilution method. The six investigating bacteria namely *Bacillus subtilis* ATCC® 6633™ (MTCC 736), *Listeria monocytogenes* ATCC® 19111™ (MTCC 657), *Staphylococcus aureus* ATCC® 700699™, *Escherichia coli* ATCC® 25922™, *Salmonella typhimurium* ATCC® 23564™ (MTCC 98) and *Klebsiella pneumoniae* ATCC® 15380™ (MTCC 109) were freshly cultured in nutrient broth (NB) and $1 \times 10^5$ CFU/ml concentrated dilutions were prepared. Reactions were performed in 96 well plate consisting of 200 µl of NB, 20 µl of inoculum and dilutions of methanol extract. Following incubation for 24 h at 37°C, 40 µl of iodonitrotetrazolium chloride dye (0.2 mg/ml) was added to each well and incubated for another 30 min. Concentration that inhibited 50% bacterial growth in comparison with positive control was calculated as MIC value. Streptomycin was used as a standard drug (Khatua et al. 2017a, c).

Evaluation of antioxidant potential

To determine total antioxidant capacity, reaction mixture was prepared by mixing 3 ml of reagent (0.6 M sulphuric acid, 4 mM ammonium molybdate and 28 mM sodium sulphate) and 0.3 ml sample. The resultant solution was incubated at 95 °C for 90 min and absorbance was measured at 695 nm. The activity was expressed as number of equivalent of ascorbic acid (Prieto et al. 1999). Rest of the methods were performed in 96 well plates and absorbance was recorded using Bio-Rad iMark™ Microplate Reader (USA). Superoxide radical ($O_2^-$) scavenging activity of methanol extract (100–2000 µg/ml) was evaluated using 200 µl reaction mixture consisting of 26 µl methionine (2 nM), 20 µl EDTA (0.1 nM), 17 µl nitroblue tetrazolium (0.1 nM) and 4 µl riboflavin (1 nM). The plate was illuminated for 10 min and absorbance was noted at 595 nm. In addition, quenching potential of 2,2-diphenyl-1-picrylhydrazyl (DPPH·) was also explored by mixing methanol extract at various concentration (100–2000 µg/ml) with 0.004% DPPH solution in a reaction mixture of 200 µl. The plate was incubated for 30 min followed by detection of final colour at 595 nm. Moreover, the ability of investigated formulation to chelate ferrous ion was also estimated and for that 5 µl ferrous chloride (3 nM) was mixed with methanol formulation at different level (50–400 µg/ml). Then, 10 µl ferrozine (0.12 nM) was added in the microtiter plate and absorbance was recorded at 595 nm. Finally, a modified method of reducing power was considered where 10 µl of extract solution at different concentration

(1000–3000 µg/ml) was mixed with 25 µl sodium phosphate buffer and 25 µl potassium ferricyanide (1%). After incubation for 20 min, 85 µl water and 8.5 µl ferric chloride were added followed by determination of optical density at 750 nm (Khatua et al. 2017b). To compare the effects of extract with a standard, ascorbic acid was used in all assays except chelating ability of $Fe^{2+}$ method where EDTA was adopted as a positive control. The sample concentrations exhibiting 50% of antioxidant activity or 0.5 of absorbance were determined from graphs of antioxidant activity percentages and regarded as half maximal effective concentration ($EC_{50}$).

Cell culture

Hep3B human liver cancer cell line, purchased from National Centre for Cell Science (NCCS), Pune, India, was maintained in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 0.25% amphotericin B (250 µg/ml) and 0.5% PenStrep (5000 IU/ml penicillin and 5 mg/ml streptomycin). Cell line was maintained at 37 °C in a humidified atmosphere with 5% $CO_2$.

Determination of cytotoxic effect

Briefly, about 1000 cells were seeded in 96 well plate before methanolic extract dissolved in sterile dimethyl sulfoxide (DMSO) was added at different concentrations. After 24 h treatment, 20 µl water soluble tetrazolium (WST) reagent was added and absorbance was measured at wavelength of 450 nm.

Clonogenic cell survival assay

About 1000 cells were seeded in 6 well plate followed by treatment of extract at various doses (50, 100, 300 and 500 µg/ml). After 24 h, media was completely removed and replaced with new media to allow the cells to divide. After about 2 weeks, colonies were visualized by fixing the cells in 75% ethanol and staining with 0.1% crystal violet (Munshi et al. 2005).

Wound-healing assay

Hep3B cells were cultured in a monolayer up to 90% confluence in 12 well plate. Cells were scraped in a straight line to create a scratch with the help of sterile 10 µl microtip and detached cells were removed by phosphate buffered saline (PBS). Healing of the wound in control and treated sets (50, 100, 300 and 500 µg/ml of methanol extract) were assessed at different time intervals of 0, 12 and 24 h using an inverted microscope (FLoid Cell Imaging Station, Life Technologies, India) (Liang et al. 2007).

Cell cycle analysis by flow cytometry

Hep3B cells were seeded in 6 well plate and treated with different concentrations of methanol extract (50, 100, 300 and 500 µg/ml). After 24 h, cells were collected by trypsinization, fixed in 75% ethanol and kept at − 20 °C overnight. The fixed cells were centrifuged and washed with cold PBS twice. RNase A (20 µg/ml) and propidium iodide staining solution (50 µg/ml) were added to cells and incubated for 30 min at 37 °C in dark. Cell cycle distribution was determined by flow cytometry (BD Bioscience, San Jose, CA, USA) and analysed by BD CellQuest Pro software.

Determination of phenotypic changes in nucleus by 4,6-diamidino-2-phenylindole (DAPI)

Cells were treated with various concentrations of extract (50, 100, 300 and 500 µg/ml) and allowed for incubation of 24 h. The adherent cells were fixed with 4% paraformaldehyde for 10 min, washed with PBS. The fixed cells were permeabilized with 0.2% Triton X-100 in PBS and incubated with 1 µg/ml of DAPI solution for 10 min in dark (Lin and Liu 2006). Nuclear morphology was viewed and photographed using fluorescent microscope.

Assessment of cellular morphology by acridine orange/ethidium bromide (AO/EB) dual staining

Cells seeded in 6 well plate were treated with fresh media containing extract (50, 100, 300 and 500 µg/ml). After 24 h, the cells were suspended with 100 µl of AO/EB solution followed by incubation for 15 min and images were captured with the help of fluorescent microscope (Liu et al. 2015).

### Measurement of reactive oxygen species (ROS)

Cells were seeded in 6 well plate and incubated with extract at various doses (50, 100, 300 and 500 µg/ml). Intracellular ROS levels were measured after 30 min of incubation using 2′,7′-dichlorofluorescin diacetate (DCFDA) dye (Wu et al. 2011) with flow cytometry.

### Detection of mitochondrial membrane potential (MMP)

After 24 h treatment with the methanol extract (50, 100, 300 and 500 µg/ml), cells were trypsinized, washed with PBS once and incubated with 100 nM 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$) for 15 min at 37 °C (Wu et al. 2011). The stained cells were detected by flow cytometry and mean fluorescence intensity was quantified in comparison with that of untreated cells.

### Analysis of apoptosis related gene expression

RNA was extracted after 24 h incubation using TRIzol reagent and reverse transcription was carried out with 1 µg of total RNA using RT-and GO Mastermix according to the manufacturer's protocol (MP Biomedicals, Santa Ana, California, USA). The reaction was carried out at 42 °C for 60 min followed by 70 °C for 10 min. To analyse the expression of specific gene, reverse transcriptase polymerase chain reaction (RT-PCR) was performed. The sequence of PCR primers are listed in Table 1. The PCR cycle conditions were as follows: 95 °C for 4 min, then 35 cycles of 94 °C for 20 s, annealing temperature (Tm) for specific primer for 30 s and 72 °C for 45 s with a final extension step of 7 min at 72 °C in a thermal cycler (Applied Biosystems, Foster City, CA, USA). The PCR products were separated in agarose gel, visualized under UV transilluminator and then photographed.

### Statistical analysis

All values are expressed as mean ± standard deviation (SD) of triplicate values. Statistical analysis was implemented using Student's $t$ test and $P < 0.05$ was denoted as being statistically significant. The treatment was carried out using Microsoft® Office Excel (Microsoft®, USA).

## Results and discussion

### Physico-chemical characterization of methanol extract

Crude organic extracts consist complex mixture of phenols that are soluble in certain solvents. In that note, methanol has a polarity index of 5.1 which is higher than ethanol, acetone, ethyl acetate and among others facilitating more solubility of phenolic compounds. Thus, methanol is mostly used for isolation of various polar compounds (Boeing et al. 2014). Likewise, the process resulted herein a yellowish-brown coloured organic formulation with high recovery percentage (14.4 ± 2.4%). The major chemical component was detected to be phenol (13.27 ± 3.06 µg gallic acid equivalent/mg of extract) followed by flavonoid (8.35 ± 1.75 µg quercetin equivalent/mg of extract). In addition, ascorbic acid was also

**Table 1** The primer sequences used in semi-quantitative reverse transcriptase-PCR technique

| Gene of interest | Sequence of primers | Tm (°C) | Reference |
|---|---|---|---|
| Bax | F: 5′TGCCAGCAAACTGGTGCTCA3′ | 58 | Biswal et al. 2017 |
| | R: 5′GCACTCCCGCCACAAAGATG3′ | | |
| Bcl2 | F: 5′CGCATCAGGAAGGCTAGAGT3′ | 57 | |
| | R: 5′AGCTTCCAGACATTCGGAGA3′ | | |
| Caspase3 | F: 5′AGAAGATCACAGCAAAAGGAGC3′ | 55 | |
| | R: 5′TCAAGCTTGTCGGCATACTG3′ | | |
| Caspase9 | F: 5′GCTCTTCCTTTGTTCATCTCC3′ | 60 | |
| | R: 5′CATCTGGCTCGGGGTTACTGC3′ | | |
| GAPDH | F: 5′GAGTCAACGGATTTGGTCGT3′ | 56 | |
| | R: 5′GACAAGCTTCCCGTTCTCAG3′ | | |

determined to be present in higher extent i.e. 4.69 ± 0.52 µg/mg of extract. However, insignificant amount of β-carotene and lycopene were estimated such as 0.04 ± 0.01 µg/mg and 0.03 ± 0.01 µg/mg of dry extract respectively. Extraction of these bioactive molecules solely not dependent on the type of solvent but also reliant on the nature of biological sample. Accordingly, methanolic extract of *R. alatoreticula* was found to be enriched with constituents than that of *Macrocybe lobayensis* (Khatua et al. 2017c), *Russula delica* (Gursoy et al. 2010), *Pleurotus djamor* (Acharya et al. 2017a, b), *Grifola frondosa* (Acharya et al. 2015a, b) and *Macrocybe crassa* (Acharya et al. 2015a, b).

## HPLC profiling

Further, HPLC profile was determined to procure phenolic fingerprint and predict the molecular constituents. As presented in Fig. 1, the chromatogram was found to be composed of minimum nine components of which three were tentatively recognized. Among them, pyrogallol was estimated as the major phenolic compound (4.19 ± 0.13 µg/mg of dry extract); followed by cinnamic acid (0.18 ± 0.01 µg/mg of extract). Besides, *p*-coumaric acid was also detected, although it was present in trace (0.08 ± 0.01 µg/mg of extract). Our observation was found to be in accordance with *R. delica*, *Russula lepida*, *Russula mustelina*, *Russula caerulea* and *Russula sardonia* where cinnamic acid was also noticed as the common ingredient (Kouassi et al. 2016; Alves et al. 2013). On the other hand, *p*-coumaric acid has been identified in *Agaricus arvensis*, *Agaricus silvicola, Lepista nuda*, *Boletus edulis*, *Tricholoma populinum*, *Armillaria tabescens*, *Psathyrella candolleana* and *Helvella leucopus* (Barros et al. 2009; Dundar et al. 2015). Moreover, pyrogallol was discerned in *Agaricus bisporus*, *Cantharellus cibarius*, *Craterellus cornucopioides*, *Calocybe gambosa* and *Lactarius deliciosus* (Palacios et al. 2011).

## Estimation of antibacterial action

Methanolic extract from *R. alatoreticula* expressed potent antibacterial activity against all investigating microbes where MIC values ranged from 99 to 2673.74 µg/ml concentration (Table 2). The most sensitive species was found to be *S. aureus*, while the least susceptible one was detected as *K. pneumoniae*. The outcome suggested that the fraction exhibited better potential than methanol extracts of *Amanita rubescens*, *Cantharellus cibarius*, *Lactarius piperatus* as well as *Russula cyanoxantha* (Kosanic et al. 2013) and that could be explained by presence of bioactive components in higher amount in *R. alatoreticula*. Besides, the studied extract also presented superior efficacy than *Agaricus arvensis*, *Cantharellus cibarius*, *Agaricus bisporus*, *Lactarius deliciosus*, *Fistulina hepatica*, *Sarcodon imbricatus*, *Leucopaxillus giganteus*, *Lactarius salmonicolor*, *Mycena rosea*, *R. delica*, *Ramaria botrytis*, *Tricholoma portentosum*, *Boletus lupinus*, *Flammulina velutypes*, *Sarcodon imbricatus*, *Phellinus igniarius*, *Xerocomus ichnusanus* and *Tricholoma aurantium* (Alves et al. 2012; Nedelkoska et al. 2013).

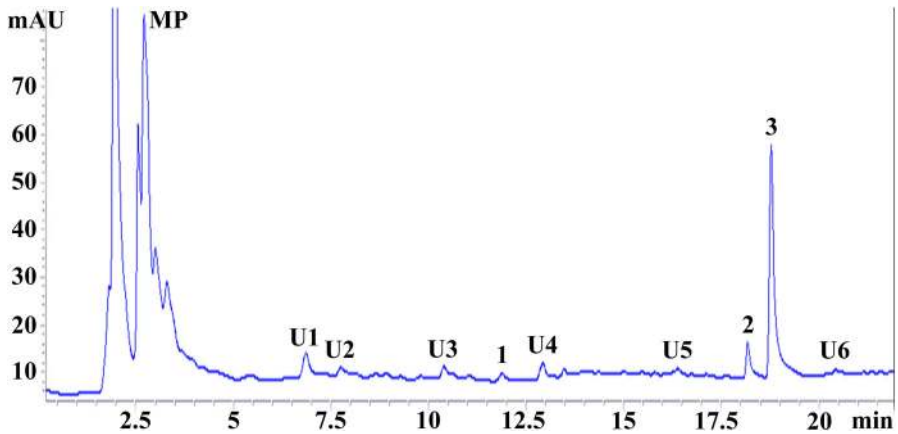


**Fig. 1** HPLC chromatogram of methanol extract from *Russula alatoreticula.* Peaks MP: mobile phase, 1: *p*-coumaric acid, 2: cinnamic acid, 3: pyrogallol; U1-6: undetermined

**Table 2** Antibacterial activity of methanol extract from *Russula alatoreticula*

| Type of bacteria | Name of bacteria | Methanol extract | Streptomycin |
|---|---|---|---|
| Gram positive | *Listeria monocytogenes* | 255.11 ± 52.32[a] | 4.68 ± 0.17[b] |
| | *Staphylococcus aureus* | 99.56 ± 4.01[a] | 6.29 ± 0.16[b] |
| | *Bacillus subtilis* | 222.19 ± 87.27[a] | 5.61 ± 0.01[b] |
| Gram negative | *Escherichia coli* | 468.07 ± 138.89[a] | 5.41 ± 0.11[b] |
| | *Salmonella typhimurium* | 961.23 ± 3.46[a] | 5.09 ± 0.03[b] |
| | *Klebsiella pneumoniae* | 2673.74 ± 30.23[a] | 5.29 ± 0.14[b] |

The activity was estimated by determining minimum inhibitory concentration value (μg/ml) (mean ± SD; n = 3). In each row different letters mean significant differences ($P < 0.05$)

## Evaluation of antioxidant activity

Total five in vitro systems were followed herein that are dependent on change in colour of reaction mixture in presence of antioxidative substance and the overall effects have been summarized in Table 3. Firstly, $O_2^-$ quenching activity assay was performed as the radical is known to produce other harmful components inside human body (Sanchez 2017). In the present study, methanol extract from *R. alatoreticula* was found to be a notable scavenger as it inhibited the radical at rate of 29.52, 40.9 and 51% in presence of 1000, 1500 and 2000 μg/ml respectively (Fig. 2a). Besides, the antioxidant efficacy was also evaluated by adopting the technique of DPPH· scavenging activity. Results indicated that the fraction inhibited 23.8 and 48.5% radicals at 500, 1000 μg/ml concentrations respectively that increased to 65% at the level of 1500 μg/ml (Fig. 2b). Further to that, $Fe^{2+}$ chelating effect was accomplished as the transition metal promotes radical generation and thus required to be chelated to combat against oxidative stress (Sanchez 2017) Fig. 2c depicted dose dependent potential of the fraction demonstrating 16.9, 38.6 and 54.7% binding capacity at 100, 200 and 300 μg/ml concentrations respectively. Moreover, the assay of reducing power was also performed depicting electron donation ability of investigating drug. As presented in Fig. 2d, the formulation at level of 1000, 2000 and 3000 μg/ml demonstrated reducing power of 0.176, 0.378 and 0.608 respectively. Finally, total antioxidant capacity was investigated and outcome indicated that 1000 μg of the methanol formulation acted equivalent to 0.62 ± 0.03 μg of ascorbic acid.

Since the last decade, natural products consisting cinnamoyl moiety have attracted much attention due to their broad spectra of therapeutic potential and less toxicity. Among the different types, *p*-coumaric acid represents strong antioxidant effect being direct scavenger of ROS (Pontiki et al. 2014). In addition, synergistic activity of phenolics with other antioxidants like ascorbic acid and carotene have also been

**Table 3** Antioxidant activity of methanol extract from *Russula alatoreticula*

| Antioxidant assays | Methanol extract | Standard |
|---|---|---|
| $EC_{50}$ (μg/ml) | | |
| Scavenging ability of superoxide radical | 1940 ± 263[a] | 34 ± 1[b] |
| Scavenging activity of DPPH radical | 1083 ± 23[a] | 4.3 ± 0.3[b] |
| Chelating ability of ferrous ion | 263 ± 9[a] | 2.54 ± 0.59[b] |
| Reducing power | 2382 ± 23[a] | 14.5 ± 5[b] |
| Total antioxidant capacity (μg ascorbic acid equivalent/mg of extract) | 0.62 ± 0.03 | NA |

Ascorbic acid was used for comparison in all assays except chelating ability of ferrous ion method where EDTA was adopted as a positive control. In each row, different letters mean significant differences between sample and standard ($P < 0.05$)

*NA* Not applicable

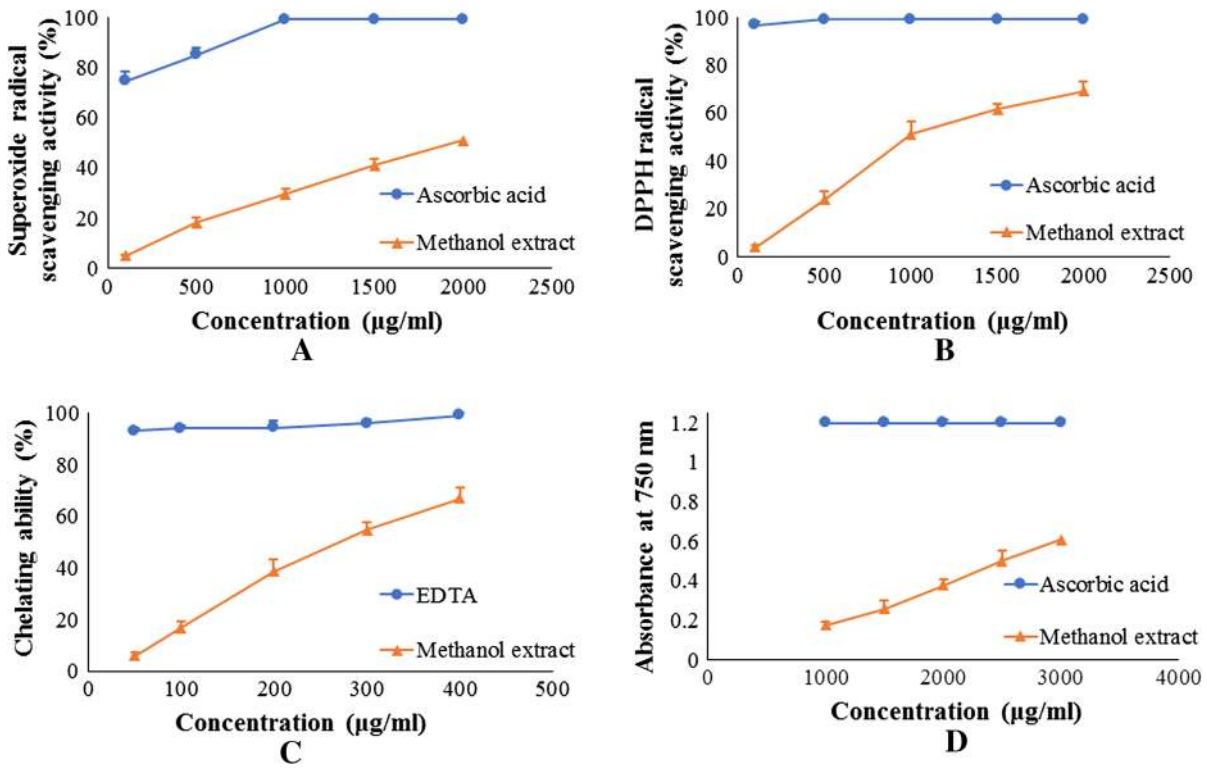


**Fig. 2** Antioxidant activity of methanol extract from *Russula alatoreticula*. **a** Superoxide radical scavenging activity, **b** DPPH radical scavenging activity, **c** chelating ability of ferrous ion, **d** reducing power

described (Pereira et al. 2009). Therefore, total amount of both phenols and vitamin C could be regarded as the major contributors to strong antioxidative capacity of the studied extract. Consequently, the fraction from *R. alatoreticula* exhibited more pronounced antioxidant effect than *A. rubescens*, *C. cibarius*, *L. piperatus*, *R. delica*, *Russula vesca* and *Termitomyces eurrhizus* (Kosanic et al. 2013; Singdevsachan et al. 2014). In contrast, the formulation presented lesser potential than methanol fraction from *Laetiporus sulphureus* (Acharya et al. 2016) and *Ramaria subalpine* (Acharya et al. 2017a, b).

## In vitro cell growth inhibition

Cytotoxic consequence of the methanol fraction on Hep3B cells was assessed using WST reagent that produces orange coloured formazan in direct proportional to amount of living cells. As portrayed in Fig. 3a, the fraction inhibited cellular growth in a dose dependent manner with $GI_{50}$ (growth inhibition by 50%) value at 358.57 ± 7.96 µg/ml. In a recent study, Youn et al. (2008) presented cytotoxic activity of *Inonotus obliquus* on Hep3B cells, although the macrofungus executed lower potential than *R. alatoreticula*.

## Clonogenic cell survival assay

It is a basic tool that determines capacity of cell to retain reproductive ability forming large colony (Munshi et al. 2005). As presented in Fig. 3b, in control sets cells divided many times and formed colony. On the other hand, in treated conditions cells gradually lost ability to proliferate. While, the highest concentration of extract (500 µg/ml) indicated a significant reduced proliferation rate where survived cells did not divide even in 2 weeks span. The same assay was adopted by Xu et al. (2012) to examine antiproliferative effects of *Pleurotus pulmonarius* against Hep3B cells where cell growth was significantly inhibited in treatment of 200 µg/ml concentration.

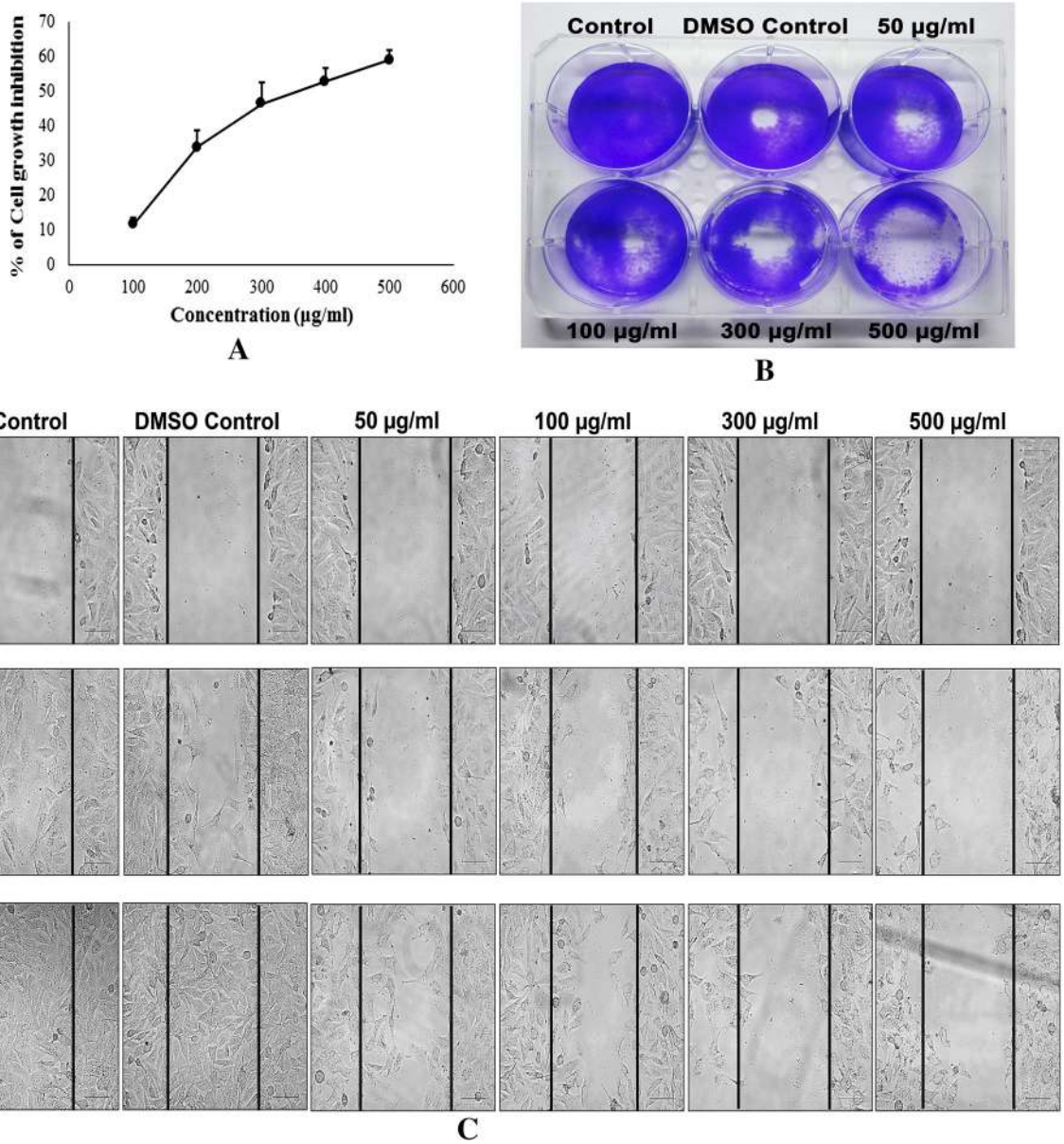


**Fig. 3** Anti-proliferative activity of methanol extract from *Russula alatoreticula* against Hep3B cells. **a** Cytotoxic effect was determined after 24 h incubation using WST reagent, **b** colony-formation assay was performed by treating cancer cells with increasing concentrations of the extract for 24 h. Result demonstrated a dose-dependent decrease in proliferation of Hep3B cells in presence of the fraction. **c** Cells were cultured in a monolayer in 12 well plate and an artificial gap was created. Rate of cell migration to close the scratch was analysed at different time intervals and photographed under a microscope (×20 magnification). The observation showed proliferation rate of Hep3B cells decreased with increase of extract. The bold lines define areas of artificial wound

## Wound-healing assay

The assay is a simple and easy method to determine rate of cell migration as well as interactions (Liang et al. 2007). Results showed that DMSO control cells migrated 67.5 ± 9.65% of damaged area after 12 h while cells challenged with 500 µg/ml concentration filled up only 15.94 ± 2.89% of area. Within 24 h, control cells proliferated to the opening of scratch completely and closed the area. In contrast, cellular

migration was slower in treated conditions as cells with the highest dose of extract moved only 30.73 ± 1.69% of area even after 24 h (Fig. 3c). However, from migration rate it could be assumed that Hep3B cells needed approximately 39 h to close 50% gab in presence of 500 µg/ml concentration of extract.

Cell cycle analysis by flow cytometry

To confirm induction of apoptosis, cell cycle progression in treatment of methanolic extract was analysed. An increase in G2/M phase population and sub-G1 peak (apoptosis %) were observed after incubation with sample for 24 h in a dose-dependent manner (Fig. 4). As the proportion of cells in the sub-G1 peak increased the damaged DNA may have induced blocking of entry of cells into mitotic stage through G2/M cell cycle checkpoint. Thus, from the observation it could be said that the fraction induced arrest of cell cycle at G2/M phase.

Determination of phenotypic changes in nucleus by DAPI

DAPI, a fluorescent dye, intercalates between minor grooves of double-stranded DNA. Therefore, staining of DNA with this fluorochrome is mostly applied for dead cell detection as their nuclear structure presents morphological hallmarks (Preet et al. 2012). In this assay, results showed that untreated cells displayed uniform chromatin staining. While the extract at 50 and 100 µg/ml concentrations induced morphological characteristics of apoptosis. At the highest concentration (500 µg/ml) tested, severe fragmentation of nuclei was detected (Fig. 5a).

Assessment of cellular morphology by AO/EB dual staining

AO and EB emit green and orange colour respectively after intercalating in DNA. AO permeates all cells

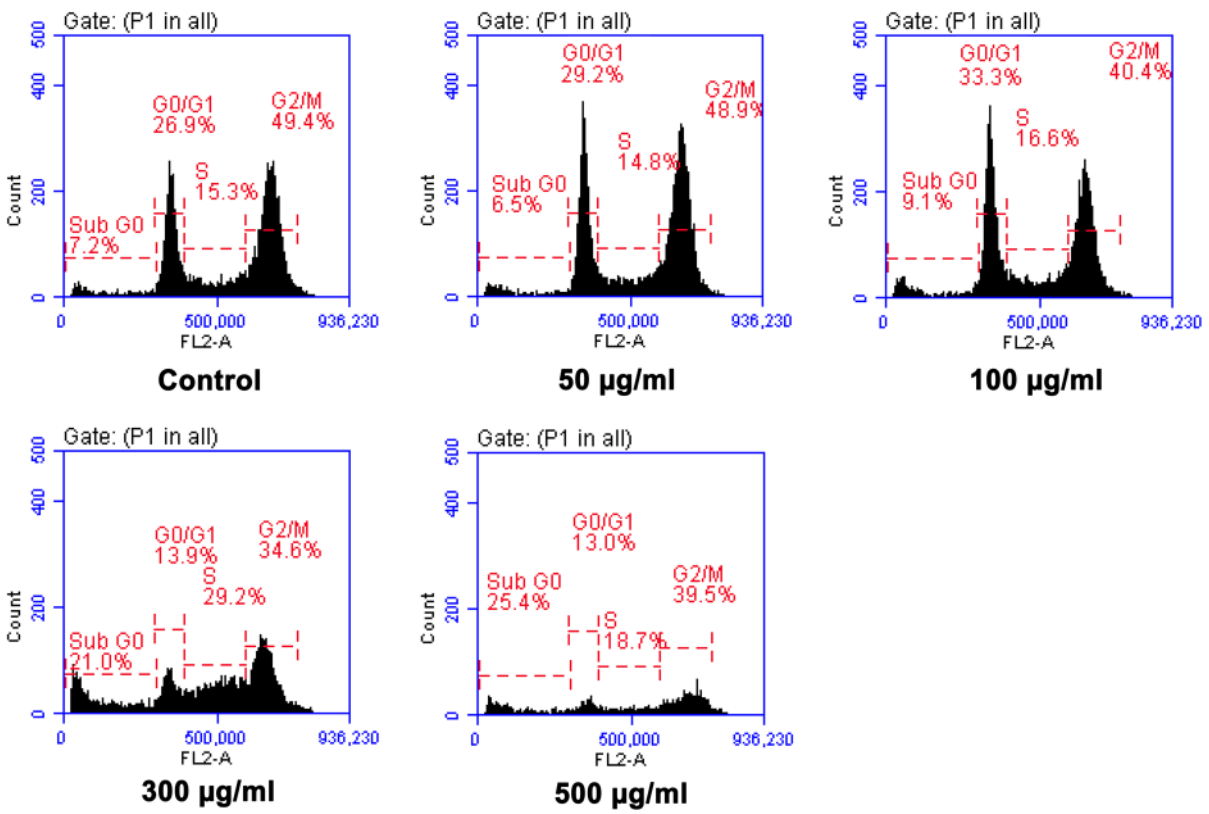


**Fig. 4** Effect of the methanol extract from *Russula alatoreticula* on cell cycle distribution was determined by flow cytometry. Result indicated after 24 h the fraction caused accumulation of Hep3B cells in sub-G1 stage and an increase at G2/M phase indicating arrest of cells in G2/M phase

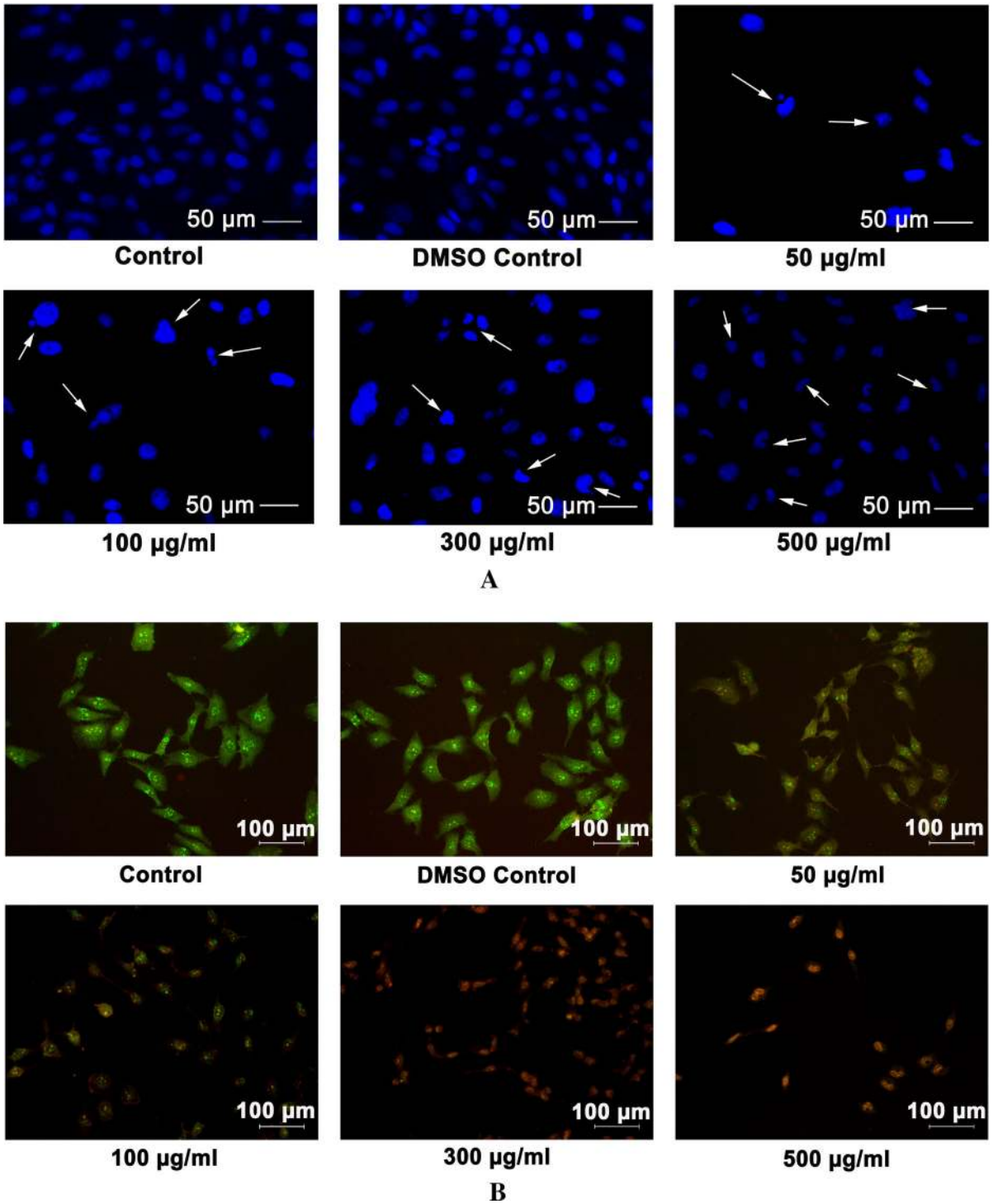


**Fig. 5** Methanol extract from *Russula alatoreticula* induced morphological changes of Hep3B cells. **a** After 24 h treatment, effect of extract on nuclear morphology was determined by DAPI staining. The arrows show nuclear condensation and fragmentation. **b** Cells were placed in 6 well plate and treated with the extract in various concentrations. After 24 h, changes in morphology of Hep3B cells that are hallmark for apoptosis were detected by AO/EB dual staining

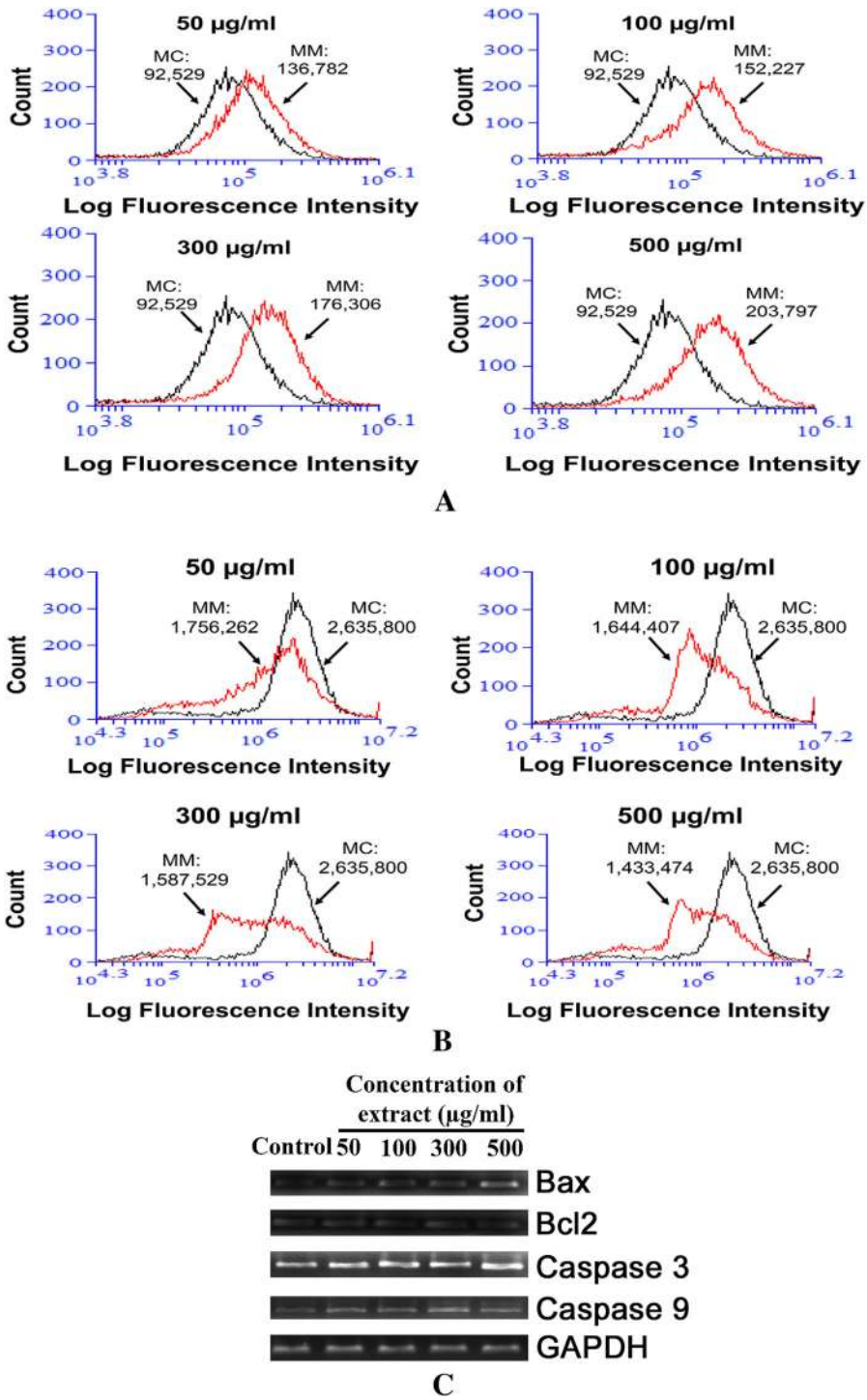


**Fig. 6** Anticancer activity of methanol extract from *Russula alatoreticula* might be mediated through mitochondrial intrinsic pathway. **a** ROS generation was determined by flow cytometry using DCFDA dye following treatment for 30 min. Data are presented as log fluorescence intensity. MC means relative fluorescence intensity of control; MM means relative fluorescence intensity of the methanol extract treatment. **b** Cells were exposed in presence of various concentrations of the fraction for 24 h. The cells were stained with $DiOC_6(3)$ and fluorescence was detected by flow cytometry. Shifting of the peaks to left indicated that the fraction induced depletion of MMP in Hep3B cells. **c** Total RNA was isolated from Hep3B cells after 24 h treatment and semi-quantitative reverse transcriptase PCR was performed to analyse the expression of four different genes

while EB cannot enter into cells until cytoplasmic membrane integrity is lost which is the characteristic of dead cell. Therefore, both stains are used simultaneously for discrimination of live from dead cells on the basis of membrane integrity (Ribble et al. 2005; Kashibhatla et al. 2006). Figure 5b showed that in control sets, live cells were represented by round and intact nucleus with green fluorescence; whereas cells

treated with 50 and 100 μg/ml concentrations of extract exhibited bright greenish yellow staining indicating early apoptotic phase. However, the morphology of cells transformed to cytoplasmic shrinkage, membrane blebbing and nuclear chromatin condensation in presence of 300 μg/ml concentration. The sign of apoptosis was more prominent in treatment of the highest dose, where cell numbers were drastically reduced and the adhered cells were noticed with deformed nucleus.

### Measurement of intracellular ROS

ROS has been suggested as an important mediator for apoptosis and its elevated level is sufficient to trigger cell death. DCFDA, a ROS indicating reagent, has ability to diffuse into cells and undergoes deacetylation followed by oxidation by ROS into 2′,7′-dichlorofluorescein (DCF). DCF is a highly fluorescent compound and the intensity can be quantified using flow cytometry (Wu et al. 2011). As indicated in Fig. 6a, DCF fluorescence increased (peaks shifted to the right) after exposure to the methanol extract suggesting induction of ROS production in Hep3B cells. Analysis revealed that the fraction elevated ROS generation by 47.83, 64.52, 90.54 and 120% at 50, 100, 300 and 500 μg/ml concentrations respectively in respect to control cells.

### Detection of MMP

Initiation of pro-apoptotic signal is reflected by depletion of MMP suggesting loss of mitochondrial membrane integrity that was detected herein using $DiOC_6(3)$. This green-fluorescent dye accumulates in mitochondria due to their large negative membrane potential (Wu et al. 2011). As shown in Fig. 6b, MMP reduction was encountered in presence of the methanol extract in a dose dependent manner evident by gradual decrease in fluorescence (MMP peak shifted to left). Results presented that in case of 50 μg/ml concentration MMP decreased by 33.37%, while in case of 500 μg/ml concentration MMP was lost by 45.61% in respect to control cells.

### Analysis of apoptosis related gene expression

Generally, Intrinsic pathway is the most common mode of apoptosis in response to stress initiated by damage-inducing agents like cytotoxic drugs. Briefly, cells are protected against apoptosis when Bcl-2 is present in excess, while cells are susceptible to programmed death when Bax exceeds certain level. Therefore, the ratio of Bcl-2/Bax determines the fate of a cell. Bax translocation to the mitochondria has been shown to activate caspase 9. Caspase 9 consecutively triggers activation of caspase 3 that is essential for nuclear changes associated with apoptosis (Park et al. 2003; Hsu et al. 2007; Golbano et al. 2008). In this study, the methanol extract resulted in a significant increase in pro-apoptotic Bax protein and decrease in levels of anti-apoptotic Bcl2 protein, thus shifting the Bax/Bcl2 ratio in favour of apoptosis. Furthermore, pronounced induction of caspase 9 and caspase 3 were also demonstrated (Fig. 6c). Thus, the present result indicated that methanol extract positively regulates liver cancer cell apoptosis.

## Conclusion

Overall, the work provides insights into bioactive components of methanol extract from *R. alatoreticula* that included phenols, flavonoids, ascorbic acid, β-carotene and lycopene. These elements might have played active roles behind noticeable antibacterial effect of the formulation where investigating microbes exhibited susceptibility in order of *S. aureus* > *B. subtilis* > *L. monocytogenes* > *E. coli* > *S. typhimurium* > *K. pneumoniae*. Besides, the fraction was also able to scavenge free radicals, chelate ferrous ion and donate hydrogen atom exhibiting strong antioxidant potency. Further, results also provided new hope for chemotherapy of hepatoma cancer as the organic formulation inhibited growth, elevated ROS, decreased MMP, caused G0/G1-phase arrest, alleviated caspase 9 as well as caspase 3 resulting apoptosis in Hep3B cells. However, studies on the isolation of active component for development of new generation therapeutic agent will be pursued in future work.

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**Compliance with ethical standards**

**Conflict of interest** The authors declare that they have no conflict of interests.

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