Malaysian Journal of Analytical Sciences (MJAS)
Published by Malaysian Analytical Sciences Society

QUANTIFICATIONS OF LINOLEIC ACID AND β-GLUCAN IN *Lignosus rhinocerotis* EXTRACT AND ITS CYTOTOXICITY ANALYSIS IN HUMAN LUNGS EPITHELIAL BEAS-2B CELLS

(Kuantifikasi Asid Linoleik dan β-Glukan dalam Ekstrak *Lignosus rhinocerotis* dan Analisis Kesitotoksikan dalam Sel Epitelium Bronkus Manusia BEAS-2B)

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Received: 26 February 2023; Accepted: 23 July 2023; Published: 22 August 2023

Abstract

Lignosus rhinocerotis, a polypore found in the tropical forests of Malaysia is traditionally used by the locals to treat various diseases. The objectives of the study were to quantify linoleic acid and β-glucan, characterize polysaccharide structure as well as to evaluate the cytotoxicity effects of *L. rhinocerotis* extracts. The linoleic acid and β-glucan concentrations in six different extracts of *L. rhinocerotis* were quantified using HPLC and β-glucan assay kit, respectively and the polysaccharide structure were analyzed using FTIR and XRD. The proliferation rate of human bronchial epithelial BEAS-2B cells treated with the *L. rhinocerotis* extracts were also analyzed. The highest linolic acid content and β-glucan composition were found in petroleum ether extract and hexane residue, respectively. Cold water and petroleum ether extracts showed cytotoxic effect with 30% reduction of cell viability at 72 hours at the concentration of 62.5-250 µg/mL while the other extracts are not cytotoxic. Quantification of linoleic acid and β-glucan as well as characterization of polysaccharide structure in *L. rhinocerotis* extracts have been successfully determined. The observed effects on BEAS-2B cells are likely attributable to the immunomodulatory properties exhibited by the active compounds of linoleic acid and β-glucan.

Keywords: *Lignosus rhinocerotis*, linoleic acid, beta-glucan, high performance liquid chromatography, cytotoxicity

Abstrak

Lignosus rhinocerotis merupakan polipor yang terdapat di hutan tropika Malaysia telah digunakan secara tradisi oleh penduduk tempatan untuk merawat pelbagai penyakit. Kajian ini bertujuan untuk mengukur tahap asid linoleik dan β-glukan, mencirikan struktur polisakarida serta menilai kesan sitotoksisiti ekstrak *L. rhinocerotis*. Tahap asid linoleik dan β-glukan di dalam enam ekstrak *L. rhinocerotis* yang berlainan masing-masing diukur dengan kaedah HPLC dan kit ujian mezayme yis dan cendawan β-glukan. Struktur polisakarida juga dianalisa menggunakankan kaedah FTIR dan XRD. Titisan sel epitelium bronkus manusia

BEAS-2B dikultur dan dirawat dengan ekstrak *L. rhinocerotis* dan ujian kesitotoksikan telah dijalankan. Petroleum eter menghasilkan jumlah asid linoleik yang paling tinggi, manakala ekstrak residu heksana menghasilkan komposisi β-glukan tertinggi. Ekstrak air sejuk dan petroleum eter menunjukkan kesan sitotoksik ringan pada kepekatan 62.5-250.0 µg/mL dengan penurunan kadar kebolehhidupan sel adalah tidak melebihi 30% manakala kebanyakan ekstrak lain tidak sitotoksik. Tahap asid linoleik dan β-glukan serta pencirian struktur polisakarida di dalam ekstrak *L. rhinocerotis* telah berjaya ditentukan. Kesan ekstrak ini terhadap titisan sel epitelium bronkus manusia BEAS-2B juga telah berjaya dikaji dan berkemungkinan disebabkan oleh sifat imunomodulator yang ditunjukkan oleh sebatian aktif, iaitu asid linoleik dan β-glukan.

Kata kunci: *Lignosus rhinocerotis*, asid linoleic, β-glukan, kromatografi cecair berprestasi tinggi, kesitotoksikan

Introduction

Lignosus rhinocerotis (*L. rhinocerotis*) or domestically known as tiger milk mushroom by the local community in Malaysia, is a polypore found in the tropical rainforests in Malaysia. *L. rhinocerotis* is traditionally used to reduce body temperature, as a postpartum medicine for women after childbirth, as a contraceptive and in the treatment of breast and cervical cancers [1], asthma, cough, joint pain, food poisoning, liver-related illnesses as well as a general tonic [2].

Besides, *L. rhinocerotis* is believed to possess anti-inflammatory activities against inflammatory mediators during inflammation. This action may be contributed by the high amounts of active compounds found in this fungus, primarily the polysaccharide of β-glucans and the polyunsaturated fatty acid of linoleic acid. β-glucans are mostly derived from the fruiting bodies of mushrooms, and it is believed to be an immune system booster where it has demonstrated to effectively stimulate the host immune response against microbial infections [3]. Additionally, β-glucan is also used as an adjuvant to enhance the effectiveness of modern medicine [4]. Meanwhile, linoleic acid which is an omega-6 polyunsaturated fatty acid; a precursor for arachidonic acid which is often associated with inflammation [5]. The low levels of pro-inflammatory cytokines such as TNF-α and IL-6 and the high levels of anti-inflammatory cytokines including IL-10 was reported in corresponding to the presence of polyunsaturated fatty acid in the plasma, suggesting its effect in inflammation [6]. In our previous study, the volatile compositions of *L. rhinocerotis* in hot water extract showed that linoleic was the one with the most abundant polyunsaturated fatty acids when analyzed through gas chromatography-mass spectrometry (21.35%) [7].

Numerous studies have reported on the cytotoxicity of L. *rhinocerotis* extracts. According to Fauzi et al. [8], *L. rhinocerotis* is reported to induce apoptosis in HCT 116 colon cancer cells. *L. rhinocerotis* was also found to induce apoptosis that led to cell death in the human breast carcinoma cell line, MCF-7 and the human lung carcinoma cell line, A549 [9]. In this study, human lungs epithelial BEAS-2B cells was chosen because of its role in conserving the normal airway function by acting as defensive barrier and secreting the inflammatory mediator in response to the inflammation. BEAS-2B is widely used as a pulmonary epithelium model in many types of in vitro studies including toxicology testing [10], respiratory injury and remodeling [11].

Thus, the objectives of the study were to quantify linoleic acid and β-glucan as well as to characterize polysaccharide and crystalline structure in *L. rhinocerotis* extracts. The cytotoxicity of the extracts in human lungs epithelial BEAS-2B cells was also evaluated.

Materials and Methods

Sample preparations

The samples were prepared by dissolving *L. rhinocerotis* powder in methanol. Water-based extract such as hot water, cold water, hexane residue and petroleum ether residue extracts were prepared at 8 mg/mL while the concentration for solvent-based extracts such as hexane and petroleum ether extracts were at 100 µg/mL. Table 1 shows three different extraction methods used for the extraction of *L. rhinocerotis*.

HPLC analysis for linoleic acid

A reversed phase high performance liquid chromatography (HPLC) was performed on an Agilent

1260 Infinity System (Agilent Technologies, Santa Clara, USA). Instrument control and data collection were carried out using an Agilent OpenLab ChemStation Edition C.01.05 software. The column used was COSMOSIL 5C18-MS-II (250 X 4.6 mm I.D: 5 µm) (Nacalai Tesque, Inc, Japan) coupled with a guard column (10 X 4.6 mm I.D: 5 µm) (Nacalai Tesque, Inc, Japan) with similar packing material. The detection was done by an ultraviolet detector at 208 nm. The mobile phase used was a combination of acetonitrile, methanol, and hexane in the ratio of 90:8:2 with the addition of 0.2% acetic acid at a flow rate of 1.0 mL/min. Indomethacin was used as internal standard. The total analytical time was approximately 10 minutes. HPLC method validation were performed according to FDA guideline [11] to evaluate calibration curves, linearity, precision, accuracy, and recovery.

Quantification of β-glucan

Quantification of β-glucan in *L. rhinocerotis* extracts was conducted using mushroom and yeast Beta-Glucan Assay Kits (Megazyme, Bray, Ireland) where both total and alpha glucans were measured. Concentrations of β-glucan were determined by subtracting the concentrations of alpha glucan from the total values.

Table 1. Different extraction methods for the extraction of *L. rhinocerotis*

Extraction Method	Soxhlet	Maceration	Ultrasound-Assisted Extraction
Step by step procedures	1. TMM powder (50 mg) was transferred into an extraction thimble. 2. Distilled water (500 ml) was put into a round bottom flask. Soxhlet extraction set was set up. 3. The extract was then concentrated using a rotating evaporator. 4. The concentrated extract was dried, weighed, and stored in a tightly capped container for further use.	1. TMM powder (50 g) was macerated in 500 ml of water for 24 hours in cold room (4 °C) with agitation. 2. After 24 hours, the extract was centrifuged at 2500 g for 10 min. 3. The supernatant was collected and dried using freeze dryer.	1. TMM powder (50 mg) was dissolved in 500 ml of solvents and transferred into a water bath sonicator for 30 min at 40 °C. 2. The solution was centrifuged at 2500 g for 10 min. Then, the supernatant was concentrated, dried and stored. 3. The pellet was resuspended in 500 ml of water before being subjected to ultrasound in a water bath sonicator for another 30 min prior to centrifugation at 2500 g for 10 min. 4. The supernatant was collected, concentrated, dried, weighed and stored.
Product	Hot water extract	Cold water extract	Step 1-2: hexane and petroleum ether extract. Step 3-4: hexane residue and petroleum ether residue extract.

Determination of polysaccharide structural analysis

Fourier-transform infrared spectroscopy (FTIR) was used to determine polysaccharide structural analysis in *L. rhinocerotis* extracts. Infrared radiation (IR) spectra of *L. rhinocerotis* crude extract was performed using KBr-disk method where the sample was ground with KBr powder and pressed into 1 mm pallet. The pallet was analyzed using FTIR spectrometer (Tensor 27, Bruker, Germany) spectrometer in the range 4000-400 cm^{-1}.

Determination of crystalline structure

The X-ray diffraction (XRD) of *L. rhinocerotis* crude extract was analyzed using diffractometer (D2 Phaser, Bruker, Germany). Copper lamp radiation (Cu Kα) was applied to the sample at 10mA and 30 kV with rotation speed of 15.0 s. The diffraction angle was shifted from 10º to 90º to identify the crystalline structure.

BEAS-2B cell culture

BEAS-2B was purchased from American Type Culture Collection (Manassas, USA) (resource number: ATCC® CRL 9609™). The cells were cultured in a complete Dulbecco Modified Eagle's Medium (DMEM) at 37oC and 5% carbon dioxide humidified atmosphere. Complete DMEM was prepared by adding 1% penicillin-streptomycin and 10% fetal bovine serum. Cells from passage 6 to 8 were used in all experiment.

Proliferation assay

BEAS-2B cells were seeded at 2 x 103 cells per well. After 24 hours, the cells were treated with six different extracts: hot water, cold water, hexane, petroleum ether, hexane residue and petroleum ether residue extract at various concentrations (250, 125, 62.5, 31.25 and 15.625 µg/mL, respectively) for 24, 48 and 72 hours. An untreated negative control was included in the study. Proliferation assay was carried out using MTS assay kit (Promega, USA) following the manufacturer's instruction.

Statistical analysis

Statistical analyses were performed using the Statistical Package of Social Science software, version 21.0. A one-way ANOVA was used to analyze the findings. All statistical analyses were performed at significance level of $p < 0.05$.

Results and Discussion

Chromatography

A typical chromatogram of the analysis of linoleic acid in *L. rhinocerotis* extracts obtained on COSMOSIL 5C18-MS-II column, is shown in Figure 1. The calibration curve for linoleic acid was linear in the concentration range of 5 to 200 µg/mL. The inter- and intra-day calibration curves showed consistent linearity, as seen in consistency of intercept, slope and coefficient of correlation. A typical concentration curve for linoleic acid had a slope of 0.0053, an intercept of 0.0005 and r^2 = 0.9978 (Figure 2).

The precision of the test was evaluated by determining the relative standard deviation (RSD) of the measured peak area ratios for different drug concentrations. RSD was calculated as follows: $\bar{x}$ = mean of peak area ratio and s = standard deviation of peak area ratio. Based on the FDA guideline [12], the acceptable precision for each concentration is RSD < 15% except for the limit of detection concentration which is at 20%. Accuracy was expressed as the mean percentage of analyte recovered in the assay with a targeted acceptable range within 80-120%. Our data indicated that the method is both precise (relative standard deviation less than 15%) and accurate (mean percentage recovery of between 80-120%).

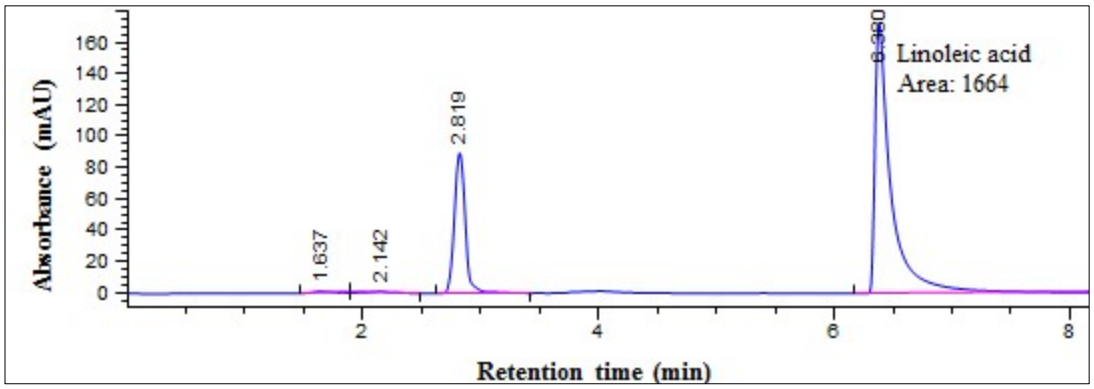

Figure 1. Chromatogram of linoleic acid (6.680 min) and internal standard, indomethacin (2.819 min) when using combinations of acetonitrile, methanol and hexane for mobile phase in the ratio of 90:8:2 with the addition of 0.2% acetic acid at a flow rate of 1.0 mL/min. The detection was done by an ultraviolet detector at 208 nm.

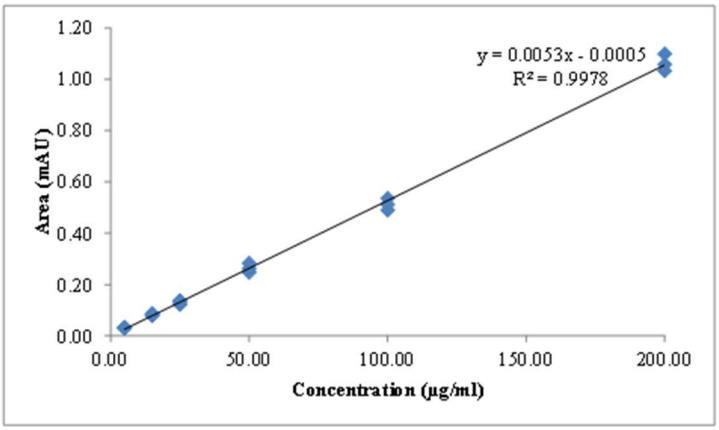

Figure 2. A typical concentration curve for linoleic acid

Measurement of linoleic acid in *L. rhinocerotis* extracts

Table 2 shows the amount of linoleic acid in 100g of the extracts. Based on the results, water-based extracts such as hot water, cold water, hexane residue and petroleum ether residue extracts recorded a low amount of linoleic acid which is approximately 0.05g in 100g of extract. The non-polar extracts such as hexane and petroleum ether yielded significantly higher amounts of linoleic acid which are 16.79 and 17.55g in 100g of extract, respectively.

Table 2. Measurement of linoleic acid in *L. rhinocerotis* extracts

Extract	Mean Linoleic Acid Content in Extract (g) ± Standard Deviation
Hot water	0.049 ± 0.277
Cold water	0.049 ± 0.115
Hexane	16.790 ± 0.629*
Petroleum ether	17.550 ± 0.221*
Hexane residue	0.046 ± 0.113
Petroleum ether residue	0.048 ± 0.133

*p <0.05

Analysis of β-glucan in *L. rhinocerotis* extracts

Analysis of β-glucan composition in water-based *L. rhinocerotis* extracts indicated that it is present at 29.0, 33.6, 39.8 and 32.1g in 100g extracts of hot water, cold water, hexane residue and petroleum ether residue extracts, respectively. On the other hand, the amount of β-glucan in non-polar extracts such as hexane and petroleum ether extracts were 31.5 and 24.1g, respectively. Hexane residue is the best extract for β-glucan (Table 3).

Table 2. Measurement of β-glucan in *L. rhinocerotis* extracts

Extract	Mean β-Glucan Content in Extract (g) ± Standard Deviation
Hot water	29.0 ± 0.001*
Cold water	33.6 ± 0.006*
Hexane	31.5 ± 0.001*
Petroleum ether	24.1 ± 0.001*
Hexane residue	39.8 ± 0.002*
Petroleum ether residue	32.1 ± 0.004*

*p <0.05

Analysis of the FTIR spectrum

The FTIR spectrum of *L. rhinocerotis* crude extract was shown in Figure 3a. A broad band observed at 3423 cm^{-1} is attributed to –OH group stretching vibration in the polysaccharide or moisture of sample [13]. The band 2931 cm^{-1} is attributed to the C-H stretching vibration which include CH, CH_2 and CH_3 stretching, which are the characteristic absorptions of polysaccharides. The band between 1000 and 1200 cm^{-1} region indicated the presence of C-O stretching vibration of C-O-H and C-O-C glycosidic bonds are also characteristic of polysaccharides. An intense band in the region 1639 cm^{-1} is associated to the C=O stretching vibration of amide I group [14].

X-ray diffraction (XRD)

The crystallinity of the *L. rhinocerotis* crude extract was evaluated and the diffractogram pattern is shown in the Figure 3B. The sample shows diffraction peak at 13.8, 20.5º, 21.2, 25.7 and 33.9º 2θ (Degrees) of the crystallinity. The computed crystallinity is at 19.5% and amorphous is at 80.5% within 10 to 90º 2θ.

The effect of extract on cell viability

Analysis of cell proliferative activity was conducted by MTS in BEAS-2B treated with various concentrations (250, 125, 62.5, 31.25 and 15.625 µg/mL) of *L. rhinocerotis* extracts after 24, 48 and 72 hours of incubation is shown in Figure 4. The viability of cell treated with hot water extract, hexane residue extract and petroleum ether residue extract did not show any significant different after 3-day treatment. However, petroleum ether extract at the concentration of ⩾ 31.25 µg/mL showed significant reduction throughout the 3-day treatment. Cell treated with cold water extract at the concentration of 125 and 250 µg/mL also significantly reduced, starting from the second day of the treatment. Reduction in cell viability could also be seen in cell treated with hexane extract (125 and 250 µg/mL) on day 3.

Mushrooms are rich in polyunsaturated fatty acids especially linoleic acids. Our results indicated that linoleic acid concentration was significantly lower in water-based extracts than in solvent-based extracts which extracted a higher number of compounds. Hexane and petroleum ether which are non-polar, can pull out non-polar compounds [15] in extraction of fatty acids from flaxseed oil. The reason is that the organic solvents used for the extraction process were specific for the extraction of fatty acids. It is plausible that the non-polar solvents such as hexane and petroleum ether attract the long hydrocarbon alkane chain present in fatty acids. The effect was similarly shown in another study on the extraction of fatty acids from flaxseed oil where non-polar solvents such as hexane and petroleum ether fatty acids were found to be better at extracting fatty acids as compared to polar solvents [15].

Linoleic acid is the most abundant polyunsaturated fatty

acid in *L. rhinocerotis* and represents about 21.35% of total volatile compounds [7]. A study on fatty acid analysis from nine wild edible mushrooms (*Aleurodiscus vitellinus, Cortinarius magellanicus, Hydropus dusenii, Cyttaria hariotii, Fistulina antarctica, F. endoxantha, Grifola gargal, Lepista nuda and Ramaria patagonica*) from Argentina indicated that linoleic acid was the predominant fatty acid found in the investigated mushroom species [16]. Another study on the phytochemical analysis of the *Rhizopogon luteolus* showed that eight fatty acids were detected in *R. luteolus* with linoleic acid, making up 45.8% of the total fatty acids present [17]. Twenty-eight fatty acids were detected in 16 strains of *Pleurotus ostreatus* mushroom lipids where the linoleic acid was predominant in all samples (56.8–80.5%) [18], indicating that it is an important constituent to be analyzed in mushroom samples.

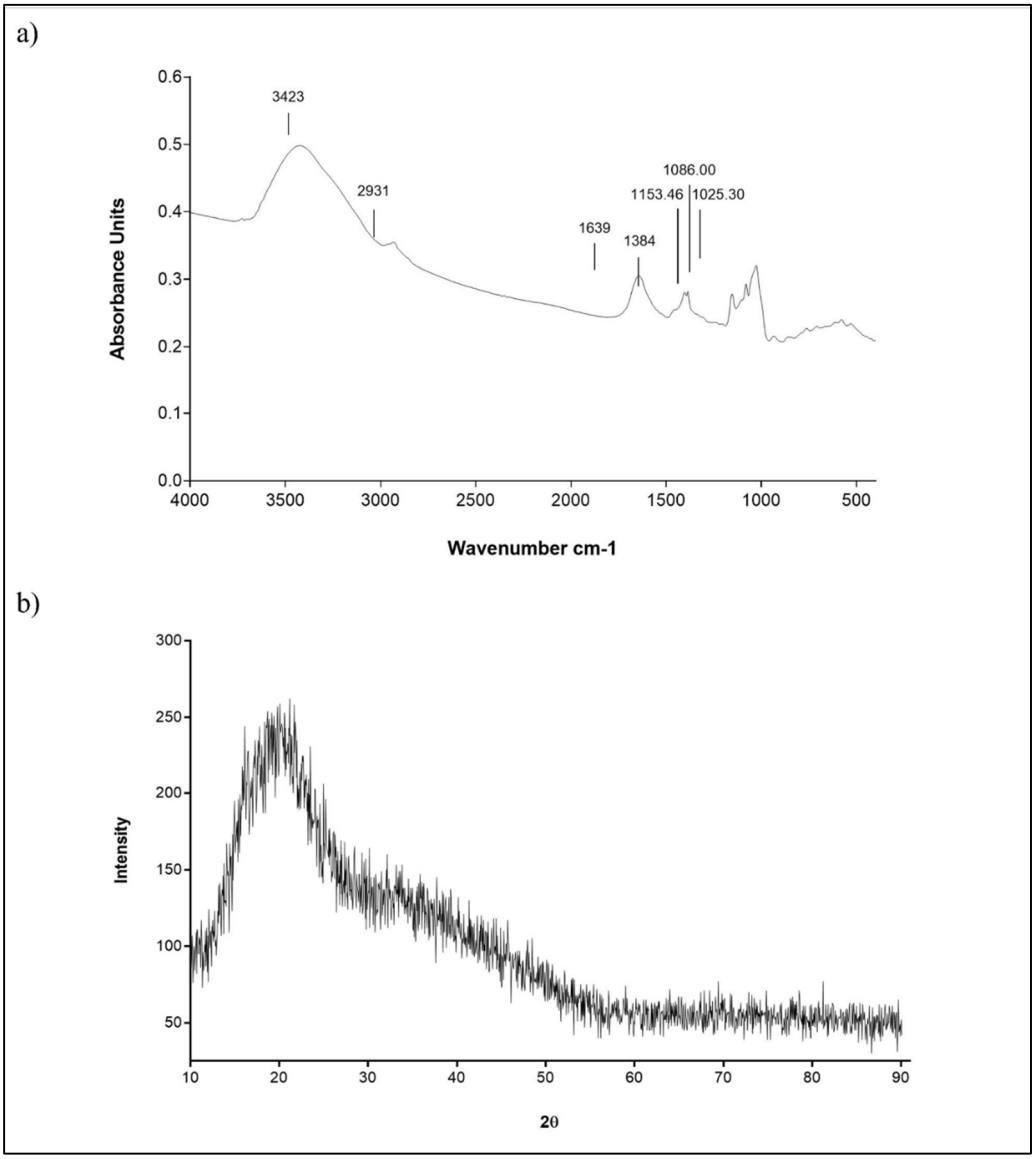

Figure 3. Analyses of (a) FTIR spectra and (b) XRD of *L. rhinocerotis* crude extract

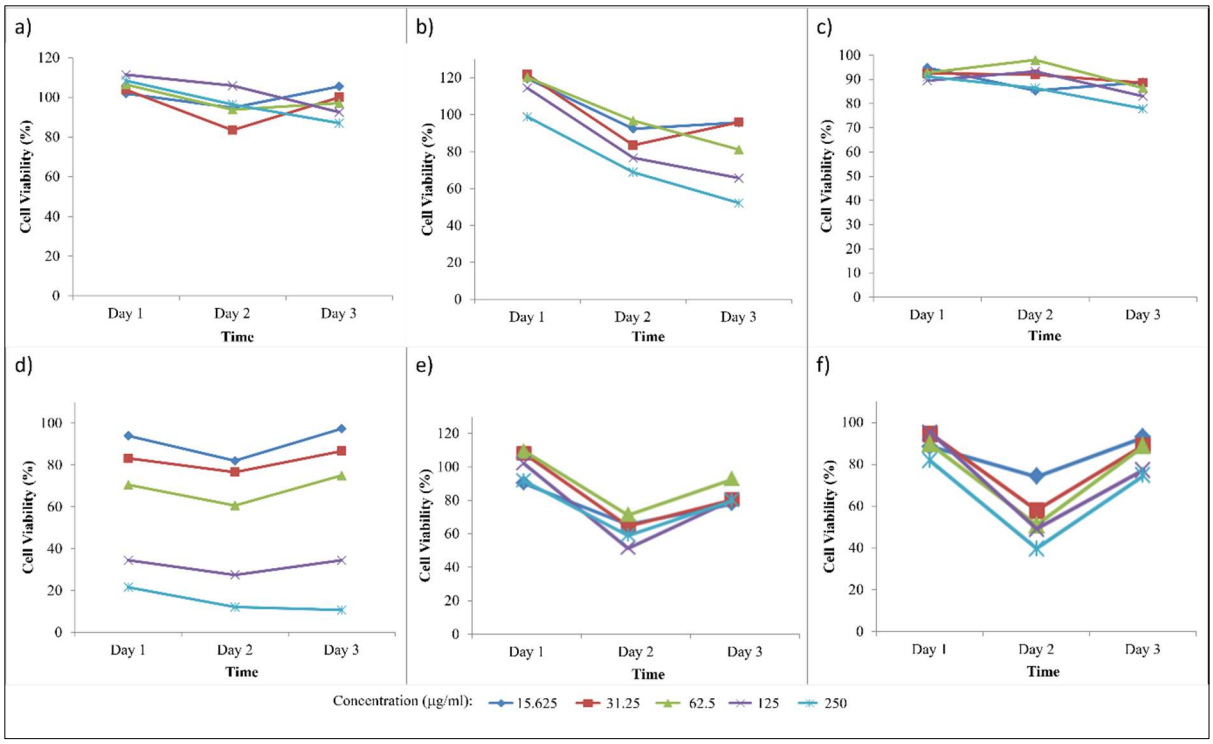

Figure 4. The effects of various concentrations of a) hot water, b) cold water, c) hexane, d) petroleum ether, e) hexane residue and f) petroleum ether residue extracts on viability of BEAS-2B cell line. Untreated cells were used as a control. The differences between concentrations of each extract were tested statistically using Kruskal-Wallis. Data are presented as mean ± standard deviation for three independent experiments. *$p < 0.05$ vs control.

There are several studies reported on the β-glucan composition in *L. rhinocerotis* with conflicting data. Jamil et al. [19] reported that β-glucan constituted more than 50% of glucan content in *L. rhinocerotis* while another study Lau et. al. [1] reported that β-glucan constituted the majority of glucan, ranging from 82 to 93% (w/w). However, another study on the β-glucans content stated that β-glucan constituted only 9% of total glucan compositions [20]. Meanwhile, the data obtained from this study shows that β-glucans make up more than 20% of total glucan in all extracts. Nevertheless, the difference in β-glucan content may be attributed to the different extraction method used in each study.

The peak characteristic of FTIR spectrum suggested the presence of both polysaccharide and protein. The absorption bands of O-H, C-H and C-O groups represent the typical polysaccharide characteristic in FTIR [21]. The amide group is characterized by the peptide bond of secondary structure of protein [22]. The absorption bands of O-H, C-H and C-O groups represent the typical polysaccharide characteristic in FTIR [21]. Previous studies reported the presence of polysaccharides as the major constituents in *L. rhinocerotis* extracts [23]. The XRD result confirms the existence of both broad peak amorphous behavior and a small crystalline structure region in *L. rhinocerotis* crude extract which is very common in nature. The profile suggested the sample is composed of monodisperse particle. Several studies also reported the similar profile pattern [9,24,25].

In this study, cold water and petroleum ether extracts between 62.5 and 250.0 µg/ml show cytotoxic effect since the reduction of cell viability at 72 hours is more than 30%. Other extracts did not show cytotoxic effect against BEAS-2B cell line. The data is consistent with a previous study on the anti-proliferative effect of the cold sclerotia of *L. rhinocerotis* [9]. Lee et al. [9]

hypothesized that the cytotoxic effect of the cold-water extract was due to the presence of thermolabile compounds in cold water extract which were absent in hot water extract. The study reported that cytotoxic cold-water extracts contained more proteins than non-cytotoxic hot water extract, suggesting that the cytotoxic activity might be attributed to the additional proteins in the cold-water extract. In the case of cytotoxic effect shown by petroleum ether extract, it might be attributed to the presence of alkaloids and terpenoids that might be present in the extract since petroleum ether has the ability to extract these compounds [26]. In fact, many plants that are reported to have toxic properties usually contain certain terpenoid and alkaloids [27]. Interestingly, *L. rhinocerotis* extracts did not confer any apoptotic effect on normal lungs epithelial cell line, BEAS-2B. It is plausible that the *L. rhinocerotis* extracts used in the study do not cause harmful effect to the normal cell line.

Conclusion

In conclusion, quantification of linoleic acid in hot water, cold water, hexane, petroleum ether, hexane residue and petroleum ether residue extract of *L. rhinocerotis* has been successfully determined using HPLC. In addition, the amount of β-glucan in *L. rhinocerotis* extract has also been successfully quantified using the optimized method. The characterization of polysaccharide and crystalline structure in *L. rhinocerotis* extracts have been also successfully determined. All extracts (especially at high concentration, 125 and 250 µg/mL) showed reduced proliferative activities as compared to control at 72 hours; although the majority of the extracts were not cytotoxic to BEAS-2B cell line. The observed effects are likely due to the high content of immunomodulatory active compounds, such as linoleic acid and β-glucan, present in the extract.

Acknowledgement

This study was supported by the Universiti Sains Malaysia Research University Grant (Grant No: 1001/PPSK/8012344).

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