This Issue is Dedicated to
Professor Dr Wilhelm Fleischhacker
On the Occasion of his 85th Birthday

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Identification and *in vitro* Evaluation of Lipids from Sclerotia of *Lignosus rhinocerotis* for Antioxidant and Anti-neuroinflammatory Activities

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**Lignosus rhinocerotis** (Cooke) Ryvarden (Tiger milk mushroom) is traditionally used to treat inflammation triggered symptoms and illnesses such as cough, fever and asthma. The present study evaluated the *in vitro* antioxidant, cytotoxic and anti-neuroinflammatory activities of the extract and fractions of sclerotia powder of *L. rhinocerotis* on brain microglial (BV2) cells. The ethyl acetate fraction had a total phenolic content of 0.30 ± 0.11 mg GAE/g. This fraction had ferric reducing capacity of 61.8 ± 1.8 mg FSE/g, ABTS•+ scavenging activity of 36.8 ± 1.8 mg TE/g and DPPH free radical scavenging activity of 21.8% ± 0.7. At doses ranging from 0.1 µg/mL – 100 µg/mL, the extract and fractions were not cytotoxic to BV2 cells. At 100 µg/mL, the crude hydroethanolic powder of *L. rhinocerotis* had ferric reducing capacity of 61.8 ± 1.8 mg FSE/g, ABTS•+ scavenging activity of 36.8 ± 1.8 mg TE/g and DPPH free radical scavenging activity of 21.8% ± 0.7. At doses ranging from 0.1 µg/mL – 100 µg/mL, the extract and fractions were not cytotoxic to BV2 cells. At 100 µg/mL, the crude hydroethanolic extract had the highest ferric reducing ability of 68.7% and 58.2%, respectively. Linoleic and oleic acids were the major lipid constituents in the ethyl acetate fraction based on FID and GC-MS analysis. Linoleic acid reduced nitric oxide production and down regulated the expression of neuroinflammatory iNOS and COX2 genes in BV2 cells.

**Keywords:** Anti-neuroinflammation, *Lignosus rhinocerotis*, BV2 Cells, Lipid component, Linoleic acid, Oleic acid, Antioxidant.

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Mushrooms are consumed globally and are valued not only for their unique taste and flavor but also for their high medicinal and nutritional properties [1]. In recent years, the search for mushrooms is focused on ethnomedicinal knowledge. In Malaysia, the indigenous communities use many species of mushrooms such as *Amauroderma* sp., *Lignosus rhinocerotis* (Cooke) Ryvarden, *Pycnoporus sanguineus* (L.) Murrill and *Termitomyces clypeatus* (R.) Heim as food and/or medicine [2]. Many of these species are used to treat a number of ailments related to inflammation such as fever, cough, cold, epilepsy and asthma [2].

*L. rhinocerotis* can be found in small geographic regions encompassing South China, Thailand, Malaysia, Indonesia, Philippines, Papua New Guinea, New Zealand, and Australia [3]. In Malaysia, this mushroom is also known as “cendawan susu rimau”, which translates to tiger milk mushroom. *L. rhinocerotis* has more than 15 traditional uses including treatment or prevention of cancer, fever, cough, asthma, hunger, food poisoning, wounds and it is also used as a general tonic [4]. Asthma, fever and cough are attributes of inflammation.

Recent *in vitro* and *in vivo* studies supported the medicinal properties including the anti-inflammatory activities of the aqueous extract *L. rhinocerotis* (Table 1). To date, however, there are no reports on the anti-neuroinflammatory activities of solvent extracts. Therefore, this study aimed to investigate the hydroethanolic extraction of the sclerotia of *L. rhinocerotis* and its fractions. The extracts and fractions were examined to determine their chemical composition, antioxidant activities, cytotoxicity and effect on nitric oxide production in microglial cells.

<table>
<thead>
<tr>
<th>Medicinal properties</th>
<th>Active extracts</th>
<th>Cell line</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-cancer</td>
<td>cold aqueous</td>
<td>breast (MCF 7) and lung (A549 cancer leukemia (HL-60, K562 and THP-1))</td>
<td>[5]</td>
</tr>
<tr>
<td>Immunomodulating</td>
<td>Polysaccharides</td>
<td>Immune cells</td>
<td>[7]</td>
</tr>
<tr>
<td>Neurite outgrowth</td>
<td>hot aqueous</td>
<td>PC 12</td>
<td>[8,9]</td>
</tr>
<tr>
<td>Antioxidant</td>
<td>cold aqueous, hot aqueous and methanol</td>
<td>N2a and BALB/3T3</td>
<td>[10]</td>
</tr>
<tr>
<td>Anti-inflammatory</td>
<td>cold aqueous, hot aqueous and methanol</td>
<td>carrageen induced paw edema in Sprague Dawley rats</td>
<td>[12]</td>
</tr>
</tbody>
</table>

The highest TPC was observed in the crude hydroethanolic extract (0.4 ± 0.1 mg GAE/g extract). However, the *n*-hexane and ethyl acetate fractions contained low TPC of 0.1 ± 0.0 and 0.3 ± 0.1 mg GAE/g extract, respectively. The FRAP assay showed that the hydroethanolic extract had the highest ferric reducing ability of 122.6 ± 4.8 mmol FSE/g extract. The ABTS•+ scavenging activity was expressed in terms of TEAC. The higher the TEAC value, the more potent the radical scavenging effect. The most potent radical
scavenger was the hydroethanolic extract with 86.5 ± 4 mg TE/g and the least potent extract was the n-hexane fraction with only 30.9 ± 5.3 mg TE/g. At a concentration of 5 mg/mL, the hydroethanolic extract showed the highest DPPH radical scavenging activity of 29.4 ±1.7% followed by the ethyl acetate fraction (21.8±0.7%) and n-hexane fraction (17.2 ±1.2%).

This study demonstrated that the hydroethanolic extract and its fractions (n-hexane and ethyl acetate fractions) possessed free radical reduction and scavenging activities. However, each extract showed different in vitro assay patterns, probably due to the different mechanisms involved in the steps of the oxidation process. Some studies found a correlation between the phenolic content and the antioxidant activities, while others did not [13-15].

In this study the hydroethanolic extract and ethyl acetate fraction showed high ABTS+ (36-86 mg TE/g extract) and DPPH (21-30%) scavenging activities and free radical reduction (61-122 mg FE/g extract), but the antioxidant activities were not correlated with its total phenolic content. Hassimotto et al. [13] also reported that the antioxidant activity of vegetable and fruit extracts did not correlate with either phenolics or vitamin C content. Thus, other non-phenolic compounds such as fatty acids may also be responsible for the antioxidant activity observed in the hydroethanolic extract and ethyl acetate fraction. Li et al. [16] also reported that the ethanolic extract of Corpinus comatus mushroom possessed higher antioxidant activity compared with its hot water extract.

The effects of various concentrations of the hydroethanolic extract and fractions of L. rhinocerotis on the viability of BV2 cells determined by the MTS assay are given in Figure 1. The cell viability of the positive control (untreated BV2 cells) was denoted as 100%. The crude extract/fractions were not cytotoxic to BV2 cells at concentrations up to 100 μg/mL. However, at 1000 μg/mL all extracts tested were cytotoxic to the BV2 cells. A dose-dependent increase in the viability of cells treated with the extracts was observed at concentrations ranging from 0.1 to 100 μg/mL followed by a dose-dependent decrease from 100 to 1000 μg/mL. An increase of 4-10% in viable cell number was seen in BV2 cells treated with 10 μg/mL of each extract tested. However, there was no significant (p<0.05) difference in cytotoxic effects at concentrations 0.1 μg/mL – 100 μg/mL compared with the positive control. Hence, in all subsequent assays, 1000 μg/mL concentration of extract/fractions was omitted.

Lee et al. [5] demonstrated that L. rhinocerotis cold water extract (LR-CW) did not show significant cytotoxic effect on human normal breast and lung cell lines (184B5 and NL 20) at concentrations ranging from 15.6 to 1000 μg/mL. However, anti-proliferative activity against both MCF-7 and A549 cancer cell lines was exhibited. The concentrations used were similar to the range of concentration used in the present study. Further, 0.1-100 μg/mL concentrations were used in this study to determine the anti-inflammatory effects on BV2 cells.

The effect of L. rhinocerotis crude extract/fractions (0.1 to 100 μg/mL) on NO production by LPS stimulated BV2 cells is presented in Figure 2. The LPS stimulation of the cells resulted in an increase in NO production (39.1 ± 0.8 μM) compared with the unstimulated cells basal levels (0.7 ± 0.2 μM). L-NNAME, a commercial nitric oxide suppressant, was used as a positive control at 200 μM. It was able to suppress 50% of NO production in the LPS induced BV2 cells. A dose dependent inhibition of NO production from 14% to 69% in BV2 cells occurred when cells were treated with the hydroethanolic extract at concentrations from

In general, inflammation is a naturally occurring reaction in the body in response to trauma, infection and tissue injury [17]. Although activated microglia scavenge dead cells from the CNS and secrete different neurotrophic factors for neuronal survival, overproductions of activated microglia may lead to neuronal death and brain injuries [18]. The activated cells also increase the secretion of various pro-inflammatory mediators such as nitric oxide (NO), prostaglandin E2 (PGE2), and cytokines. Nitric oxide, a short-lived free radical produced from L-arginine by nitric oxide synthase (NOS), mediates [19] a variety of pathophysiological actions ranging from vasodilatation, neurotransmission, inhibition of platelet adherence and aggregation, as well as the macrophage- and neutrophil-mediated killing of pathogens [20]. Overproduction of these mediators is responsible for inflammation. Therefore, inhibition of proinflammatory mediator(s) is beneficial in attenuating an inflammatory disorder. In the last few decades, evidence suggests that excessive NO production may play a role in neurodegenerative diseases.
The hydroethanolic extract and its ethyl acetate fraction significantly (\( p < 0.05 \)) inhibited (> 60%) NO production compared with the control. These extracts also showed significant (\( p < 0.05 \)) antioxidant properties. A study with *Tricholoma matsutake* Sing (pine mushrooms) showed that the ethyl acetate fraction exhibited the highest inhibition (61.6%) of NO by BV2 cells [21], similar to the findings in this study. Further, *Houttuynia cordata*, a traditional plant used as folk medicine for treating several ailments including allergic inflammation and anaphylaxis showed that HC-EA (ethyl acetate fraction) inhibited the LPS-stimulated increase of NO release by BV2 cells in a concentration-dependent manner [22].

Eight major lipid components, comprising 41.7% of the total components detected in the ethyl acetate fraction, were identified using GC-MS (Table 2). Two other components amounting to 38.2% of the total oil were not identified. The three major components were identified as linoleic acid (23.3%), ethyl linoleate (8.1%) and oleic acid (2.1%). About 25% of the total identified components in the ethyl acetate fraction were linoleic acid and oleic acid. The presence of these fatty acids as major components instead of phenolic compounds may have contributed to the inhibition of NO production. This correlates with the antioxidant findings in the present study in that the ethyl acetate fraction has a high antioxidant activity but relatively low TPC levels.

The dominant components; linoleic acid, ethyl linoleate and oleic acid were tested individually to measure their effect on viability and nitrite production in BV2 cells. The results are shown in Figure 3. The positive control (untreated BV2 cells) for cell viability was denoted as 100%. The unstimulated cell basal levels (0.2 \( \mu \text{M} \pm 0.6 \)) of NO were detected while LPS stimulation of the cells resulted in an increase in NO production (81.2 \( \mu \text{M} \pm 2.1 \)). The major compounds were not cytotoxic to BV2 cells at concentrations up to 100 \( \mu \text{g/mL} \). Increase in the concentrations of linoleic acid led to a decrease in NO production. There was a significant (\( p < 0.05 \)) reduction of 57% in NO production when compared with the control (cDNA of unstimulated BV2 cells), as shown in Figure 4. Treatment with linoleic acid significantly (\( p < 0.05 \)) decreased both iNOS and COX2 expression by 1.2 fold compared with the LPS (negative control).

Unsaturated fatty acid components are able to penetrate into the cells to reduce NO production of glial cells by down-regulating the expression of iNOS. The major lipid component, linoleic acid, caused a significant (\( p < 0.05 \)) reduction of iNOS and COX2 gene expression. Linoleic acid downregulated the expression of the proinflammatory genes, iNOS (30%) and COX2 (15%), lower than in aspirin treated cells. Aspirin is a non-steroidal anti-inflammatory drug (NSAID) known to exert its effects through inhibition of COX2. Pharmacological inhibition of COX2 can provide relief from the symptoms of inflammation and pain. In this study, linoleic acid was demonstrated to mimic aspirin in reducing inflammation via the COX2 mediated pathway. Yu et al. [27] had shown that linoleic acid reduced NO production by LPS activated cells and decreased the IFN\( \gamma \)-dependent expression of inducible NOS (iNOS) and iNOS promoter activity. Linoleic acid is also one of the fatty acids responsible for the inhibition of COX2 catalysed prostaglandin biosynthesis, as shown in *Plantago major L.* (Plantaginaceae) [28]. Excess NO may be produced by a higher promoter activity of iNOS and to induce also COX2 in various *in vitro* and *in vivo* models causing chronic inflammation [29].

### Table 2: Chemical constituents of the ethyl acetate fraction of *L. rhinocerotis*.

<table>
<thead>
<tr>
<th>RF (min)</th>
<th>Chemical constituent</th>
<th>Molecular formula</th>
<th>Molecular weight</th>
<th>Area (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>24.717</td>
<td>Palmitic acid</td>
<td>( \text{C}<em>{16}\text{H}</em>{32}\text{O}_{2} )</td>
<td>284.5</td>
<td>84.2</td>
</tr>
<tr>
<td>25.393</td>
<td>Ethyl palmitate</td>
<td>( \text{C}<em>{18}\text{H}</em>{36}\text{O}_{2} )</td>
<td>280.5</td>
<td>82.9</td>
</tr>
<tr>
<td>27.471</td>
<td>Methyl linoleate</td>
<td>( \text{C}<em>{18}\text{H}</em>{36}\text{O}_{2} )</td>
<td>280.5</td>
<td>74.2</td>
</tr>
<tr>
<td>27.573</td>
<td>Methyl oleate</td>
<td>( \text{C}<em>{18}\text{H}</em>{32}\text{O}_{2} )</td>
<td>296.5</td>
<td>7.0</td>
</tr>
<tr>
<td>28.305</td>
<td>Linoleic acid</td>
<td>( \text{C}<em>{18}\text{H}</em>{32}\text{O}_{2} )</td>
<td>280.5</td>
<td>842.9</td>
</tr>
<tr>
<td>28.788</td>
<td>Ethyl linoleate</td>
<td>( \text{C}<em>{20}\text{H}</em>{36}\text{O}_{2} )</td>
<td>308.5</td>
<td>829.1</td>
</tr>
<tr>
<td>28.873</td>
<td>Oleic acid</td>
<td>( \text{C}<em>{18}\text{H}</em>{36}\text{O}_{2} )</td>
<td>282.5</td>
<td>74.2</td>
</tr>
<tr>
<td>29.316</td>
<td>Ethyl stearate</td>
<td>( \text{C}<em>{20}\text{H}</em>{40}\text{O}_{2} )</td>
<td>312.5</td>
<td>2.1</td>
</tr>
<tr>
<td>37.927</td>
<td>Unidentified</td>
<td><strong>-</strong></td>
<td><strong>-</strong></td>
<td><strong>-</strong></td>
</tr>
<tr>
<td>48.370</td>
<td>Unidentified</td>
<td><strong>-</strong></td>
<td><strong>-</strong></td>
<td><strong>-</strong></td>
</tr>
</tbody>
</table>

Real time PCR was performed to investigate the effect of linoleic acid on LPS stimulated iNOS and COX2 genes which are involved in the proinflammatory response. After LPS stimulation for 24 h, the expression of iNOS and COX2 increased by 1.5 fold and 2-fold, respectively, when compared with the control (cDNA of unstimulated BV2 cells), as shown in Figure 4. Treatment with linoleic acid significantly (\( p < 0.05 \)) decreased both iNOS and COX2 expression by 1.2 fold compared with the LPS (negative control).
In conclusion, the EA fraction has the potential to suppress inflammation by reducing NO/iNOS and COX2 proinflammatory genes and to suppress inflammation via NF-κB and the STAT3 pathway. The presence of linoleic acid as the major bioactive lipid component may have contributed to the anti-oxidant and anti-inflammatory activities. However, the synergistic effect of the components in the ethyl acetate fraction may have also played a role in inhibiting neuroinflammation. To our knowledge this is the first report on the chemical constituents of the sclerotia of *L. rhinocerotis* and its in vitro anti-neuroinflammatory activity on BV2 cells.

**Experimental**

**Materials:** Freeze dried powdered sclerotia of *L. rhinocerotis* (TM02; commercial cultivar) were purchased from Ligno Biotech, Selangor, Malaysia, and linoleic (L1367) and oleic acids (O1008) from Sigma, USA.

**Extraction and fractionation of sample:** The powdered sclerotia were extracted and fractionated for biological screening according to a method described earlier [31]. Freeze dried powder (2.5 kg) was soaked in hydroethanol 80% and kept at room temperature for 2 days. The extract was filtered using a vacuum filter and the filtrate was concentrated on a rotary evaporator at 45°C (Buchi, Switzerland) under reduced pressure. This process was repeated 5 times; the filtrate from each extraction was concentrated and combined to obtain the crude hydroethanolic extract. This was further fractionized with *n*-hexane to yield a hexane soluble and hexane insoluble fractions, which were further partitioned with ethyl acetate: water mixture (1:1) by a counter current technique. The ethyl acetate soluble fraction was separated from the aqueous layer.

**Total phenolic content (TPC) estimation:** The TPC assay was conducted using the method outlined by Cheung et al. [32]. The absorbance of the sample was measured at 750 nm in a microplate reader (BioTek Instruments, USA). Gallic acid, up to 100 µg/mL, was used as a standard. The TPC results are mean values of triplicate assays and are expressed as gallic acid equivalents (GAE) per g mushroom (mg GAE/g mushroom extract).

**Antioxidant activity:** The antioxidant potential of the hydroethanolic extract of *L. rhinocerotis* and its *n*-hexane and ethyl acetate fractions was investigated using the following standard assays.

**Ferric reducing antioxidant power (FRAP) assay:** The FRAP assay was performed using the method described by Benzie and Strain [33]. FRAP reagent was prepared by mixing 50 mL of 300 mM acetate buffer, 5 mL of 10 mM 2,4,6-tripryridyl-3-triazine solution (TPTZ) in 40 mM of hydrochloric acid (HCl) and 5 mL of 20 mM ferric chloride (FeCl₃•6H₂O) in the ratio of 10:1:1. FRAP reagent (300 µL) was added to 10 µL of mushroom extract plated in a 96 well plate and absorbance was measured at 593 nm after 4 min in microplate reader. The standard used was iron sulfate (FeSO₄). FRAP results are mean values of triplicates assays and are expressed in mM FeSO₄ equivalent (FSE) per g mushroom (mmol FSE/g mushroom extract).

**Trolox equivalent antioxidant capacity (TEAC) assay:** TEAC was determined by using the method outlined by Re et al. [34]. The absorbance of the reaction mixture was measured at 734 nm in a microplate reader. Trolox was used as the standard. TEAC values are mean values of triplicates assay and expressed as mg Trolox equivalent (TE) per g mushroom extract (mg TE/g mushroom extract).

**Diphenyl-1-picryl-hydrazyl (DPPH) radical scavenging assay:** DPPH radical activity was determined using the method of Brand-Williams et al. [35]. Ascorbic acid at different concentrations was used as standard. Mushroom extract (5 µL) was mixed with 195 µL of a methanolic solution of DPPH radical in a 96 well plate. The mixture was shaken vigorously and left to stand for 3 h in the dark, and the absorbance was measured at 515 nm. The assay was carried out in triplicate. DPPH activity was expressed in DPPH inhibition percentage (%).

**BV2 cell culture:** BV2 cells were maintained in Dulbecco Modified Eagle’s medium (DMEM) supplemented with 5% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin, 1 mL/L gentamycin, 250 µg/mL fungizone, 1X non-essential amino acids, 2 mg/mL insulin and 1.5 g/L sodium bicarbonate. Cultures were maintained at 37°C in 95% humidified air and 5% CO₂. Cells were harvested by treating with 0.25% trypsin in 1 mM ethylenediaminetetraacetic acid (EDTA) for 5 min at 37°C.

**Cell viability assay:** The cytotoxic effects were determined by using a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxyphenyl)-2H-tetrazolium (MTS) assay. This assay was carried out according to the method of Tan et al. [36]. In a 96 well flat-bottomed microplate, 5×10⁴ cells were seeded per well and incubated at 37°C overnight for attachment. Different concentrations of the extract were then added. After 24 h incubation, MTS solution was added and further incubated for 2 h. The absorbance was measured at 490 nm with a microplate reader (Dynex MRX II microplate reader, USA). Each assay was performed in triplicate. Absorbance of all wells was deducted from the absorbance of complete growth medium which served as background reading. Cell number was calculated in comparison to untreated cells.
Nitric oxide determination assay: Nitric oxide was measured in the culture medium as an indicator of NO production based on the Griess reaction. The BV2 cells were plated in a 96 well plate at a density of 5 × 10⁴ cells/well and incubated overnight. Cells were then incubated with different concentrations of the extract (0.01-1000 μg/mL) and 1μg/mL of Escherichia coli (O55:B5) lipopolysaccharide (LPS) (Sigma, US). After 24 h, the culture supernatant was collected for nitrite measurement. Fifty μL of the spent medium were plated in a 96 well plate and 50 μL of Griess reagent (0.1% N-(1-naphthyl)ethylene diamine-diHCl and 1% sulfanilamide and 2.5% H₃PO₄) was added. The plate was incubated for 15 min, and the absorbance measured at 530 nm using a microplate reader (Dynex MRX II microplate reader, USA). The amount of NO was calculated using a sodium nitrate standard curve [36].

GC-FID and GC-MS analysis: The sample was analyzed using Agilent Technologies gas chromatography (GC) 7890A with FID equipped with a fused silica capillary column HP-5ms, 5% phenylmethylsiloxane (30.0 m by 0.25 mm ID by 0.25-lm film thickness). Purified nitrogen was used as carrier gas at a flow rate of 1 mL per min, a split ratio of 1:10 and 2 μL injection volume. The column temperature was programmed initially at 100°C, then increased by 5°C per min to 300°C and was kept isothermally for 20 min. The temperature of the injector port and FID jet was 250°C and 230°C, respectively. Identification of constituents was performed on an Agilent GC 7890A equipped with a 5975C inert mass selective detector (70 eV direct inlet) using the same capillary column as the GC-FID, HP-5ms. The carrier gas was purified helium at a flow rate of 1 mL per min, a split ratio of 1:10 and 1 μL injection volume. The column temperature was programmed as for the GC-FID. The temperatures of the injector port and interface of the MS were 250°C and 270°C, respectively. The constituents were identified by comparison with constituents identified in the mass spectral database (Wiley Registry 9th Edition / NIST 2011 Library, 2011).


Altitude Variation in the Composition of Essential Oils, Fatty Acid Methyl Esters, and Antimicrobial Activities of Two Subspecies of *Primula vulgaris* Grown in Turkey
Nurettin Yaylı, Gonca Tosun, Buşra Yaylı, Zeynep Gündoğan, Kamil Coşkunçelebi and Şengül Alpay Karaoğlu

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