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ORIGINAL ARTICLE

Antioxidation and immune-stimulatory actions of cold alkali extracted polysaccharide fraction from *Macrocybe lobayensis*, a wild edible mushroom

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Abstract

Mushroom β-glucans are presently gaining widespread attention, being one of the promising healthy compounds with excellent antioxidative and immunomodulatory activities. Conventionally, hot water extraction procedure is followed to isolate the polymers where the residue is discarded after filtration. However, the remnants still contain plenty of bioactive components that could provide a unique opportunity for the discovery of novel therapeutic agents. In this backdrop, the present study was aimed to expand utilization of a popularly edible mushroom, *Macrocybe lobayensis*, by re-cycling left-over material that has passed through traditional aqueous process. For that, the residue was immersed in alkaline solution followed by ethanol precipitation and repeated washing resulting preparation of a water soluble and partially purified polysaccharidic fraction (ML-CAP). Chemical and molecular characterization by FT-IR, HPTLC, GC–MS, GPC and spectroscopy unveiled that ML-CAP was consisted of a homo-polymer with Mw of ~ 122 kDa. The backbone was mainly composed of β-glucan where galactose was identified as the second most abundant unit. Subsequently, the fraction exhibited potent antioxidant activity in terms of radical scavenging, chelating ability and reducing power. Furthermore, strong immune enhancing property was also recorded as the polymer, particularly at the concentration of 100 µg/ml, triggered murine macrophage functionality in terms of cell proliferation, phagocytosis, pseudopods formation and nitric oxide production. The study thus advocates for potential application and further extraction of hot water extracted mushroom residue in drug development and nutraceutical industries, as the example of ML-CAP showed promising biological effects.

Keywords Chemical characterization · Cold alkali extraction · Radical scavenging property · RAW264.7 cell line

Abbreviations

ABTS	2′-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid)
EDTA	Ethylenediaminetetraacetic acid
FT-IR	Fourier transform infrared
HPTLC	High performance thin-layer chromatography
GC-MS	Gas chromatography-mass spectrophotometry
GPC	Gel-permeation chromatography
LPS	Lipopolysaccharide
Mw	Molecular weight
NO	Nitric oxide
WST	Water-soluble tetrazolium

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Introduction

Medicinal mushrooms have been treasured for centuries in Asian and other traditional health-care practices; although their therapeutic prospects remained unexplored to the wide scientific community for a long time. The most common active ingredients in these higher fungi are their extracellular and intracellular or cell wall polysaccharides involved in several biological processes such as antioxidation, immune-modulation, anti-tumor, antiviral and so forth (Maity et al. 2021). Recently, β-glucans (chain of D-glucan monomers connected by β-glycosidic bonds) have drawn the attention of chemists and immunobiologists as the polymers can stimulate the immune system (Frioui et al. 2018). The compounds are known to activate antigen presenting cells

such as macrophages which are the powerful phagocytes and key players in innate immune system linking in-born as well as adaptive defense mechanisms. Thus, searching for mushroom polysaccharides that can elicit immune responses could be beneficial, particularly in immune-therapy of patients suffering from various tumor types (Han et al. 2020). Another widely studied bioactivity of macrofungal polymers is antioxidative effect facilitating the human being to confront oxidative stress. The condition is induced by free radicals that can damage biomolecules such as DNA, proteins, lipids and carbohydrates contributing many diseases such as cardiovascular disorders, cancer, arthritis and inflammation. Because of public concern with the adverse effects of synthetic chemical antioxidants on our health, there has been increasing interest in the discovery and application of natural antioxidants where mushroom derived polysaccharides have shown significant radical scavenging activities based on various in vitro and in vivo assays (Khatua et al. 2021a, b; Kozarski et al. 2015; Siu et al. 2014).

Most of these functional polysaccharides are water soluble, and thus, hot aqueous condition is conventionally used to separate the polymers discarding the filtrate (Leong et al. 2021). Despite being simple and safe, the technique bears some disadvantages such as high temperature, long extraction time, low recovery percentage, and the need for repetitions to procure considerable amount of crude polysaccharides (Barbosa et al. 2020). More importantly, the residue after filtration still contains enough bioactive molecules, as shown in our previous works, that could further be re-cycled for maximum utilization of the bio-resource and minimize waste production (Khatua et al. 2021a; Khatua and Acharya 2017, 2019, 2021b). In this context, NaOH reflux has been reported as an efficient method to obtain fractions with high yield and rich in therapeutic primary metabolites (Chen et al. 2014). However, there are no reports on alkaline extracted polysaccharides from many wild edible mushrooms, so far.

Macrocybe lobayensis is one of such matrices, originally described from Central African Republic. Later, the mushroom has been reported from Ivory Coast, Ghana, Nigeria, India (Bhale et al. 2019) and China (Liu et al. 1996). In India, Madhya Pradesh (Verma et al. 2017), Kerala (Vrinda and Pradeep 2006), Maharashtra (Bhale et al. 2019) and West Bengal (Khatua et al. 2019, 2017b; Khatua and Acharya 2018) have been attributed to nurture natural growth of the taxon. The specimen is traditionally praised as a gourmet cuisine and consumed by parboiling as well as cooking (Vrinda and Pradeep 2006; Khatua and Acharya 2021a). Despite that, research on estimation of health benefits of the edible mushroom is still lacking. Recently, Liu et al. (2015) illustrated antioxidant and immune-regulatory activities of the Basidiomycete where the bioactive polymers were isolated by traditional water extraction. Similar observation has also been demonstrated in our previous research where the fruit body powder was subjected to hot aqueous condition (Ghosh et al. 2019). Extending the earlier work, the present study was aimed to further utilize residue of the conventional method by subjecting the left-over material to cold alkali process. The effort resulted isolation of a partially purified polysaccharidic fraction from *M. lobayensis* which was further subjected to molecular characterization. Alongside, antioxidant activity and immunomodulatory potential were also elucidated for downstream practical application of the polymers.

Materials and methods

Mushroom collection and preliminary treatment

The basidiocarps were collected from coastal region of West Bengal, India and identified following standard literature (Pegler et al. 1998). A specimen has been deposited in Calcutta University Fungarium (CUH AM 483). The fruit bodies were cleaned to remove soil debris, dried with the help of field drier and crushed through a grinder.

Polysaccharide extraction

Small-molecular-weight components were removed by dipping the powdered fruit bodies in ethanol for overnight. The samples were then filtered and immersed in distilled water for 7 h under boiling condition. The suspension was filtered and the residue of conventional hot water process was subjected to 100 ml of 10% NaOH solvent. After 24 h incubation at 4 °C, the remainder was separated and the extract was neutralized by glacial acetic acid. Four volume of absolute ethanol was added in the solvent and left overnight at cold temperature. The polysaccharides were isolated by centrifugation (Rotavapor R3, Butchi, Switzerland) at 11,000 rpm for 10 min at 4 °C and dissolved in water repeatedly (Khatua and Acharya 2019, 2017). Finally, water-soluble fraction of cold alkaline extracted polysaccharide from *M. lobayensis*, designated as ML-CAP, was prepared. The sample was kept at 4 °C until further use.

Polysaccharide structure determination

Total sugar and protein content were estimated with the help of phenol sulfuric acid method and Bradford reagent, respectively. Furthermore, Mushroom and Yeast β-Glucan Assay Kit (Megazyme Institute Wicklow, Ireland) was followed at per the manual to calculate total glucan, β-glucan and α-glucan quantities. Moreover, the polymers and KBr were pressed into pellets and recorded on FT-IR spectrophotometer in the frequency range of 400–4000 cm^{-1}. Homogeneity of the fraction as well as Mw of the existed

polymer were estimated by GPC on a Seralose 6B column (1.6 cm × 60 cm) using water as eluent and Dextrans (110, 70 and 40 kDa) as standards. Finally, molecular composition of ML-CAP was estimated with the help of HPTLC as well as GCMS as described in our previous publications (Khatua et al. 2021b; Ghosh et al. 2019; Khatua and Acharya 2017).

Estimation of antioxidant potential

Ability of the macromolecules to chelate ferrous ion was determined using a chelating agent such as ferrozine and the effect was compared with EDTA. Moreover, potential against ABTS radical was evaluated using ML-CAP at different levels and the absorbance was detected at 750 nm. Finally, the assay of total antioxidant capacity was carried out and the activity was expressed as number of equivalent of ascorbic acid (Khatua and Acharya 2018; Khatua et al. 2017a).

Determination of immune-stimulatory activity

RAW264.7 murine macrophages were purchased from National Centre for Cell Science, Pune, India. Effect of ML-CAP on macrophage viability and phagocytic uptake was determined using WST and neutral red reagents, respectively, after 24 as well as 48 h incubation. Following one day treatment, NO production by treated and untreated monocytes was estimated by Griess reagent. Cellular morphology of both stimulated and unstimulated macrophages was observed after 24 h incubation and photographed using fluorescent microscope (Ghosh et al. 2019; Khatua and Acharya 2017).

Statistical analysis

The outcomes demonstrated herein are conveyed as mean ± SD of three independent experiments. The analysis 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).

Results and discussion

Physico-chemical characterization

The present work was aimed to re-use left-over residue of *M. lobayensis* that has passed through hot water process and for that a multi-step isolation method was followed. Ultimately, a brown-colored fraction was extracted using cold alkali solvent which was further washed repeatedly to prepare a water-soluble fraction (ML-CAP). The protocol lead to moderate extractive yield (Table 1) where the value was comparatively better than hot water extracted polysaccharides from *Armillaria mellea*, *Cantharellus cibarius* (Zavastin et al. 2018), *Lentinus squarrosulus* (Ayimbila and Keawsompong 2021), and *Termitomyces eurhizus* (Chatterjee et al. 2013). The observation could be justified by ability of NaOH to readily destroy bonds between polymers and cell wall or intracellular components. As a result, alkali solution promoted release of carbohydrate chains resulting preparation of a fraction with appreciable yield (He et al. 2016). Subsequently, several assays were performed to elucidate structural and molecular characteristics of ML-CAP, as depicted in Table 1. At first, total carbohydrate content was estimated where the amount was found to be better than water extracted polysaccharides from *Auricularia auricula*, *Lentinus edodes*, and *Poria cocos* (Khaskheli et al. 2018). Alongside, small amount of protein was also recorded and the quantity was found to be superior to hot water extracted polymers from *Russula alatoreticula* (Khatua et al. 2017a) but lower than alkali extracted polysaccharides from *Russula senecis* (Khatua and Acharya 2017).

The observation was further verified by FT-IR to measure molecular vibrations, corresponding to covalent polysaccharide bonds (Fig. 1a). The spectrum exhibited a broad as well as intense hydroxyl group stretching band at 3435 cm^{-1} and a weak C–H-stretching peak at around 2922 cm^{-1}, which

Table 1 Extractive yield and chemical characterization of ML-CAP

Parameters	ML-CAP
Yield (%)	2.63 ± 0.63
Carbohydrate (g per 100 g of dry polysaccharide)	63.35 ± 0.61
Protein (g per 100 g of dry polysaccharide)	8.3 ± 0.3
Total glucan (g per 100 g of dry polysaccharide)	43.54 ± 1.41
α-Glucan (g per 100 g of dry polysaccharide)	0.08 ± 0.01
β-Glucan (g per 100 g of dry polysaccharide)	43.46 ± 1.42
Homogenity and molecular weight	Homopolysaccharide, ~ 121.88 kDa
Monosaccharide composition	Xylose: rhamnose: mannose: glucose: galactose = 7:3:7:50:33

are characteristics of polysaccharides (Zhang et al. 2018). The absorbance at 1641 cm^{-1} could be assigned to amides I, related to the elongation vibrations of groups C=O and CN (Barbosa et al. 2020). The signal at 1154 cm^{-1} was might be due to C–O–C asymmetric stretching of glycosidic linkage. While, the band at 1072 cm^{-1} indicated C–O stretching of β-glucans (Ma et al. 2018). The weak absorption near 893 cm^{-1} might be due to asymmetric retractive vibration of β-pyranose signifying β-configuration of sugar units (Nie et al. 2019). Overall, ML-CAP was found to be mainly consisted of carbohydrate conjugated with minute amount of protein. Interestingly, such polysaccharide–protein complexes are often noted for their immunomodulatory benefits (Ayimbila and Keawsompong 2021).

The homogeneity of ML-CAP was further tested following GPC, a powerful analytical technique used to separate dissolved molecules by size. As presented in Fig. 1b, the profile showed a dominant, sharp and single peak at tube number 22 indicating the fraction to be consisted of homo-polymer. Moreover, the Mw was calculated as around 122 kDa according to the calibration curve equation. The observation was in accordance to *Boletus edulis* where the average Mw of polysaccharides ranged from 10^2 to 10^3 kDa (You et al. 2014). In contrast, Mw of the polysaccharide obtained from *Lentinus fusipes* and *Pleurotus sajor-caju* was 60 and 79 kDa, respectively (Seedevi et al. 2019). Liu et al. (2014) isolated water and alkali soluble polysaccharides from *Russula vinosa* and enumerated average Mw as 87.1 and 107 kDa, respectively, indicating NaOH solution aids in extraction of higher Mw polymers.

To estimate glucan content, a spectrophotometric assay was performed and the results portrayed pre-dominance of β-glucan in ML-CAP. The findings were further verified by performing sophisticated chromatographic techniques. Both HPTLC (Fig. 1c) and GC–MS (Fig. 1d), chromatograms supported the spectroscopic findings where the gas chromatographic profile revealed presence of additional sugar monomers apart from glucose. As such, galactose was enumerated as the second most abundant unit followed by xylose and mannose; while rhamnose was identified to be present in minute amount. The monosaccharide profile was similar to hot water extracted polysaccharides from *M. lobayensis* (Ghosh et al. 2019) and *Macrocybe gigantea* (Khatua and Acharya 2016).

Estimation of antioxidant potential

To estimate antioxidant ability of ML-CAP, the polymer was subjected to three in vitro systems and the outcome has been summarized in Table 2. At first, the method of chelating ability of ferrous ions was performed as iron, a catalyst, can generate free radicals via Fenton and Haber–Weiss methods. Presence of a chelating medium such as polysaccharides interrupts the formation of iron (II)–ferrozine complexes and diminishes the purple tone indicating antioxidant effect of the investigated drug (Khatua et al. 2017b). As can be

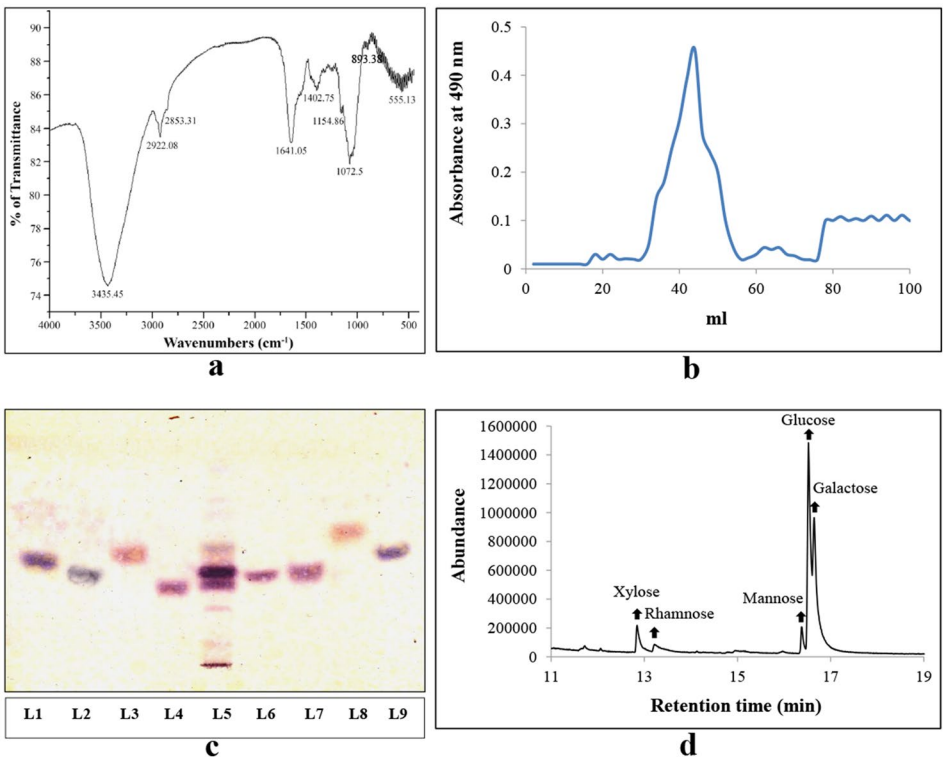

Fig. 1 Enumeration of structural features of ML-CAP. **a** FT-IR spectroscopy. **b** Estimation of homogeneity and molecular weight. **c** Identification of monomers by HPTLC (lanes are as follows: 1, L-arabinose; 2, D-fructose; 3, D-fucose; 4, D-galactose; 5, ML-CAP; 6, D-glucose; 7, D-mannose; 8, D-rhamnose; and 9, D-xylose). **d** GC–MS

seen from Fig. 2a, ML-CAP displayed a dosage-dependent metal ion-binding affinity. At the concentrations of 100 and 300 µg/ml, the chelating ability of ML-CAP was enumerated as 13.93% and 30.8%, respectively, which increased to 50.69% in presence of 500 µg/ml. Thus, EC_{50} value of the extract was found to be lower than the polysaccharides isolated by four different methods from *Lactarius vividus* (Xu et al. 2019) indicating better potential of ML-CAP.

Besides, ABTS radical scavenging assay was also performed being a widely followed method to determine antioxidant capacity of an investigating drug. The radical ($ABTS^{\bullet+}$) is generated by treating ABTS solution with potassium persulfate resulting dark blue-colored solution. In presence of the antioxidative sample, color intensity of the mixture is changed to colorlessness, and thus, absorption decreases (Khatua and Acharya 2016). As shown in Fig. 2b, the radical scavenging activities of ML-CAP also increased in a concentration-dependent manner. At the level of 1, 2, and 3 mg/ml, the polymers quenched 20.32%, 44.36%, and 60.72% $ABTS^{\bullet+}$, respectively. Comparison with previous reports revealed lower activity of *M. lobayensis* than water extracted crude polysaccharide fraction from *Oudemansiella radicata* (Wang et al. 2018).

Moreover, total antioxidant capacity was also determined for quantitative estimation of antioxidant capacity, through formation of phosphomolybdenum complex. The protocol is based on reduction of Mo (VI) to Mo (V) by the sample under investigation and subsequent formation of a green phosphate Mo (V) complex at acidic pH (Alam et al. 2013). At per the result, ML-CAP exhibited moderate activity which was found to be better than *Russula pesudocyanoxantha* (Khatua et al. 2021b). In our previous work, hot water extracted polysaccharide from *M. lobayensis* presented 5.3 µg ascorbic acid equivalent bioactivity/mg of extract (Ghosh et al 2019) depicting its higher activity than that of the studied polysaccharide fraction. Literature survey also unveiled that ML-CAP presented lower activity than *M. gigantea* (Khatua and Acharya 2016) as well as *Termitomyces medius* (Mitra et al. 2021) in terms of total antioxidant capacity assay.

Determination of immune-stimulatory activity

Macrophages act as the front line of host defense and can kill pathogens immediately after recognition. Ingredients that can provoke function of these phylogenetically conserved cells are considered as effective immune booster agents (Khatua et al. 2022). Keeping this in mind, RAW264.7 cells were treated with ML-CAP at a range of concentrations to investigate whether the polymers can influence functionality of the monocytes. WST results (Fig. 3a) showed that the fraction at the concentrations of 50 and 100 µg/ml can augment cell viability by 105.29% and 116.44%, respectively, over negative control within one day of incubation. Further treatment for another 24 h amplified the propagation rate by 146.89% and 144.83% at the above-mentioned levels indicating time-dependent effect of the isolated fraction. However, the rate of cell proliferation dropped somewhat when the concentration was incremented to 200 µg/ml. On the other hand, LPS, an acknowledged stimulant for immune system, alleviated 128.36% and 148.65% proliferation frequency after 24 and 48 h treatment, respectively. Overall,

Table 2 Antioxidant activity of ML-CAP

Antioxidant parameters		ML-CAP	Standards
EC_{50} values (µg/ml)	Chelating ability of ferrous ion	480 ± 12^{a}	2.51 ± 0.43^{b}
	ABTS radical scavenging assay	2320 ± 28^{a}	3.2 ± 0.08^{b}
Total antioxidant activity (µg ascorbic acid equivalent per mg of dry extract)		2.82 ± 0.94	Not applicable

Ascorbic acid and EDTA were considered as standards in ABTS radical scavenging and chelating ability of ferrous ion methods, respectively

[a, b] In each row, dissimilar letters designate significant alterations between ML-CAP and standard ($p < 0.05$)

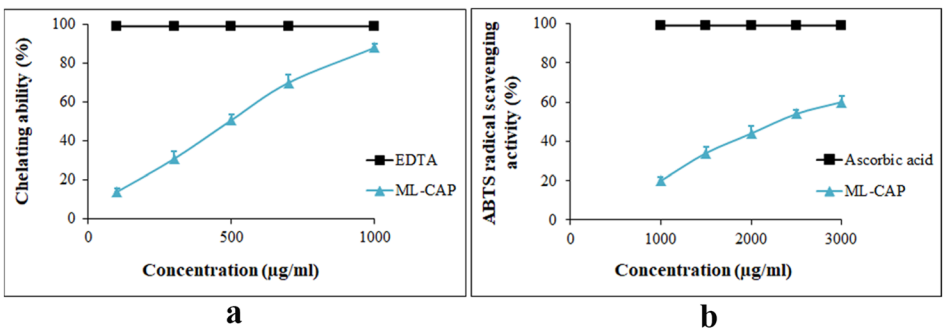

Fig. 2 Antioxidant activity of ML-CAP was determined as follows: **a** chelating ability of ferrous ion and **b** ABTS radical quenching effect

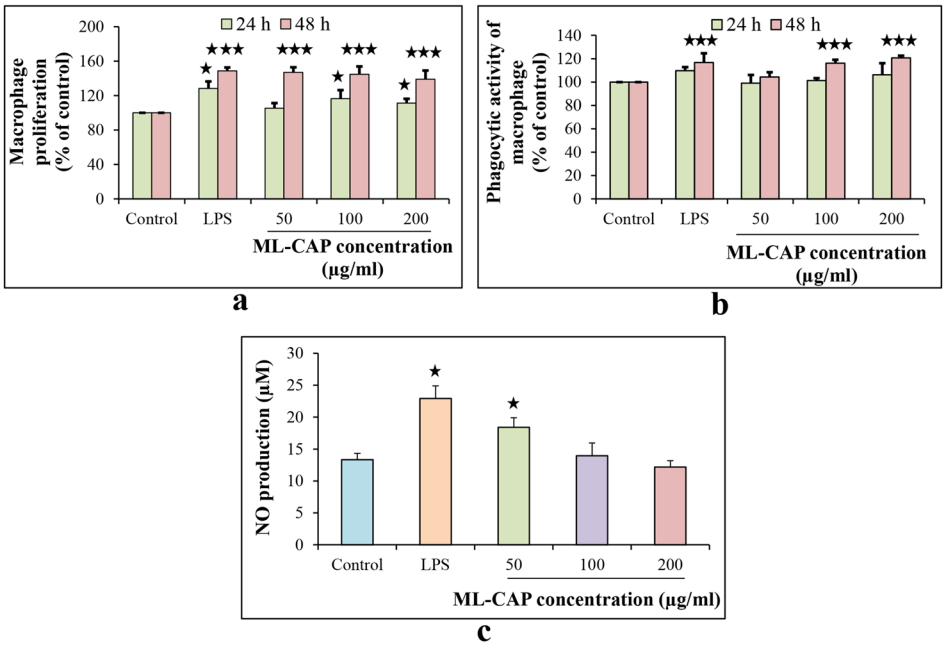

Fig. 3 The influence of ML-CAP on **a** proliferation, **b** phagocytic uptake, and **C** NO production by RAW 264.7 cells was monitored after treatment of polysaccharides at different concentrations. In all assays, 5 µg/ml of LPS was used as a positive control. $*p < 0.05$ and $***p < 0.001$ (unpaired *t* test)

the outcome clearly suggested that ML-CAP at the investigated doses were non-toxic to RAW264.7 cells and may possess immune eliciting activity. Comparative study with previous literature indicated better activity of ML-CAP from *M. lobayensis* than the polysaccharide from *Russula griseocarnosa* (Chen et al. 2018).

Alongside, neutral red assay was also performed to determine effect of ML-CAP on phagocytic effect of the macrophage cells. As such, activated monocytes are known to produce membrane protrusions surrounding the pathogens and absorb the foreign particles into phagosome (Hirayama et al. 2017). Thus, increase in the ingestion power of macrophage cells represents a definite sign of immune-boosting effect of the drug under investigation. As shown in Fig. 3b, treatment of ML-CAP at the levels of 100 and 200 µg/ml augments engulfment capacity by 101.23% and 106.17%, respectively, over negative control within 24 h. When the incubation period was extended for another 24 h, the effect was recorded to increase by 104.35%, 116.08% and 120.6% in presence of 50, 100 and 200 µg/ml concentrations, respectively signifying a dose- and time-dependent activity of ML-CAP. Intriguingly, the outcome was found to be superior to the cold alkali extracted polysaccharide fraction from *R. alatoreticula* (Khatua and Acharya 2019) advocating strong potential of ML-CAP as an immune booster.

Apart from phagocytosis, activated macrophages can also resist pathogens by synthesizing certain pro-inflammatory factors such as NO. Level of this important regulator and mediator thus can reflect the status of immune response (Lan et al. 2021). In this context, Griess reagent was used to elucidate effect of ML-CAP on NO production by RAW264.7 cells within 24 h. Results showed that a minimum level of NO production, i.e., 13 µM in the culture supernatants of macrophages, incubated with medium alone. Whereas, when treated with ML-CAP, particularly at the level of 50 µg/ml, the level of NO was significantly increased compared with that of negative control (Fig. 3c). However, the higher doses could not trigger NO production and the observation was in according to Pacheco-Sánchez et al. (2007) showing polysaccharide from *Collybia dryophila* reduced NO production at higher doses.

In the process of pathogen elimination, morphology of macrophage cells is changed due to formation of pseudopods from cell surface. To determine effect of ML-CAP on structural features of the monocytes, RAW264.7 cells were incubated with the polymers for 24 h and then observed under microscope. As presented in Fig. 4, the unstimulated cells appeared regular in size with smooth surface and round shape. However, incubation of the fraction at the concentration of 50 µg/ml caused increase in cell size with irregular shape and production of several microvilli-like structures from cell surface which was probably in relation to more adherence and activation of macrophages. The effect was more prominent at higher doses, particularly in presence of 100 µg/ml representing a hallmark of immune-boosting effect of ML-CAP. The result was in accordance to several previous findings (Khatua et al. 2022, 2021a, b; Mitra et al. 2021; Ghosh et al. 2019).

Conclusion

In sum, a carbohydrate enriched fraction was successfully isolated using residue of *M. lobayensis* that has passed through hot water process to widen application of the

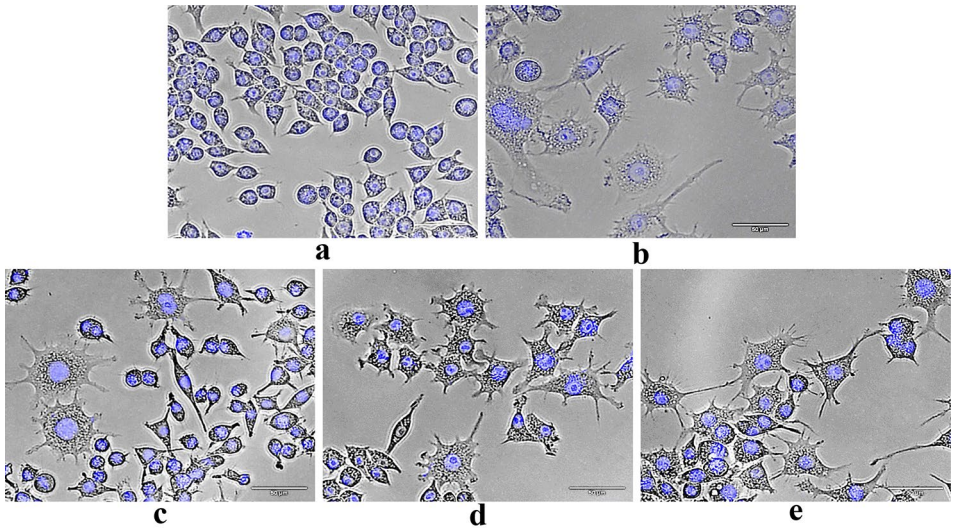

Fig. 4 The effect of ML-CAP on morphology of murine macrophages was visualized under a fluorescence microscope. **a** Negative control. **b** LPS (5 µg/ml) and ML-CAP at the concentrations of **c** 50 µg/ml, **d** 100 µg/ml, and **e** 200 µg/ml. Scale 50 µM

traditionally edible mushroom. Consequently, the fraction executed good antioxidant and promising immune-boosting effect. Research on chemical account unveiled presence of a single polymer of relatively high Mw where β-linked glucose was the chief component. Overall, the work suggests that ML-CAP possesses significant health benefits that should further be investigated to improve the quality of life.

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Author contributions SK: data curation; formal analysis; investigation; methodology; writing original draft; writing—review & editing. KA: conceptualization; resources; supervision; validation; writing—review & editing.

Declarations

Conflict of interest The authors declare that there are no conflicts of interest.

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