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Understanding the Medicinal Prospects of Methanolic Extract from a Recently Explored Mushroom of Tribal Delicacy

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Abstract

At present, wild edible mushrooms are experiencing a curious renaissance as they offer a range of functional benefits. In that connection, West Bengal, India constitutes a wealth of macrofungal flora where many species are being harvested by locals as food and medicine. One of such matrices has recently been reported by our team that in turn appeared as a novel species (*Russula pseudocyanoxantha* Paloi, K. Acharya & S. Khatua). The present study thus aimed to screen bioactive potential of the neglected tribal cuisine and explore related metabolite profile for downstream applications. For that the dried basidiocarps were subjected to methanolic extraction and the fraction was found to be mainly composed of phenolics (pyrogallol> cinnamic acid) along with ascorbic acid and carotenoids. Consequently, the preparation emerged as a potent radical scavenger, metal ion chelator and electron donor where EC_{50} values ranged from 621 to 1491 µg/ml. Additionally, antimicrobial activity tests were also performed using microdilution technique against six bacterial strains where MIC values ranged from 88.65 to 1559 µg/ml. Besides, the extract was able to inhibit Hep3B cell proliferation as well, evident by cytotoxicity (IG_{50} 376.21 µg/ml) and scratch assays. The findings thus suggest that *R. pseudocyanoxantha* may be used as a valuable resource of natural antioxidant, antibacterial and cytotoxic ingredients to control various human diseases.

Keywords: Hep3B, HPLC, Human pathogens, Radical scavenging activity, Wild edible mushroom.

1. Introduction

India is one of the most mega-diverse countries in the world where an appreciable portion of the total land area is under forest and tree cover (Krishna *et al*., 2015). The subcontinent includes virtually all the major climate zones of the globe cradling a large number of fungal species and their natural beauty. Indeed, scientists have suggested that one third of the universal myco-diversity exists in India, and thus presence of novel species in the nation, is not a rare event (Tripathi *et al*., 2017; Khatua *et al*., 2019). In this regard, it is worth mentioning that West Bengal, a state in eastern India, constitutes a wealth of macrofungal flora (Singha *et al*., 2017). The monsoon season makes the highly prized mushrooms to flourish on forest floors where many species play a key role in food security for tribals. Inherently, the local people value these nature derived resources, and the wisdom collected by forefathers is orally passed to the next generations (Khatua *et al*., 2017a; Khatua *et al*., 2019). During our recent foray, one of such matrices was fortunately being discovered growing under *Shorea robusta* at lateritic regions of West Bengal. The investigation based on morphological characters, DNA barcoding and phylogenetic analysis revealed novelty of the specimen which was further entitled as *Russula pseudocyanoxantha* Paloi, K. Acharya & S. Khatua. The mushroom is colloquially known as “Jam Patra” and enjoyed as seasonal health promoting food by locals indicating potential to be used as a functional nutrient. Despite that, city people have refrained from consuming the macrofungus due to lack of proper knowledge, and thus detailed study should be carried out to increase awareness, before the species disappears from the natural habitat (Khatua *et al*., 2021a).

Nowadays, people are predisposed to various diseases due to modern lifestyle associated with exposure to a wide range of chemicals, processed food and lack of exercise (Sharifi-Rad *et al*., 2020). As a result, people are gradually moving towards functional foods that are purported to provide optimal nutrition and reduce the risk of disease occurrence (Granato *et al*., 2020). In this context, mushrooms are widely acknowledged for their tremendous nutritional value and pleasant taste. They have also been continually used in traditional Asian medical systems to treat many diseases and promote longevity (Ho *et al*., 2020). Current research has comprehended that macrofungi are the natural reservoirs of potent pharmaceuticals and new interface for drug discovery (Zeb and Lee, 2021). Basidiomycetes possess a variety of biologically active compounds such as phenolics that offer valuable therapeutic effects including antioxidant (Khatua *et al*., 2017c), antibacterial (Khatua and Acharya, 2021) and anticancer properties (Khatua *et al*., 2021b). There is thus an increasing interest with medicinal effects of mushroom extracts enriched in secondary metabolites and

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potential for its use as functional foods (Abdelshafy *et al*., 2021). The present study henceforth was aimed to determine health beneficial effects of *R. pseudocyanoxantha,* and for that a methanol extract was isolated.

2. Materials and methods

2.1. Fungal material collection

Several field trips were conducted at the lateritic areas of West Bengal, and fruit bodies were collected. The fruit bodies were identified following macroscopic study, anatomical characterization, DNA barcoding and phylogenetic placement analysis. The voucher specimen is conserved at Calcutta University Herbarium under accession number of CUH AM652 (Khatua *et al*., 2021a).

2.2. Preparation of crude methanol extract

Dried fruit bodies were first pulverized using an electric blender and sieved through 160 mesh. For extract preparation, 10 g of the powder was soaked in 200 ml of methanol for 24 h with frequent shaking. The fraction was then isolated using Whatman filter paper. The preparation was further dried by evaporation (Rotavapor R-3, Butchi, Switzerland) at 40°C (Khatua *et al*., 2019).

2.3. Determination of major bioactive compounds

For estimation of total phenolic compounds, Folin-Ciocalteu (FC) assay was followed where the isolated methanol extract from *R. pseudocyanoxantha* was mixed with FC reagent and sodium carbonate solution. Further, absorbance was recorded at 725 nm and gallic acid (10–40 µg) was used as a standard (Khatua *et al*., 2019). The amount of total flavonoid was quantified by mixing the extract with aluminium nitrate and potassium acetate. Following 40 min incubation, absorbance was measured at 415 nm. Quercetin (5–20 µg) was considered as a reference. Further, the amount of ascorbic acid was quantified following a modified titration method where vitamin C was mixed with oxalic acid and titrated against 2,6-dichlorophenolindophenol dye (Khatua *et al*., 2017c). Contents of carotenoids were determined by mixing the extract with acetone-hexane solution and recording absorbance at three different wavelengths such as 453, 505 and 663 nm. Finally, the extract was analyzed for estimation of phenolic composition with the help of high performance liquid chromatography (HPLC) (Agilent, USA) (Khatua *et al*., 2015).

2.4. Evaluation of antioxidant activity

A revised version for reducing power assay was considered where variable doses of the preparation under investigation were mixed in 96 well plate and the absorbance at 750 nm was recorded (Bio-Rad iMarkTM Microplate Reader, USA). Nevertheless, ability of the fraction to chelate ferrous ions was also estimated in the microtiter plate using ferrozine, and ferrous chloride and absorbance at 595 nm was estimated (Dehimat *et al*., 2021). Methanol solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical was evaluated against various dosages of the studied extract in 96 well plate and absorbance at 595 nm was determined (Thakur *et al*., 2021; Khatua *et al*., 2017b). The method of total antioxidant capacity was carried out in the present study and activity of the methanol extract was expressed as a number of equivalents of ascorbic acid (Khatua *et al*., 2018).

2.5. 2.5. Estimation of antibacterial action

Listeria monocytogenes ATCC® 19111™, *Bacillus subtilis* ATCC® 6633™, *Staphylococcus aureus* ATCC® 700699™, *Escherichia coli* ATCC® 25922™, *Klebsiella pneumoniae* ATCC® 15380™ and *Salmonella typhimurium* ATCC® 23564™ were cultured overnight in nutrient broth and used for the investigation. After 24 h incubation with the methanol extract, antibacterial effect was assessed by computing minimum inhibitory concentration (MIC) values (Khatua and Acharya, 2021).

2.6. Estimation of anti-proliferative activity

To determine cytotoxicity, Hep3B human liver cancer cells were seeded in 96-well plate overnight, and the studied methanolic extract dissolved in sterile DMSO was added at a range of concentrations. After 24 h incubation, 20 µl water-soluble tetrazolium (WST) reagent was added in each well and absorbance was measured at 450 nm (Khatua *et al*., 2017c). Further, wound healing assay was performed by creating a scratch in Hep3B monolayer and incubation of the cells with the studied fraction. Healing of the wound was estimated after 24 h incubation and photographed (Khatua *et al*., 2021b).

2.7. Statistical analysis

The results presented herein are expressed as mean ± SD of three independent experiments. The analysis of statistical data was procured with Student's t-test by $p<0.05$ as the minimal level of significance using IBM SPSS Statistics, v. 23.0. (IBM Corp., Armonk, New York, United States).

3. Results and discussion

3.1. Determination of major bioactive compounds

The phenol content of an extract depends on the type of sample and solvent used for extraction. In general, methanol is identified as the most effective medium for the preparatory process, resulting in high extraction yield as well as appreciable content of phenolic compounds (Do *et al*., 2014). Keeping this in mind, the solvent was selected in the present study to isolate secondary metabolites from *R. pseudocyanoxantha* that in turn resulted satisfactory recovery percentage (21.07 ± 0.93%). Literature survey revealed that the yield was comparatively better than that of *Auricularia polytricha*, *Tremella fuciformis* and *Auricularia fuscosuccinea* (Lin *et al*., 2013). Spectroscopic analysis depicted that the studied preparation was enriched mainly with phenols (11.37 ± 0.94 µg gallic acid equivalent/mg of extract) and the amount was found to be superior to alcoholic extract from *Auricularia auricula* (Yuwa-Amornpitak et al., 2020). Alongside, flavonoid was also detected in the fraction under investigation in moderate extent (7.14 ± 3.33 µg quercetin equivalent/mg of extract) which was higher than the methanol fraction of *Pleurotus ostreatus* (González-Palma *et al*., 2016). In contrast, trace amount of ascorbic acid was recorded (0.83 ± 0.28 µg/mg of extract) which was quantified lower than *Pleurotus djamor* (Acharya *et al*., 2017). Similar range of carotenoids including 0.55 ± 0.08 µg β-carotene and 0.43 ± 0.07 µg lycopene per mg of extract was also found in the

studied preparation where the quantities were enumerated to be better than *Macrocybe lobayensis* (Khatua *et al*., 2017c).

Further, HPLC was performed following a standardized protocol to procure a phenolic fingerprint of the methanolic preparation from *R. pseudocyanoxantha*. The chromatogram revealed presence of minimum five phenolics in the extract; amongst them two components were tentatively recognized. Comparatively, pyrogallol was detected as the chief component presented at the level of 3.96 ± 0.04 µg/mg of extract. Besides, cinnamic acid was also detected where the amount was calculated as 0.16 ± 0.03 µg/mg of extract. The observation was in contrast to Kouassi *et al*. (2016) reporting presence of a range of phenolic compounds in *Russula delica*, *Russula lepida* and *Russula mustelina*.

3.2. Evaluation of antioxidant activity

Free radicals, oxygen-containing molecules with an uneven number of electrons, are the natural by-product of chemical processes. As a result, they can react so easily with other molecules causing damage to cells, proteins and DNA (Neha *et al*., 2019). These reactions can lead to a vast number of diseases including cancer, atherosclerosis, Alzheimer's disease, Parkinson's disease and many others. Antioxidants are molecules that can donate an electron to the highly reactive free radicals stabilizing them (Khatua *et al*., 2017d). In this line, several synthetic antioxidants are available in market; however, they are prone to cause side-effects. At the same time, nature-derived antioxidants are gaining more and more attention due to food safety aspects (Lourenço *et al*., 2019).

To determine the antioxidant potential, ferric reducing assay was executed as it delivers vital information regarding hydrogen atom donation capacity. The technique is based on the principle that antioxidative substances can react with potassium ferricyanide resulting formation of potassium ferrocyanide, which subsequently forms ferric-ferrous complex with λ_{max} of 750 nm. The study demonstrated that the fraction from *R. pseudocyanoxantha* owns effective reducing power that increased steadily with the increase of concentrations (Figure 1a). The preparation at the concentrations of 1000 and 1500 µg/ml exhibited reducing power of 0.35 and 0.57 respectively which reached to 0.9 in presence of 2000 µg/ml dosage. In this connection, the studied formulation could be considered as a better reductant than methanol extract from *Russula alatoreticula* (Khatua *et al*., 2019). Further, a ferrozine based assay was followed to depict affinity of the studied fraction towards metal ion as excess amount of Fe^{2+} is prone to generate free radicals. The method is based on the formation of violet coloured ferrozine-Fe^{2+} complex which is disrupted in presence of antioxidative compound resulting decrease in colour. As presented in Figure 1b, the preparation under investigation exhibited 7.45%, 43.09% and 80.48% Fe^{2+} chelating abilities at the levels of 100, 500 and 1000 µg/ml respectively. Literature survey revealed that methanol extract from *R. pseudocyanoxantha* might possess higher affinity to metal ions than that of *M. lobayensis* (Khatua *et al*., 2017c). To confirm antioxidant potency of the studied methanol extract, $DPPH^{\bullet}$ scavenging assay was performed being an accurate, easy and economic technique to estimate the bioactivity. The protocol is based on reduction of the violet-colored radical by antioxidative molecules via a hydrogen atom transfer mechanism ensuing formation of stable yellow-colored diphenylpicrylhydrazine. Our results showed a dose-response curve of $DPPH^{\bullet}$ scavenging activity of the methanolic fraction from *R. pseudocyanoxantha.* At a concentration of 500 µg/ml, the preparation quenched 22.81% radicals that reached to 49.87% and 78% radical inhibition in presence of 1000 and 1500 µg/ml of the formulation respectively (Figure 1c). Comparatively, the effect was found to be inferior to the standard; but superior to methanol extract from *Macrocybe crassa* (Acharya *et al*., 2015b). Finally, phosphomolybdenum assay was implemented to determine reducing ability of the antioxidative compounds converting Mo(VI) to Mo(V). Result showed that the studied fraction did not respond well as presented in Table 1 and thus the outcome was found to be inferior to the organic preparation from *Grifola frondosa* (Acharya *et al*., 2015a).

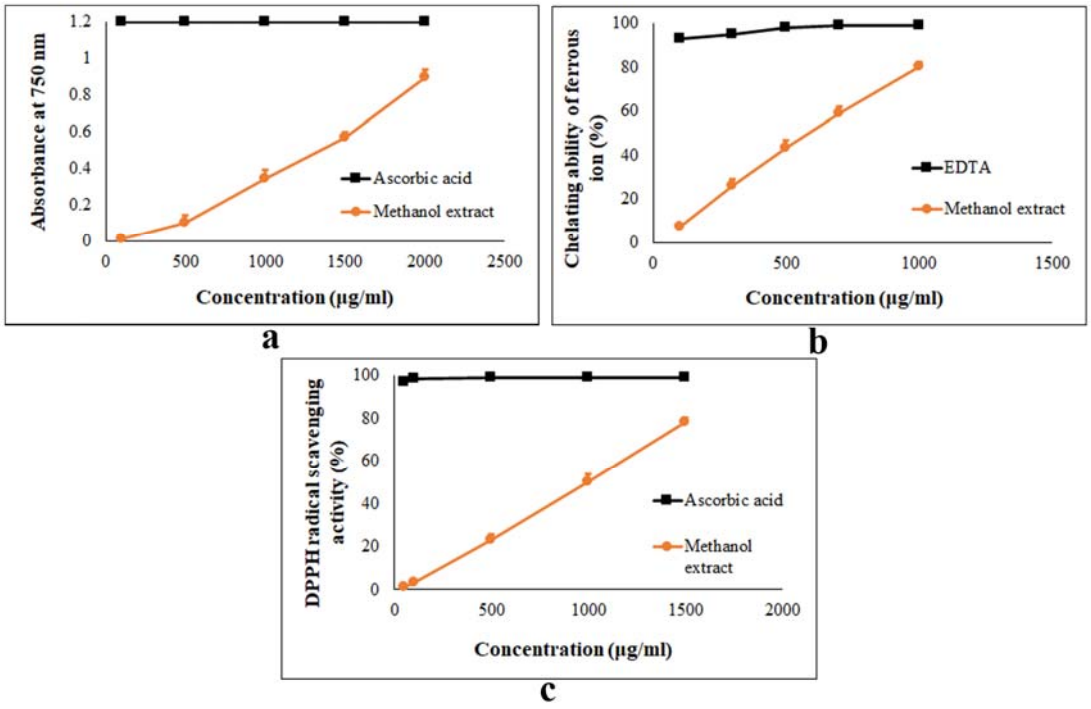

Figure 1. Antioxidant activity of *Russula pseudocyanoxantha* methanolic fraction. (a) Reducing power (b) Chelating ability of ferrous ion (c) DPPH radical scavenging activity.

Table 1. Antioxidant activity of *Russula pseudocyanoxantha* methanolic fraction. EDTA was used as standard in chelating ability of ferrous ion method, while ascorbic acid was considered as a reference for rest of the assays. Dissimilar letters in each row designate significant alterations between the sample and standard.

Antioxidant parameters		Methanol extract	Standard
EC_{50} value (µg/ml)	Reducing power	1491 ± 45[a]	17.92 ± 3.87[b]
	Chelating ability of ferrous ion	621 ± 23[a]	12.09 ± 1.67[b]
	Scavenging ability of DPPH radical	1002 ± 28[a]	8.21 ± 0.07[b]
Total antioxidant activity (µg ascorbic acid equivalent/mg of dry extract)		0.31 ± 0.03	Not applicable

3.3. Estimation of antibacterial action

Today, microbial infection is considered as the biggest challenge worldwide that threatens the health of societies causing millions of deaths every year. Many factors are known to contribute to evolution of resistance including unnecessary prescription of antimicrobials and their use in agriculture (Chassagne *et al*., 2021). In recent years, various strategies have been recommended to overcome the urgent danger. In this regard, phytochemicals have exhibited profound ability, and many researchers thus have focused on searching for natural products that can act against bacterial resistance (Khameneh *et al*., 2019).

In this context, the present study was designed to explore antibacterial activity of the methanol extract from *R. pseudocyanoxantha* against three Gram positive and three Gram negative bacteria. Amongst the tasted microorganisms, *S. aureus* was found to be the most susceptible species as evident by the lowest MIC value (Table 2). Alongside, the fraction also inhibited cellular growth of *E. coli*, *L. monocytogenes* and *B. subtilis* where the MIC data ranged from 125.63 to 350 µg/ml. On the other hand, *K. pneumoniae* appeared as the most resistant pathogen followed by *S. typhimurium* indicating more powerful activity of the fraction against Gram positive bacteria. The observation was in accord with previous publications describing difficulty to hinder growth of Gram negative pathogens by mushroom extracts (Gebreyohannes *et al*., 2019). Literature survey also revealed that the studied fraction executed better antibacterial effect than methanol extracts from *Handkea utriformis*, *H. excipuliformis* and *Vascellum pratense* (Petrović *et al*., 2016).

Table 2. Antibacterial activity of *Russula pseudocyanoxantha* methanolic fraction as determined by MIC values (µg/ml) (mean ± standard deviation; n= 3).

Type of bacteria	Name of bacteria	Methanol extract	Streptomycin
Gram positive	*Listeria monocytogenes*	276 ± 13.36[a]	5.18 ± 0.02[b]
	Staphylococcus aureus	88.65 ± 5.03[a]	5.98 ± 0.43[b]
	Bacillus subtilis	350 ± 25.05[a]	4.87 ± 0.26[b]
Gram negative	*Escherichia coli*	125.63 ± 31.59[a]	6.02 ± 0.39[b]
	Salmonella typhimurium	923 ± 27[a]	4.75 ± 0.52[b]
	Klebsiella pneumoniae	1559 ± 38.91[a]	6.42 ± 0.69[b]

3.4. Estimation of anti-proliferative activity

Cancer, abnormal cell proliferation, is the most feared ailment second only to heart disorder as a life-threatening disease. Every year, millions of people are diagnosed with cancer, often leading to death. In 2018, around 18 million

new cases of cancer were globally reported, ensuing around 10 million deaths (Khalifa *et al*., 2019). Amongst the different types, liver cancer remains a universal health challenge, and its occurrence is in steep escalation (Balogh *et al*., 2016). The majority of the anticancer agents used at clinical level are known to produce toxic effects, which limits their further usage. Scientists have suggested one realistic approach to deal with this problem in the use of natural products for effective drug development (Khazir *et al*., 2014).

In this background, Hep3B cells were used in the present study to determine growth inhibitory activity of the methanol extract from *R. pseudocyanoxantha.* The treated and untreated cells were subjected to WST assay to determine effect of the fraction on cell proliferation. The results, as shown in Figure 2a, demonstrated that the preparation was able to inhibit hepatocellular carcinoma (HCC) in a dose dependent manner. After exposure to 100, 300 and 500 µg/ml of the extract, cell viability was reduced by 10.1%, 43.9% and 59.67% respectively within 24 h. Thus, IC_{50} value was calculated as 376.21 ± 0.9 µg/ml which was found to be superior to the methanol extract from *R. alatoreticula* (Khatua *et al*., 2019). Further, wound healing or scratch assay was performed to analyze influence of the studied preparation on cell migration or invasion. As illustrated in Figure 2b, untreated HCC cells occupied the free space and filled the gap within 24 h. In contrast, wound enclosure of treated cells was suppressed upon exposure to the extract under investigation. Indeed, cells incubated with higher level i.e. 400 µg/ml failed to populate the wounded area indicating probable anti-proliferative potency of the methanol fraction from *R. pseudocyanoxantha* towards the Hep3B liver cancer cell. The observation was in accordance to our previous reports (Khatua *et al*., 2017c; Khatua and Acharya, 2021; Khatua *et al*., 2021b).

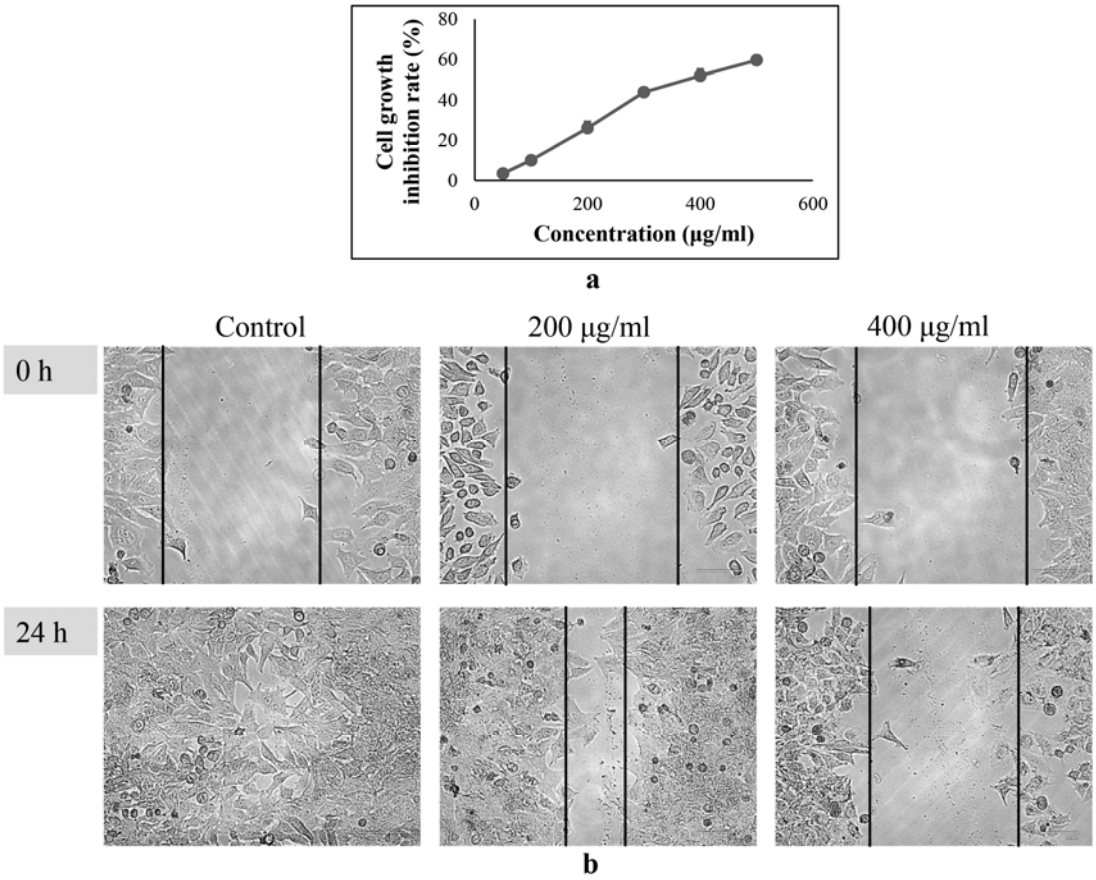

Figure 2. Anti-proliferative activity of methanol fraction isolated from *Russula pseudocyanoxantha* against Hep3B human liver cancer cells. (a) WST assay (b) Scratch assay.

4. Conclusion

In sum, the studied methanol extract exhibited potent antioxidant activity where the lowest EC_{50} value was detected in case of chelating ability of metal ion and moderate effect was noted against $DPPH^{\bullet}$ scavenging, reducing power and total antioxidant assays. Alongside, strong antimicrobial property was also evident against the targeted organisms where MIC values were in the decreasing order of *K. pneumoniae*> *S. typhimurium*> *B. subtilis*> *L. monocytogenes*> *S. aureus*. Further, the fraction was capable to inhibit Hep3B cell proliferation as well as migration within 24 h incubation. Such diverse outcomes might be related to the presence of a range of biomolecules in the fraction including phenols, flavonoids, ascorbic acid and carotenoids. Together, our results proved that the novel mushroom, *R. pseudocyanoxantha*, has a great potential in biomedical, nutraceutical and functional food applications. However, further investigation is needed

on bio-assay guided isolation of active compounds and confirmation of the therapeutic prospects *in vivo*.

Conflicts of interest

None.

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