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Original paper

Characterization of exopolysaccharide produced by *Ganoderma* sp. TP and its immunomodulatory properties

PRIYA S. VADNERKER[1], TRUPTI K VYAS[1]*, CHINTAN V KAPADIA[2]

[1]Food Quality Testing Laboratory, N M College of Agriculture, Navsari Agricultural University, Navsari – 396450, India,
[2]Department of Plant Molecular Biology and Biotechnology, Aspee College of Horticulture and Forestry, Navsari Agricultural University, Navsari. 396450. Gujarat. India

Abstract

Mushrooms have an excellent nutritional value and are referred to as healthy food. They are a good source of carbohydrates, proteins, vitamins, and minerals while they are low in fat content. Mushrooms are well known as rich sources of nutraceutical molecules also. They produce exopolysaccharides (EPS), having high antioxidant activity. Hence, the present study aims to characterize a mushroom exopolysaccharide for its antioxidant and antitumor activity. A mushroom sample was collected from the Navsari Agricultural University, Navsari campus (20.9302° N, 72.9127° E). The sample was identified by morphological and 18S rRNA as Ganoderma sp TP (Accession No. MF614913). EPS was extracted from the sample, and total carbohydrate 258.3±1.4 µg/ml content was measured. Chemical characterization by FTIR, TLC, HPLC, and NMR analysis revealed that arabinose, lactose, and ribose monomer were present in the structure. TGA analysis revealed 86.34 % weight loss after the second stage. Rheological analysis suggested it is dilatants in nature. Antioxidant activity measured by ABTS and DPPH showed 84.09 % and 86.45% antioxidant activity, respectively. Antitumor activity was examined using the MDA-MB-231 cell line and found 63.49% cell inhibition compared to control. Thus, EPS produced by Ganoderma sp TP is novel from other reported EPS and positively affects tumor cell growth reduction.

Keywords Antioxidant, Antitumor, Breast cancer, Exopolysaccharides, *Ganoderma*

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*Corresponding author: Trupti K Vyas: vyastrupti@hotmail.com, https://orcid.org/0000-0002-1168-7552

Introduction

Medicinal mushrooms are used for their health-promoting properties and nutritional value and as a possible complementary for treating diseases. Many mushrooms are well known for their medicinal properties. India, China, Japan, Korea, and some other South-Eastern countries have used it for 3000 years as a medicinal mushroom in a number of diseases[1]. Molecules, which confer medicinal and or nutritional properties to mushrooms, are called bioactive molecules. Some mushroom metabolites, including polysaccharides, glycoprotein, and proteoglycans, have the potential to modulate immune system response and inhibit tumor growth[2, 3, 4, 5], whereas; other shows potential antiviral, antibacterial, antiparasitic, anti-inflammatory, and anti-diabetic properties[6].

Exopolysaccharides (EPS), high-molecular-weight carbohydrates secreted by an organism into the surrounding environment, are composed of sugar residues. The Biochemical properties of the EPSs depend on the primary structure of the EPS. The kind of glycosidic bonds and monomer types are important in determining their medicinal properties. Many medicinal mushrooms produce exopolysaccharides which have been very well reported for antioxidant activity, superoxide radical scavenging, reducing properties, lipid peroxidation inhibition, suppression of proliferation and oxidative stress, etc. Polysaccharides isolated from different mushrooms like *Agaricus, Calocybe, Ganoderma, Grifola, Inonotus, Lentinus, Phellinus, Pholiota, Pleurotus,* etc., are also capable of providing antitumor activity[7].

Cancer remains one of the significant causes of death worldwide. The most effective treatment strategy for cancer is cytotoxic agent-based chemotherapy, which increases patient survival and has side effects that severely limit its clinical effectiveness, such as the acquisition of drug resistance. Consequently, an alternative option is required to combat the side effect produced by chemotherapy. Hence, the present study aims to characterize EPS for its antioxidant and antitumor activity.

Materials and Methods

Collection and isolation of mushroom sample:

The mushroom sample used in present studies was collected from Navsari Agricultural University, Navsari campus (20.9302° N, 72.9127° E). The isolation of mycelium was carried out on potato dextrose agar (PDA) plates. For the isolation, a small piece of mushroom was inoculated in the PDA plates in aseptic condition and plates were incubated at 25°C for 48-72 hours. Subsequently culture was purified and stored on PDA slant at 4°C until the use.

Identification of mushroom

The morphological characterization was carried out macroscopically by observing growth patterns of fungi and microscopically by wet mounting of fungi in lactophenol. For molecular characterization DNA from mushroom sample was extracted according to the methods described by Graham and coworkers [8]. Amplification of genomic DNA was done by ITS1 and ITS4 primers. PCR conditions were set as described by Kheni and Vyas [2]. Sequence was explored for homology and submitted to GenBank.

Extraction of EPS

For EPS extraction, the sample was dried at 60 °C in a hot air oven to remove water content. Fine powder was prepared from dried samples using a blender for EPS extraction. Three different methods were used for EPS extraction i.e., hot water extraction [9] ethanol extraction and methanol extraction [10]. Among the different methods used, the extraction method which produced higher EPS was used for further work.

Quantification of carbohydrate content

The carbohydrate of crude sample and extracted EPS was measured using the method mentioned by Dubois and coworker [11].

Characterization of EPS

For chemical characterization, sample was partially purified as described by Kheni and Vyas[2]. After partial purification, characterization of EPS was carried out by FTIR, TLC, HPLC, NMR and TGA analysis.

FT-IR

For characterization of EPS, dried EPS powder was ground with KBr and pressed to obtain pellets. Infrared absorption spectra (Nicolet IR200 FT-IR Spectrometer) were recorded on a FT-IR in the 4000-400 cm-1 range. KBr pellet was used as the background reference.

Thin Layer Chromatography [12]

The sugars were detected by spotting the sample on silica gel 60 (F254 Merck, Germany) using ethyl acetate: acetic acid:methanol:distilled water (60:15:15:10) as solvent 12. The TLC plate was sprayed with anisaldehyde-sulfuric acid reagent followed by heating at 100°C for 5-10 minutes for the development of colored spots. Type of sugar present in the sample was identified by comparing the Rf value of standard sugar runs along with the sample.

HPLC (High Performance Liquid Chromatography)

Hydrolyzed EPS sample was analyzed using an analytical HPLC coupled with an RI detector[2]. 0.1% H_2SO_4 in

water was used as a mobile phase and flow rate was 0.5 ml/min. Analysis. The monomers in EPS were detected by an RI detector.

NMR

To confirm the chemical structure, 1H NMR and 13C NMR analysis were carried out. Analysis was carried out at Central Salt & Marine Chemical Research (CSMCRI), Council of Scientific and Industrial Research (CSIR), Bhavnagar, India.

TGA analysis

Thermogravimetric analysis (TGA) of the sample was also carried out in the N2 atmosphere at the heating rate of 10 ºC min. The pyrolysis pattern of the EPS was investigated using a differential scanning calorimeter. The energy level was scanned from 50 to 450 ºC in N atmosphere.

Rheology

One of the important features of EPS is rheological behavior, which has great impact in manufacturing, thickening and stabilization and has potential industrial application. Flow property of EPS solution was determined using Physica MCR 301 (Anton Paar) at shear rate from 0.01 s^{-1} to 196 1000 s^{-1} was applies for 5 mins at 25 °C.

Antioxidant activity

To estimate the antioxidant activity, three different methods viz. Ferric Reducing Antioxidant Power (FRAP) assay, ABTS assay and DPPH assay were used. Antioxidant activity by FRAP assay was measured as a method described by Benzie and Strain[13] and the result was expressed as FeII µmol/gm. ABTS assay was measured as a method of Re and coworker[14] (1999). Antioxidant activity by DPPH assay was measured as mentioned by Garcia and Co-workers [15].

Antitumor Activity

Antitumor activity was measured in vitro by cell growth inhibitory activity against the human breast cancer cell lines (MDA-MB-231) by MTT assay[16]. For the assay, the cells were grown in tissue culture flasks in Leibovitz medium at 37 °C. 105cells/ml was transferred to each well in 96-well microtiter plate. The cells were allowed to grow for 24 hrs and then treated with the sample. 100 µl test sample (concentration range from 100 – 1000 µg/ml) was added to the wells and cells were further incubated for another 24 hrs at 37 °C in an incubator. 20 µl MTT (5 mg/ml in phosphate buffered saline) was added after incubation to each well and cells were further allowed to grow for 4 hrs. Media was removed afterwards and100 µl dimethyl sulfoxide (DMSO) was added to each well. The absorbance was measured on a microplate reader (Multiskan, Thermo Scientific) at 570 nm. Experiment was done in triplicate. Control comprised cell growth without a test sample. The inhibition percentage was calculated using the following formula:

$$\text{Inhibition activity (\%)} = [(\text{Acontrol} - \text{Asample})/\text{Acontrol}] \times 100$$

Where, Acontrol was the absorbance of the control reaction and Asample is the absorbance of the of the sample.

Results and Discussion

Collection and isolation of Mushrooms

A mushroom sample was collected from Navsari Agricultural University, Navsari, Gujarat, India. A portion of the mushroom sample was taken for isolation and purification of mushroom. The rest of the biomass was dried at 60 ºC. Isolation and subsequent purification were done, and pure culture was stored on a PDA slant at 4 ºC until use.

Identification of mushroom

The pure culture was used to study the morphological characteristic of the isolate. The isolate showed secondary mycelia and clam connection when observed under a microscope with lactophenol cotton blue mounting stain. The ITS region of 18S rRNA was amplified with ITS 1 and ITS 4 primers to identify the isolate. It produced a characteristic amplicon of approximately 650bp (Fig 1a). Amplified PCR product was sequenced at Saffron Life Sciences, India, and resulted in sequenced data being aligned using the NCBI blast tool. It showed homology with *Ganoderma* sp. Hence, it is named *Ganoderma* sp TP (Fig 1b). The sequence data was submitted to GenBank with accession number MF614913.

Extraction of EPS

EPS from the mushroom sample was extracted using three different solvents for higher EPS recovery. EPS was extracted by hot water, methanol, and ethanol extraction methods. Maximum EPS was extracted using ethanol ex-

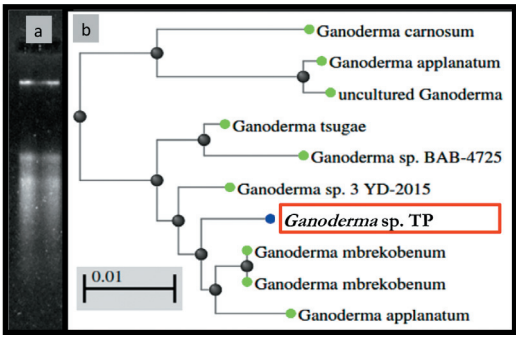

Figure 1. DNA of mushroom (a) phylogenetic tree of *Ganoderma* sp TP (b)

traction (13.47 mg/g) followed by methanol (11.63 mg/g). Hot water extraction extracted the least EPS (8.3 mg/g). Ethanol extraction showed higher EPS production; hence, it was used for further studies.

Quantification of carbohydrate content

To find out the amount of carbohydrate present in the sample, 1 mg/ml of crude mushroom sample and extracted EPS were separately analyzed by phenol sulphuric acid method for carbohydrate content. Carbohydrate content was 48.65±0.07 (µg/ml) and 258.3±1.4 (µg/ml), respectively, in mushroom and EPS samples.

Characterization of EPS

Various analyses like FTIR, TLC, HPLC, NMR TGA were carried out to know the monomers of EPS.

FTIR analysis

The FTIR spectrum of an unhydrolyzed sample of Ganoderma sp. TP EPS (Fig 2) showed the characteristic absorbance of polysaccharides. The absorption between 3426-3000 cm-1 indicates the O-H stretching vibration of the polysaccharide. The absorption band at 2924 cm-1 suggests C–H stretching vibration. The instance absorption between 1150-900 cm-1 is characteristic of carbohydrates and corresponds to stretching vibrations of C–C, C–O–C, and C–O occurring in glucopyranose structure. Thus, IR spectra confirmed the presence of sugar.

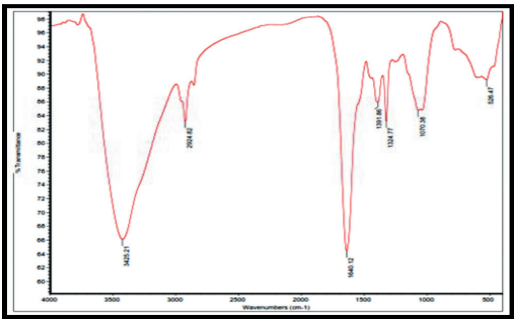

Figure 2. FTIR spectrum of EPS *Ganoderma* sp TP

TLC analysis

To identify monomers of the EPS sample, partial purification of EPS was done using acid hydrolysis. Hydrolysis was monitored using TLC analysis at 1 hr intervals. The product was completely hydrolyzed after 4 hrs of incubation in a boiling water bath. Hydrolyzed product was neutralized with sodium bicarbonate and used further for TLC analysis and HPLC analysis. Three different spots were detected on a thin layer chromatogram and had similar Rf values with standard sugar arabinose, lactose, and ribose.

HPLC analysis

A partially purified sample was analyzed for the presence of monomers in the EPS structure. Samples containing arabinose, lactose, and ribose were detected, matching standard sugars. TLC results also corroborate with HPLC analysis.

NMR

EPS sample was characterized by 1H NMR and 13C NMR analysis. In 1H NMR peak between 3.03 – 3.64 indicates the sugar proton, whereas d 4.83 indicates the anomeric carbon (Fig 3a). In 13C NMR peak at 63.657 indicated the presence of C-6. The peak at 72.577 showed a large amount of terminal C-6 branching at C-2 with β (1®3) linkage. The peak at 106.15 indicates the anomeric carbon, whereas the peak at 51.75 indicates amine and confirms the glycoprotein structure (Fig 3b).

TGA analysis

EPS produced by isolate was characterized by TGA and DSC analysis. Results of TGA analysis showed that EPS from Ganoderma sp. TP takes place in two steps. Initially, 76.84 % weight loss was observed up to 140 ºC with an onset of 91 ºC. After 140 ºC, there is slow degradation observed with continuous exotherm. The second weight loss 9.50 % was it up to 300 ºC and after 300 ºC. In the first step, high % weight loss was due to moisture content and carboxyl group, which increased bond degradation with water molecules. Second stage degradation at 300 ºC corresponds to a pyrolysis temperature. Total weight loss was 86.34 % after the second stage.

Rheology

Shear stress and viscosity of EPS produced by Ganoderma sp. TP was characterized by a rheometer. Results revealed that it is non-Newtonian shear thickening, Dilatant in nature as (n) is greater than one when fitted in the "power-law model". Hence it can also be used as a thickening agent.

Antioxidant activity

Antioxidant activity of EPS was measured using FRAP assay. If molecules have the antioxidant ability, it converts FeIII molecule to FeII. Results revealed that EPS has 15.300 mM/gm antioxidant activities. The second assay used for antioxidant activity was the ABTS assay. EPS showed 84.09 % inhibition of radical ABTS molecules. The third assay used for measuring antioxidant activity was the DPPH assay. In this assay, EPS showed 86.45% antioxidant activity.

Antitumor activity

Antitumor activity was measured by cell viability using an MTT assay. The assay is based on the cleavage of the tetrazolium salt MTT in the presence of an electron-coupling reagent. To examine antitumor activity, MDA-MB-231 cells

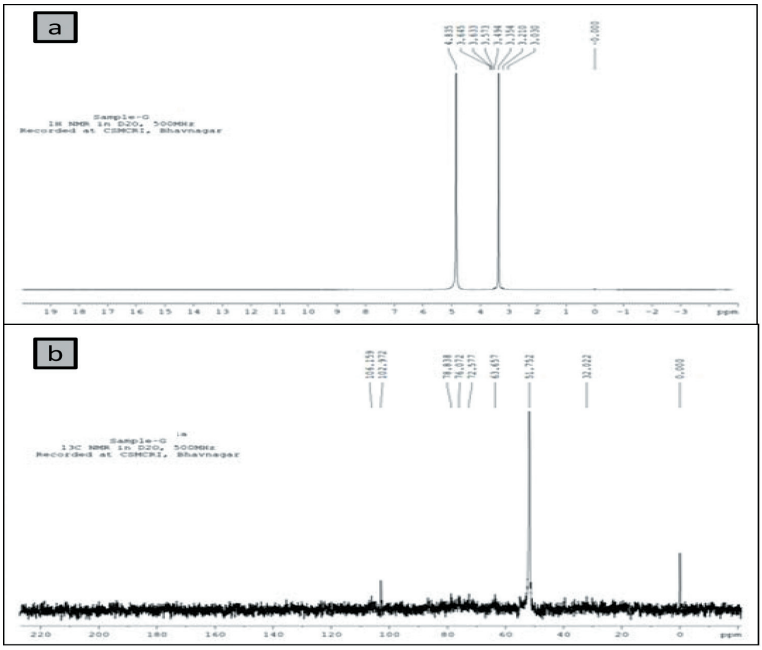

Figure 3. ^{1}H NMR (a) and ^{13}C NMR (b) spectrum of EPS produced by *Ganoderma* sp. TP

were grown in Leibovitz media (Fig 4a). For MTT assay, cells were grown in 96 well ELISA plates for 24 hrs. After 24 hrs of incubation, different concentrations of EPS (0.1 – 1 mg/ml) were added, and cells were further incubated for 24 hrs (Fig 4b). MTT solution was added in 96-well ELISA plates containing pre-grown cells with different EPS concentrations and incubated for 4 hrs. Formazan produced after this incubation period was quantified using a scanning multi-well spectrophotometer ELISA reader. The measured absorbance directly correlates to the number of viable cells. Cells were lysed after 24 hrs of incubation, as shown in Figure 4c. When most of the cells lysed, clumps of EPS were also observed after 24 hours. In control, all wells showed higher formazan production, whereas, in test samples, formazan color production decreased gradually as EPS concentration increased. Cell inhibition was started at a minimum concentration of 0.1 mg/ml of EPS concentration, and maximum cell inhibition of 63.49 % was observed at 0.9 mg/ml concentration.

Human beings are equipped with immune defense systems to neutralize free radicals as free radicals could damage macromolecules, resulting in serious diseases. Many researchers have been done exploring potential natural antioxidants to reduce oxidative damage. Mushrooms are one such source of sustainable bioactive compounds that have been consumed for thousands of years. Polysaccharides are important bioactive components produced by many mushrooms.

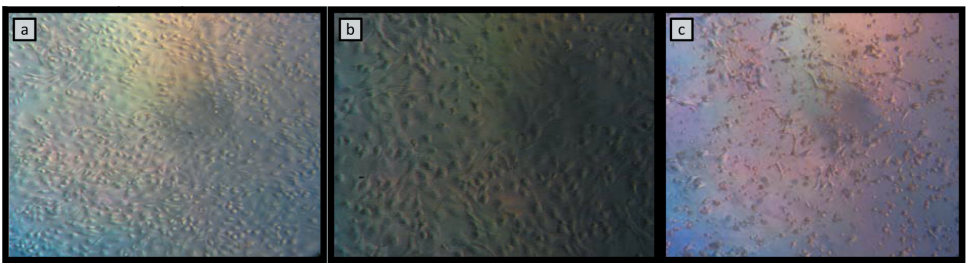

Figure 4. Normal growth of MDA-MB 231 breast cancer cell line (a), cell line with addition of EPS (b) Cell lysis in presence of EPS after 24-hour incubation (c)

In the present study, the mushroom was identified by 18S rDNA as *Ganoderma* sp. TP and submitted to Genbank. Various methods were selected for the extraction of EPS. Among three different methods used, maximum EPS was recovered by ethanol extraction followed by methanol and hot water extraction. Ethanol extraction showed a higher extractive index, and the results are in accordance with many published data [17, 18, 19]. EPS extracted using ethanol extraction method from *Ganoderma* sp. TP showed that it produced 13.47mg/g EPS. EPS contained 258.3±1.4µg/mg of carbohydrate. Sasidhara and Bakki [20] reported 300 mg/g of carbohydrates extracted from *Ganoderma lucidum*, which is higher than our results. EPS production was lower in *Ganoderma* sp. TP compared to *Ganoderma applanatum* (241.8 mg/g) whereas carbohydrate content 303 ± 1.29 mg/g is almost similar as reported by Osinska-Jaroszuk and coworker [21]. Ubaidillah and coworker [22] reported 115.89% total carbohydrate in EPS extracted from *G. neojaponicum*, which is higher than EPS from *Ganoderma* sp.

Polysaccharides are made up of monomeric sugar linked with glycosidic bonds. EPS structure may be homopolymer or heteropolymer, either linear or containing branched side chains linked with β- (1→6) glycosidic bonds [18]. Due to the versatile nature of EPS, it carries much biological information and helps identify structural diversity. These diverse structures provide regulatory mechanisms for cell-cell interactions in higher organisms. FTIR analysis helps in the identification of characteristic bonds present in carbohydrates. Various peaks present in the molecules confirm the carbohydrate structure. Our data are in accordance with the reported EPS structure by FTIR analysis [23, 24, 25].

TLC and HPLC analysis of the exopolysaccharide extracted from *Ganoderma* sp. TP, showed the presence of arabinose, lactose, and ribose monomers. The polysaccharides isolated from *Ganoderma* constitute glucose, mannose, galactose, fucose, xylose, and arabinose, with eight combinations and different types of glycosidic linkages which can be bound to protein or peptide residues [2, 26, 27, 28, 29, 30, 31]. Fraga and coworkers (2014) reported the presence of glucose, galactose, and mannose in the ratio of 69–93%, 4–19%, 2–8%, and 1–5%, respectively, in the EPS extracted from *Ganoderma lucidum* [32]. EPS extracted from *Ganoderma lucidum* contained glucose, galactose, mannose, arabinose, and rhamnose in a ratio of 332:55:32:13:3, respectively [33]. Thus, it showed a different unique structure of EPS.

EPS structure from *Ganoderma sinense* was characterized by NMR spectroscopy and revealed that it contained mannose, glucosamine, glucuronic acid, N-acetyl- ß-D-glucosamine, glucose, and galactose as EPS monomers [25]. Antioxidant activities of different mushrooms were evaluated previously by several researchers using various methods. Methods such as 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging, ferric reducing antioxidant power (FRAP), hydroxyl radical scavenging, superoxide radical scavenging, nitric oxide (NO) scavenging, and lipid peroxide inhibition assays were commonly used to determine the antioxidant activities of polysaccharides from various kinds of samples [34, 35, 36, 37(pp. 119 –132)]. The result of antioxidant activity by EPS suggests that it has almost similar activity by ABTS and DPPH assay. Mahendran and coworkers [38] reported lower antioxidant activity 63.75 ± 2.47 % inhibition by ABTS assay from *Ganoderma lucidum*, DPPH radical scavenging ability of crude EPS of *G. lucidum* (82.3 ± 3.27 %) was similar to the DPPH scavenging activity of *Ganoderma* sp. TP (84.58%) [38]. *G. lucidum* showed 50% inhibition by 0.055 mg/ml of ethanolic extracted EPS using the DPPH assay, which is lower than reported here [39].

Here, EPS extracted from *Ganoderma* sp. TP was evaluated on the MDA-MB-231 breast cancer cell line by MTT assay showed 63.49% inhibition of the tumor cell line at 0.9 mg/ml EPS concentration. Polysaccharides extracted from *G. lucidum* showed 80.8% and 77.6% reduction in tumor volume and tumor mass, respectively, of Ehrlich's ascites carcinoma cells [40]. Polysaccharides PG-1 and PG-2 from *G. lucidum* have immunomodulatory properties and showed an inhibitory effect on the growth of a human breast cancer cell line MDA-MB-23141. Their results are in accordance with our findings.

Ganoderma sinense produces a polysaccharide (GSRBPs) effective on the H1299 NSCLC cell line of non-small-cell lung cancer (NSCLC) [41]. Thus, polysaccharide GSRBPs can be effectively used to inhibit H1299 non-small-cell lung cancer. A similar study also revealed that EPS extracted from *Ganoderma* sp. had been reported for its anticancer activity against lung cancer line Murine Lewis lung carcinoma (LLC1) [42]. In one study, the sandwich structured antitumor composite was designed with three layers comprising the first top and bottom layers made from ethyl cellulose-containing triterpenes from *Ganoderma lucidum*; the second layer of polyvinyl alcohol have polysaccharide from G. lucidum [43]. Carcinoma cells, such as SGC-7901, A549, Hela, and Caco-2 were studied in vitro for antitumor activity of the synthesized sandwich drug and found to have IC50 of 51.2, 90.7, 93.0, and 21.7 µg/mL, respectively. Not only antitumor but exopolysaccharide from *Ganoderma* sp. has also been reported for their antioxidant, antiviral, anti-inflammatory, and immunomodulatory effects [44, 45]

Conclusion

Ganoderma sp TP (Accession No. MF614913) contains 258.3±1.4 µg/ml carbohydrate in extracted exopolysaccharide molecules. Chemical characterization of EPS by various analyses revealed that *Ganoderma* sp TP containing novel monomer arabinose, lactose, and ribose monomer were present in the structure. TGA analysis revealed 86.34 % weight loss after the second stage. EPS possesses higher antioxidant activity measured by ABTS and DPPH suggests that it can scavenge free radicals. EPS was able to suppress tumor cell lines, as evidenced by 63.49 % cell inhibition of the MDA-MB-231 cell line. Hence, EPS produced by *Ganoderma* sp TP which is novel than other reported EPS from *Ganoderma* sp., can be used as a potential immunomodulator. However, detailed in vivo studies required for antitumor activity and clinical trials will be further helpful in insight mechanism as an antitumor agent. Thus, EPS extracted from *Ganoderma* sp TP can be used as a food supplement to boost immunity.

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Conflicts of Interest

The authors declare no conflict of interest.

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