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Article

Lipid-rich fraction of the sclerotium of Tiger Milk Mushroom *Lignosus rhinocerotis* (Agaricomycetes) attenuates LPS-induced inflammation in BV2 cells via Nrf2 pathway

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Lignosus rhinocerotis (tiger milk mushroom) is widely used by the indigenous people of Malaysia as a traditional remedy. The present study was carried out in order to evaluate the antioxidant, cytotoxic and anti-neuroinflammatory activities of *L. rhinocerotis* extract on brain microglial cells (BV2). The antioxidant activity was evaluated by 2,2-diphenyl-1-picryhydrazyl (DPPH•), 2,2'-azinobis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS•+) scavenging assays, and ferric reducing antioxidant power (FRAP). The FRAP, DPPH and ABTS•$^{+}$ scavenging capacities of the TE3 fraction were 420.77 mg FE/g, 58.01%, and 7%, respectively. The cytotoxic activity was determined by MTS assay. The *in vitro* model of anti-neuroinflammatory property was evaluated by measuring the production of nitric oxide (NO) in lipopolysaccharide (LPS)-induced BV2 cells. The TE3 fraction showed a significant NO reduction at 1 to 100 µg/mL. The TE3 fraction down-regulated inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX2) genes while it upregulated heme oxygenase (HO-1) and NADPH quinone acceptor oxidoreductase-1 (NQO-1) genes. The nuclear factor (erythroid-derived 2)-like 2 (Nrf2) transcription was also activated. The chemical component of the active fraction (TE3) was identified by gas chromatography-mass spectrometry (GCMS). Overall, the BV2 *in vitro* model anti-neuroinflammatory activity of *L. rhinocerotis* may be caused by the lipid constituents identified in the fraction.

Keywords: Neuroinflammation. *Lignosus rhinocerotis*. Mushroom. BV2 cells. Nrf2 pathway.

INTRODUCTION

Free radicals are important in the regulation gene expression and activation of receptors. However, an excess of free radicals is toxic and able to damage functional molecules during oxidative stress (Xu *et al*., 2009). This can lead to many diseases such as cancer, age-related disorders, neurodegenerative diseases and inflammation (Diaz *et al*., 2012). Several anti-inflammatory and neuroprotective drugs have been shown to have an antioxidant and/or anti-radical scavenging mechanism as part of their activities. Functional foods that contain different types of antioxidants and their total antioxidant capacities (TAC) to scavenge free radicals, would be useful since neurodegenerative disease are caused by cellular oxidative stress.

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Inflammation of the brain is characterised by the activation of glial cells (mainly microglial and astrocytes), expression of inflammatory mediators, and release of neurotoxic free radicals (Chen *et al.,* 2016). This phenomenon is collectively known as "neuroinflammation". Emerging evidence indicates that neuroinflammation is closely associated with neurodegenerative disorders in both acute (stroke, injury) and chronic diseases (multiple sclerosis, Alzheimer's, and Parkinson diseases) (McManus, Heneka, 2017).

Lignosus rhinocerotis (Cooke) Ryvarden, better known as the "tiger milk mushroom" in Southeast Asia, has been used as a folk medicine for many years (Seow *et al*., 2015). Previous studies have attempted to validate the anti-cancer activities and its anti-inflammatory properties of sclerotium of *L. rhinocerotis*, (Lau *et al*., 2015; Lee *et al*., 2012; Nallathamby *et al*., 2018). However, there is less validation of bioactive compounds on its anti-neuroinflammatory effect on *in vitro* BV2 model. Therefore, this study is aimed towards elucidating the bioactive fraction of ethyl acetate (TE3) fraction of *L. rhinocerotis* with respect to its a) chemical components; b) cytotoxicity and the inhibition of nitric oxide (NO) production in BV2 cells; and c) effects on gene expression in the nuclear factor-erythroid 2-related factor 2 (Nrf2) pathway.

MATERIAL AND METHODS

Sample preparation

Freeze dried sclerotium of *L. rhinocerotis* commercial cultivar powder (TM02) was purchased from Ligno Biotech, Selangor, Malaysia. The freeze-dried powder (1 kg) was extracted with 80% ethanol. The ethanol extract was further fractionated with ethyl acetate as previously reported in Nallathamby *et al. (2016)*. The extract was concentrated on a rotary evaporator at 45 ºC (Buchi, Switzerland) under reduced pressure. The ethyl acetate extract (5 g) was subjected to vacuum liquid chromatography and eluted with a mixture of *n*-hexane: acetone in an increasing amount of acetone. The third fraction (TE3) out of the total seven fractions collected was further analysed (Kanagasabapathy *et al.,* 2011).

Ferric reducing antioxidant power (FRAP) assay

The FRAP assay was performed using the method described by Nallathamby *et al.* (2016). FRAP reagent was prepared by mixing 50 mL of 300 mM acetate buffer; 5 mL of 10 mM 2,4,6-tripyridyl-s-triazine solution (TPTZ) in 40 mM of hydrochloric acid (HCl) and 5 mL of 20 mM ferric chloride ($FeCl_3 \bullet 6H_2O$) in the ratio of 10:1:1. FRAP reagent (300 µL) was added to 10 µL of TE 3 fraction plated in a flat bottomed 96 well microtiter plates and absorbance was measured at 593 nm after 4 min. The standard used was iron sulfate ($FeSO_4$). The FRAP results are mean values of triplicate assays and were expressed in mM $FeSO_4$ equivalent (FSE) per gram mushroom (mmol FSE/g TE 3 fraction).

Trolox equivalent antioxidant capacity (TEAC) assay

2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic) acid ($ABTS^{\bullet+}$) is a stable radical cation used to measure TEAC. The $ABTS^{\bullet+}$ reagent was prepared by mixing 5 mL of 7 mM $ABTS^{\bullet+}$ solution with 89 µL of 140 mM $K_2S_2O_8$. It was kept in the dark at room temperature for 16 h and the absorbance of the $ABTS^{\bullet+}$ reagent was adjusted to 0.70 ± 0.05 at 734 nm prior to assay. Trolox was used as the standard. The $ABTS^{\bullet+}$ reagent (100 µL) was added to 10 µL of the TE 3 fraction in flat-bottomed 96 well microtiter plates. The absorbance was measured at 734 nm after 1 minute. TEAC values were mean values of triplicates assay and were expressed in $ABTS^{\bullet+}$ scavenged percentage (%). (Nallathamby *et al*., 2016)

Diphenyl-1-picryl-hydrazyl (DPPH) radical scavenging assay

The TE 3 fraction (5 µL) was mixed with 195 µL of methanolic solution containing DPPH radical in flat-bottomed 96 well microtiter plates. The mixture was shaken vigorously and left to stand for 3 h in the dark, and the absorbance was measured at 515 nm. Ascorbic acid was used as standard. The assay was carried out in triplicates and DPPH activity was expressed in DPPH inhibition percentage (%). (Nallathamby *et al*., 2016)

BV2 cell culture

BV2 cells were presented by Dr. Sharmili from the Immunology Laboratory in UPM, Malaysia. BV2 cells were maintained in Dulbecco Modified Eagle 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, 1× 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 EDTA for 5 min at 37 °C.

Cell viability assay

The cytotoxic effects of the fraction were determined by using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS) assay (Tan *et al.*, 2011). In a flat-bottomed 96-well microtiter plate, 5×10^4 cells were seeded per well and incubated at 37 °C overnight for attachment. To determine cytotoxic effect, cells were incubated for 24 hours with different concentrations of TE 3 fraction (0.01-1000 µg/mL). To determine the protection effect, cells were incubated for 24 hours with different concentrations of TE3 fraction (0.01-1000 µg/mL) and 1 µg/mL of *Escherichia coli* (O55:B5) lipopolysaccharide (LPS). 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 triplicates. Cell viability was calculated in comparison to the untreated cells.

Nitric oxide (NO) determination assay

Nitrite that accumulated in the culture medium was measured as an indicator of NO production based on the Griess reaction (Ozcelik, Algul, 2017). The BV2 cells were seeded in a 96-well plate at a density of 5×10^4 cells/well and incubated overnight. Cells were then incubated with different concentrations of TE3 fraction (0.01-1000 µg/mL) and 1 µg/mL of *Escherichia coli* (O55:B5) lipopolysaccharide (LPS) (Sigma, US). After 24 h, the culture supernatant was collected for nitrite measurement. 50 µL of the spent medium was plated in flat-bottomed 96 well microtiter plates and 50 µL of Griess reagent (0.1% *N*-1-[naphthyl]ethylenediamine-diHCl, 1% sulphanilamide and 2.5% H_3PO_4) was added. The plate was incubated for 15 min, and the absorbance was measured at 530 nm. The amount of NO was calculated using a sodium nitrite standard curve.

RNA extraction and quantitative real time PCR

The BV2 cells were lysed and the total RNA was extracted as recommended by the manufacturer's manual using an Ambion-RNAqueous Micro® kit (Applied Biosystems, USA). cDNA was synthesised from the purified RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). The reaction setup for all quantitative real time PCR (qPCR) reactions was performed according to the reaction setup instructions generated by the StepOne software (Ver 2.0, Applied Biosystems). The reactions were carried out with TaqMan® probes (Applied Biosystems, USA) in a reaction volume of 20 µL. Briefly, the reaction which consisted of the TaqMan® Gene Expression Master Mix and the assay mix were prepared separately. Each assay mix contained corresponding primers and probe for each targeted gene. Each reaction was assayed in triplicate. The reaction mix was mixed with either sterile ultra-pure water for no template control reactions (NTC) or the isolated cDNA. The strips were centrifuged and loaded into the real time PCR thermal cycler (StepOnePlusTM Real Time PCR System). The relative expression of the investigated genes was normalised with the endogenous control, β actin rRNA. Table I shows the list of genes investigated.

TABLE I - List of genes investigated

No	Gene name	Abbreviation	Assay ID*
1.	Nuclear factor (erythroid-derived 2)-like 2	NFE2l2/ NRF2	Mm00477784_m1
2.	Heme oxygenase-1	HO-1	Mm00516005_m1
3.	NAD(P)H quinone oxidoreductase 1	NQO-1	Mm01253561_m1
4.	Inducible nitric oxide synthase	NOS2	Mm00440502_m1
5.	Beta- actin	β-actin	Mm00607939_s1

(continuing)

TABLE I - List of genes investigated

No	Gene name	Abbreviation	Assay ID*
6.	Cyclooxigenase-2	COX-2	Mm00478374_m1

*Note: Assay ID refers to the Applied Biosystems Gene Expression Assay kits with proprietary primer and TaqMan® probe mix. Mn: *Mus musculus*

Gas chromatography-mass spectrometry (GC-MS) analysis

GC-MS analysis was performed using an Agilent Technologies 6890N (United States) gas chromatograph equipped with a 5979 Mass Selective Detector (70eV direct inlet) and a HP-5 ms (5% phenylmethylpolysiloxane) capillary column (30 m x 0.25 mm i.d. x 0.25µm film thickness). The oven temperature was initially set at 100 ºC, and then increased at the rate of 5 °C per minute to 300 °C. Helium was used as the carrier gas at a flow rate of 1 mL/min. The total ion chromatogram obtained was auto-integrated by Chemstation and the constituents were identified by comparison with those in the mass spectral library (W9N11. Mass Spectrum Library, USA, 2011).

Statistical Analysis

All data triplicates were recorded as means ± standard deviation (SD) and analysed by SPSS for Windows (version 18). One-way analysis of variance (ANOVA) and Dunnett comparisons were carried out to test any significant differences. $P<0.05$ was considered statistically significant.

RESULTS AND DISCUSION

Antioxidant activity of TE3 fraction

The ferric reduction power of the TE3 fraction was 420.77 mg FE/g TE 3 fraction. The ABTS radical scavenging capacity of the TE3 fraction was about 7% while the DPPH scavenging activity was 58.01%. The TE3 fraction possessed moderate antioxidant activities compared to the standard antioxidant compounds used. The antioxidant activities of the extract were lower than the antioxidant activities of the standard antioxidant compound used. The extract could only achieve IC_{50} at a higher concentration compared to the standard compounds. The antioxidant results of the TE3 extract and the standard compounds are shown in Table I. Undesirable oxidation increases the reactive oxygen species (ROS) creating oxidative stress which leads to inflammation if prolonged. The components in the cells (protein and lipid) will increase and defend against ROS and restore the balance in the cells (Hensley *et al*., 2000). A study by Babbar *et al.,* (2011) also showed that phenolic compounds alone are not fully responsible for the antioxidant activity. Other constituents such as ascorbates, lipids, terpenes, and pigments as well as the synergistic effect among them could possibly contribute to the totality of antioxidant activity.

Effect of TE3 fraction on cell viability of BV2 cells

The effect of the TE3 fraction of *L. rhinocerotis* on the viability of BV2 cells at various concentrations was determined by MTS assay. The cytotoxicity and protective effects of the TE3 fraction are shown in Figure 1a. The cell viability of the untreated BV2 cells was indicated as 100%. The negative control is labelled LPS and the positive control is labelled LNAME. The TE3 fraction did not exert any cytotoxic effect on BV2 cells at 0.1-100 µg/mL concentrations. The TE3 fraction also did not exert any cytotoxic effect on LPS induced BV2 cells at 0.1-100 µg/mL concentrations. The protective effects of TE3 fractions were done to show its ability to protect and prevent cell deaths of the LPS induced BV2 cells (inflamed microglial). Thus, the TE3 fraction was not cytotoxic towards the BV2 cells and was also able to protect the BV2 cells after being inflamed.

Similar to our findings, the ethanol extract of *Euphorbia hirta* (Sharma *et al*., 2014) indicated no adverse effect on RAW 264.7 cells with concentrations up to 200 µg/mL, while the conjugated linoleic acid (Cheng *et al*., 2004) had no cytotoxic effect up to 200µM, respectively.

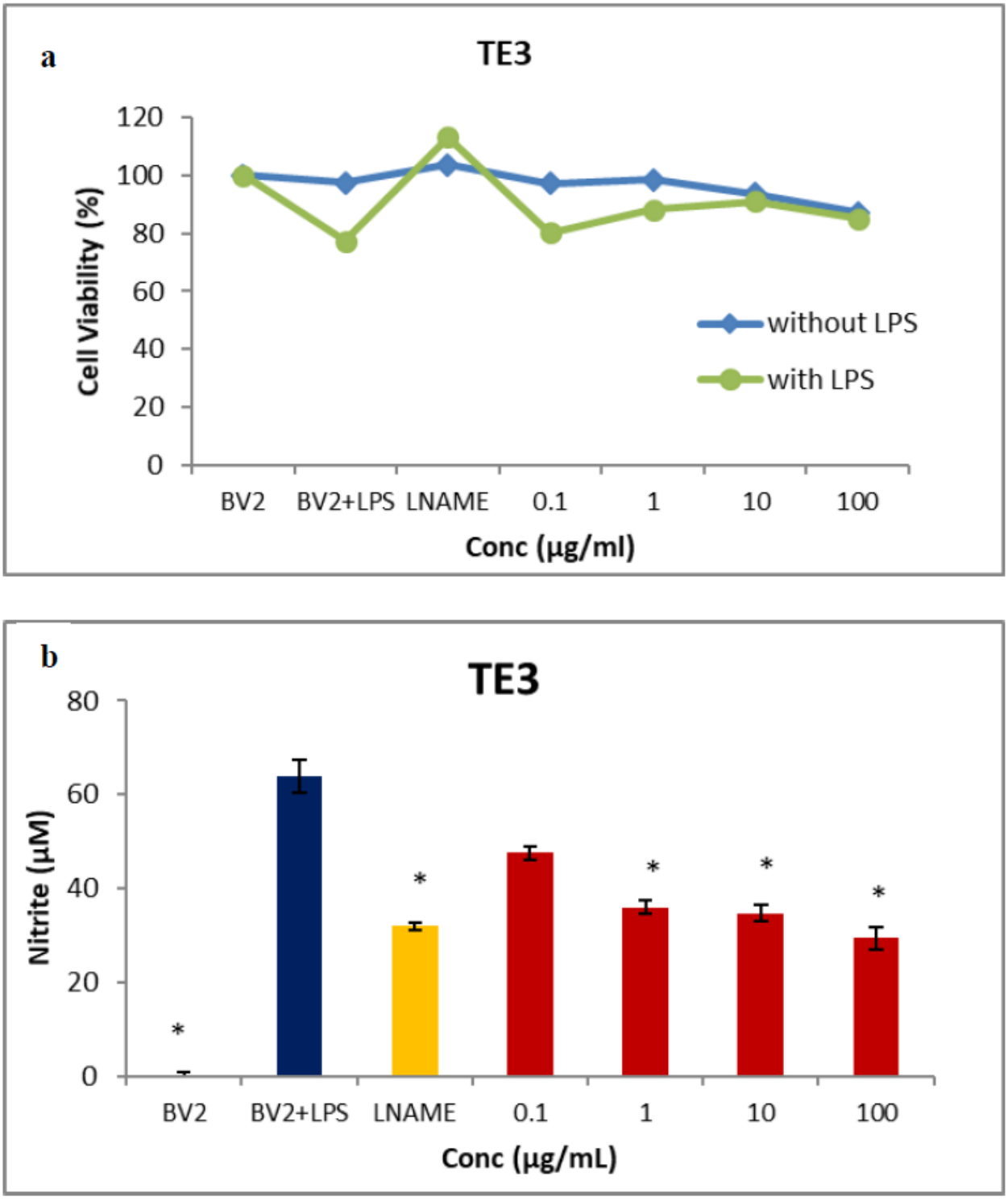

Figure 1 - The effect of treatment with the TE3 fraction of *L. rhinocerotis* on (a) BV2 cell viability and (b) nitrite production in BV2 cells. The * symbol denotes a significant difference ($p < 0.05$) from the corresponding value of the control. L-NAME= N(ω)-nitro-L-arginine methyl ester (200 µM).

Effect of TE3 fraction on nitric oxide production in LPS stimulated BV2

The effect of *L. rhinocerotis* TE3 fraction (0.1 to 100µg/mL concentrations) on NO production by LPS-stimulated BV2 cells was tested as shown in Figure 1b. In unstimulated BV2 cells, the basal levels of NO were detected while LPS stimulation of the cells resulted in an increase in NO production, which is denoted as 63.9 µM. A dose-dependent inhibition of NO production in BV2 cells was observed from 1 to 100µg/mL concentrations when treated with the TE3 fraction. A significant ($p<0.05$) reduction of NO production by BV2 cells with TE3 treatment was observed from 1 to 100 µg/mL with inhibition ranging from 43% to 54%.

Microglia are distributed in the CNS and activated cells release proinflammatory mediators such as NO and reactive oxygen species (ROS) (Gonzalez-Scarano, Baltuch, 1999). Regulation of chronic inflammation of microglia is to prevent the onset inflammation related neurodegenerative diseases. The TE3 fraction has anti-inflammatory properties that significantly ($p<0.05$) decrease NO production without exerting cytotoxic effects. The results of this study correspond to previous studies done on anti-inflammatory and anti-neuroinflammatory activities of mushrooms and plants. The results of the current study had a similar NO reduction compared with *Ganoderma lucidum* terpene extract (GLT) which significantly reduced NO levels with the IC_{50} being 11.4µg/mL (Dudhgaonkar *et al.*, 2009). Oleamide (Oh *et al.*, 2010) significantly inhibited NO production in BV2 cells, while palmitic acid (Lee *et al.*, 2010) inhibited NO in peritoneal machrophages both at 10 µM. However, the TE3 fraction inhibited NO production at a lower concentration compared to these compounds. This suggested that the TE3 fraction had higher anti-inflammatory activity than its individual compounds. This indicated a combination synergestic effect of the components.

Effect of TE3 fraction on the relative expression levels of inflammatory genes in LPS stimulated BV2 cells

In the present study, microglial BV2 cells were stimulated with lipopolysaccharide (LPS) (1 µg/mL) in the presence of non-cytotoxic concentrations of the TE3 fraction; and the level of induced nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX2) expressions were measured as shown in Figures 2a and 2b. The expression of proinflammatory genes, i.e. iNOS and COX2 after LPS stimulation increased by 1.5-fold and 1.8-fold; respectively, when compared to the controls (unstimulated BV2 cells). Treatment with the TE3 fraction significantly ($p<0.05$) decreased the iNOS gene expression by 1.18-fold and the COX2 gene by 1.19-fold. The suppression effects were higher compared to those of aspirin, which was the positive control.

The effects of the TE3 fraction on the expression of anti-inflammatory genes, i.e. heme oxygenase (HO-1) and NADPH: quinone acceptor oxidoreductase-1 (NQO-1) were determined as shown in Figure 2c and Figure 2d. The TE3 fraction upregulated the expression of HO-1 and NQO-1 genes by 3.28- and 0.99-folds, respectively. The upregulation of HO-1 and NQO-1 by the TE3 fraction was comparable to the treatment with aspirin. The effect of thevTE3 fraction on Nrf2 expression was examined as shown in Figure 2e. Aspirin showed 1.40-fold upregulation in the Nrf2 gene expression while the TE3-treated cells showed an upregulation of 1.49-fold.

Stimulation of microglial cells initiates the inflammatory responses by generating NO, iNOS, COX2, as well as other inflammatory mediators. Cumulative evidence indicates abnormalities or over-production of these mediators may play a triggering role in many chronic inflammatory diseases (Musolino *et al.,* 2017). Thus, suppression of these mediators and their functions serve as an effective therapeutic strategy for preventing or reducing inflammatory reactions and the related diseases. The transcription factor, Nrf2, is a controller of brain redox homeostasis and it regulates inflammatory conditions (Ma, 2013). It mediates the antioxidant response element (ARE) and cytoprotective genes. Nrf2-knockout-aged mice were highly sensitive to oxidative stress and more susceptible to the inflammatory response in the brain (Innamorato *et al.,* 2008). Therefore, to target Nrf2 signalling could be a good strategy to reduce neuroinflammation.

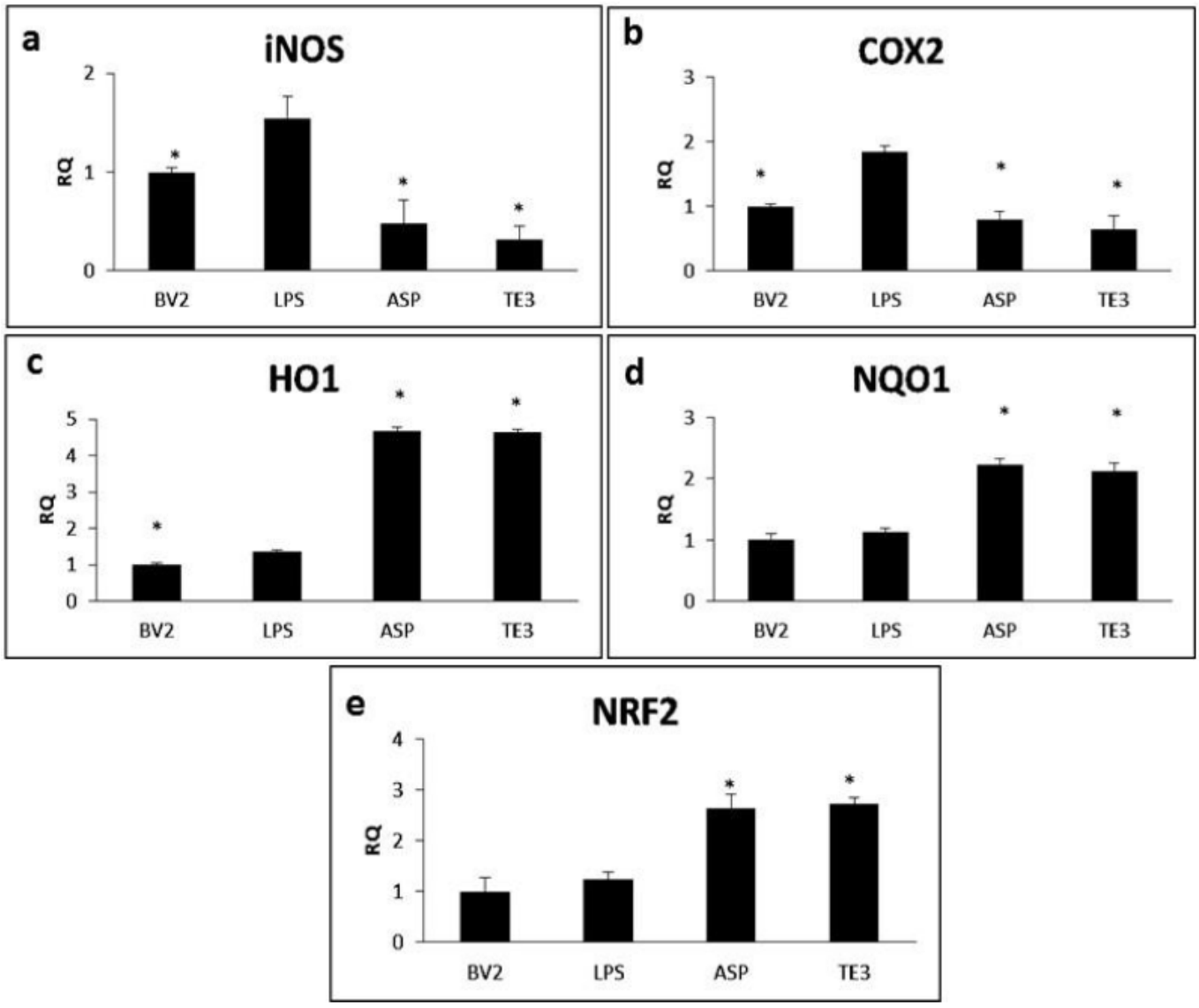

FIGURE 2A - The effect of different treatments in LPS stimulated BV2 cells on inflammatory genes; i.e. (a) iNOS, (b) COX2, (c) HO1, (d) NQO1, and (e) Nrf2. Fold increase values are calculated relative to the ACTB gene. BV2= untreated cells, LPS= LPS stimulated cells, ASP= Aspirin (100 uM), TE3= TE3 fraction (10 µg/mL) treatment in LPS stimulated BV2 cells. Data represent the mean ± SE and from a representative of three independent experiments carried out in triplicates. The * symbol denotes a significant difference ($p<0.05$) from the corresponding value of the negative control (LPS). RQ=relative quantification.

In the present study, the TE3 fraction attenuates microglial pro-inflammatory response by reducing NO, the proinflammatory and anti-inflammatory cytokines *via* Nrf2 mediated pathway. The TE3 fraction upregulated HO-1 and NQO-1 and inhibited iNOS and COX2 expression in BV2 cells *via* Nrf2 which modulates their inflammatory responsiveness. Similar to our results, mollugin from *Rubia cordifolia* also demonstrated anti-inflammatory effects by suppressing NO and down-regulating iNOS and COX2 expressions (Jeong *et al.,* 2011).

Identification of lipid constituent of TE3 fraction via GC-MS analysis

Results from the GC-MS analysis are presented in Table II and Figure 3. The constituents in the TE3 fraction were identified by matching their mass-spectra with those in the accompanying mass-spectral library. Eight compounds were identified which made up 69.28% of the total components detected in the total ion chromatogram. Linoleic acid is the major component comprising of 45.35% of the total detected components. Other components identified were palmitic acid (2.37%), oleamide (7.12%), palmitamide (3.50%), 4-hydroxybenzaldehyde (3.12%) and its analogue 4-hydroxy-3-methoxybenzaldehyde (vanillin) (1.51%), 9, 17-octadecadienal (3.36%), and dehydroergosterol (2.96%).

All mushrooms contain large amounts of essential fatty acids, especially linoleic acid. Essential fatty acids cannot be produced by animals but are required by the body for healing and good health (Wohl, Goodhart, 1968). The major component in this fraction is linoleic acid with 45.34%. Many mushrooms analysed in previous studies had linoleic and palmitic acids as the most abundant fatty acids (Gunc-Ergönül *et al.*, 2013). The results from this study are in agreement with previous reports that many mushrooms species have high abundance of unsaturated fatty acid especially linoleic acid (40%-70% of total lipid content) (Kalac, 2009; Phan *et al.,* 2014). Linoleic acid had shown good anti-inflammatory activity comparable to its derivatives, i.e. α-linolenic acid in human monocytic (THP-1) cells (Fritsche, 2015; Zhao *et al.*, 2005). Some previous reports have shown linoleic acid has the property to be transported across the blood–brain barrier, thus it may be able to exert anti-neuroinflammation in the brain region (Edmond, 2001). Palmitic acid, a saturated long chain fatty acid commonly identified in mushrooms, is reported to modulate inflammation response and reduce cytokine effect in stimulated peritoneal macrophages (Lee *et al.*, 2010).

The fatty acid amides, oleamide (7.12%) and palmitamide (3.50%) were present in this fraction. Oleamide is an amide of oleic acid; found accumulated in the cerebrospinal fluid of sleep-deprived animals and induces sedation and physiological sleep. Oh *et al.*

Table II - Chemical constituents of the TE3 fraction of ethyl acetate extract as determined by GC-MS

Chemical constituent	RT(min)	Molecular formula	Molecular weight	Area %
4-Hydroxy benzaldehyde	8.423	$C_7H_6O_2$	122.12	3.12
Vanillin	9.386	$C_8H_8O_3$	152.15	1.51
Palmitic acid	21.409	$C_{16}H_{32}O_2$	256.42	2.37
Linoleic acid	24.930	$C_{18}H_{32}O_2$	280.45	45.34
Palmitamide	25.156	$C_{16}H_{33}NO$	255.44	3.50
Oleamide	28.202	$C_{18}H_{35}NO$	281.48	7.12
9,17-octadecadienal	33.262	$C_{18}H_{32}O$	264.45	3.36
Dehydroergosterol	39.774	$C_{28}H_{42}O$	394.63	2.96
Total				69.28

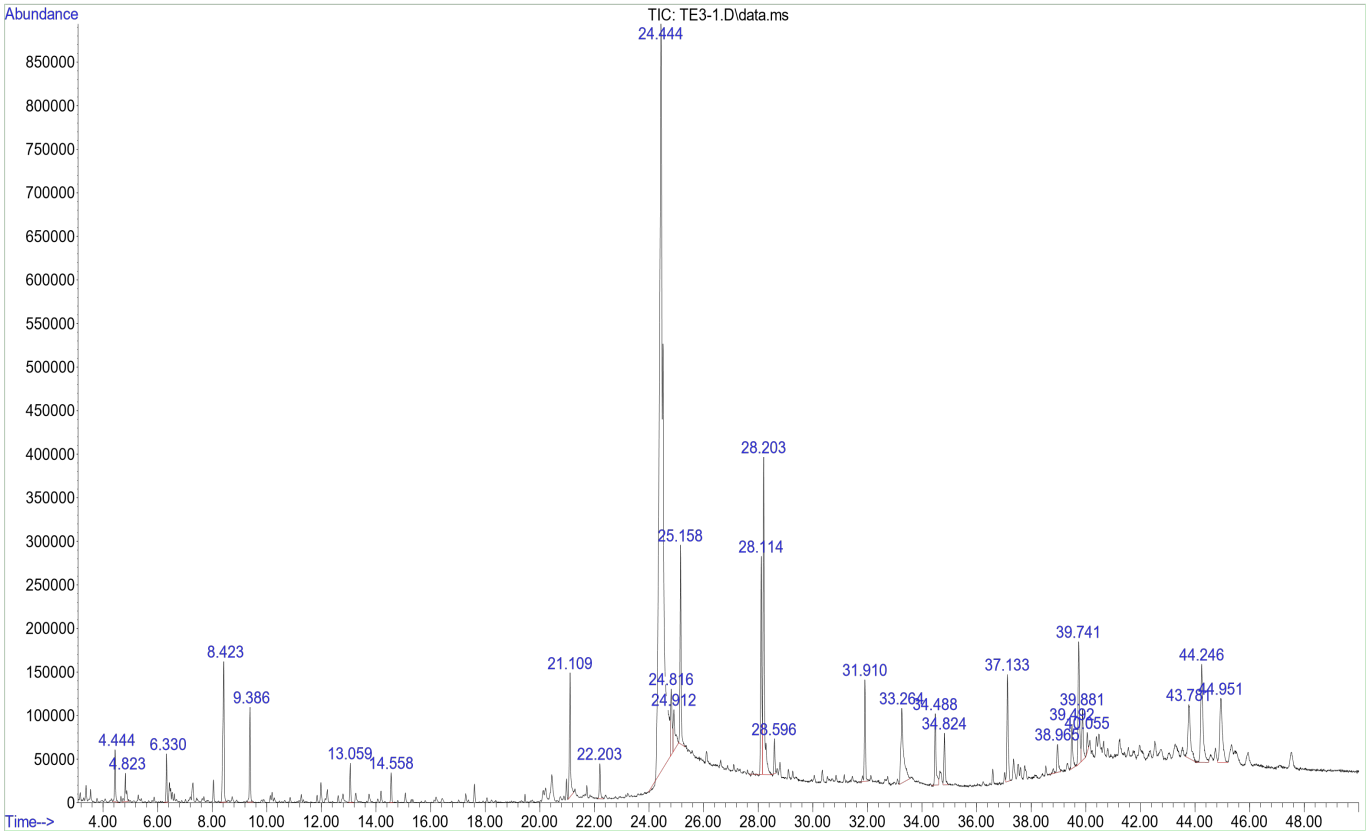

FIGURE 3 - GC-MS chromatogram of the TE3 fraction of ethyl acetate extract.

(2010) reported that oleamide suppressed LPS induced inflammation by lowering the expression of iNOS and COX-2. Palmitamide, also known as palmitic acid amide, has been shown to reduce prostate cancer progression (Liu *et al*., 2011).

The aldehyde components, 9, 17-octadecadienal, 4-hydroxy benzaldehyde, and its analogue 4-hydroxy-3-methoxybenzaldehyde (vanillin), present have been accepted to be medicinally important by earlier research. The component 9, 17-octadecadienal is an unsaturated aldehyde is known for its antimicrobial activity (Rajeswari *et al*., 2013). Components 4-hydroxy benzaldehyde and vanillin are phenolic compounds that are commonly and majorly found in *Gastrodia elata* (family of Orchidaceae) and *Vanilla planifolia*. The 4-hydroxy benzaldehyde showed anti-inflammatory activity by suppressing NO, iNOS and COX2 expressions, and ROS in murine macrophages (Lim *et al*., 2008). It has also inhibited COX2 expression and silica induced intracellular ROS (Lee *et al*., 2006). Vanillin is commonly used for its flavour and odour in food, beverages and cosmetics. Vanillin exhibits anti-inflammatory activity by inhibiting NFKB and COX2 expression (Murakami *et al*., 2007). It also displays antimicrobial, antioxidant, and chemopreventative effects on carcinogenesis models and inhibits mutagenesis and supresses the progression and migration of cancerous cells.

The dehydroergosterol is a naturally occurring fluorescent sterol analogue that mimics many of the properties of cholesterol. Recently it has been identified in Camembert and Gorgonzola cheeses fermented with *Penicillium candidum*; it inhibits cytokines and chemokines in microglial cells and reduced neurotoxicity in Neuro-2A cells (Ano *et al.,* 2015). Based on the known biological/ medicinal properties of the compounds identified in the TE3 fraction, the synergistic effect of these components contributes to the anti-inflammatory activities

CONCLUSION

The TE3 fraction of ethyl acetate extract exhibited significant ($p<0.05$) anti-neuroinflammatory activity without imposing any cytotoxic effects on *in vitro* model

of BV2 cells. The major lipid component, linoleic acid, identified in the fraction indicates that this mushroom is rich in unsaturated and essential fatty acids. The TE3 fraction gave a significant ($p<0.05$) dose-dependent NO reduction from 1-100 µg/mL treatments. The TE3 fraction downregulated the proinflammatory cytokines iNOS and COX2. It also upregulated the anti-inflammatory gene (HO-1 and NQO-1) expressions and the corresponding Nrf2 pathway activation. The fraction has potent anti-inflammatory activities in an *in vitro* BV2 model and there may be synergistic effects among the constituents present as all the individual components in the fraction had anti-inflammatory activity. To our knowledge, this is the first report on an *in vitro* BV2 model based anti-neuroinflammatory property activity of *L. rhinocerotis* solvent based lipid enriched extract and its bioactive lipid component.

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