Chemical constituents and nitric oxide inhibitory activity of supercritical carbon dioxide extracts from *Mitragyna speciosa* leaves

Norsita Tohar a, Jamil A. Shilpi a, Yasodha Sivasothy a, Syahida Ahmad b, Khalijah Awang a,c,*

a Department of Chemistry, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia
b Institute of Bioscience, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia
c Centre for Natural Products and Drug Discovery (CENAR), Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia

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**KEYWORDS**
Ketum; Traditional medicine; Griess assay; Supercritical fluid extraction; Gas chromatography–mass spectrometry; Palmitic acid

**Abstract** *Mitragyna speciosa* Korth., is a controversial plant that possesses various medicinal values and has been used from time immemorial in folk medicine. Recently, it has been widely abused as a narcotic source. The aim of the present study was to characterize the *in vitro* nitric oxide (NO) inhibition property of the supercritical CO₂ fluid extracts from *M. speciosa* leaves and to identify the chemical constituents of the extract that exhibited the highest inhibition without toxicity effect. Samples were extracted using the green technology, supercritical fluid extraction (SFE) technique via CO₂ as the mobile phase. The NO inhibitory activity was evaluated by Griess assay, which measures the formation of nitrite ion (NO₂⁻/CO) in recombinant mouse interferon gamma/lipopolysaccharide (IFN-γ/LPS) stimulated RAW 264.7 cells. Matrix 5 Step-1 (M5S1) that was extracted with pure CO₂ at 3000 psi and 60 °C exhibited the highest NO inhibitory activity (60.08%) without cytotoxicity (cell viability, 91.98%) at a concentration of 100 μg/mL. GC and GC–MS analysis revealed palmitic acid as the major constituent (34.90%) of M5S1. This study provides first evidence that M5S1, the non-alkaloidal extract obtained by supercritical fluid extraction of *M. speciosa* leaves possesses potential property in preventing inflammatory diseases mediated by excessive production of NO.

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1. Introduction

Inflammation, a healing response of the body to various insults, is a two-edged sword. While it is typically recognized as a process for the remission of diseases, the persistence of this process may lead to various diseases associated with chronic inflammation. Nitric oxide (NO) is a major signalling molecule of mammalian cells that plays an important role in host defence, homeostasis and developmental functions by either direct or indirect effects. Under normal physiological conditions, cells containing constitutive isoforms of nitric oxide synthases (NOS), i.e. endothelial NOS (eNOS) or neuronal NOS (nNOS) produce relatively small but significant amounts of NO. In inflammatory conditions, the inducible isoform of NOS (iNOS) is upregulated giving rise to cellular NO concentration more than 10 μM (Burgner et al., 1999). NO is a key signalling molecule in central and peripheral mechanisms of pain. Excessive production of NO is linked to various pathological conditions, including lipid peroxidation, DNA damage and enzyme inhibition (Ratajczak-Wrona et al., 2013; Wink and Mitchell, 1998). There are several reports on the direct role of excessive NO production by macrophages as a contributing factor in a number of chronic inflammatory diseases including arthritis, hepatitis, septic and haemorrhagic shock and certain autoimmune disorders (Boveris et al., 2002; Koulentaki et al., 2004; McDonald et al., 2003; Onur et al., 2001). To date, the over expression of iNOS and/or its catalytic and haem components has been established (Franco et al., 2004; Loibl et al., 2005; Wang et al., 2003). Therefore, NO is a pivotal chemical indicator of inflammation and inflammatory diseases.

*Mitragyna speciosa* Korth (locally known as ‘ketalum’ or ‘biak’) belongs to the family Rubiaceae and is native to the swampy regions in Asia and Africa (Shellard, 1989). This plant can be found largely in Thailand and Malaysia, reaching a height of 15 m, with a spread over 5 m. The stems are erect and branched. The flowers are yellow and spherical. The leaves are dark glossy green and their veins are either greenish-white or red. This plant has application in traditional and folkloric medicine. The leaves are used for its coca-like stimulant ability to combat fatigue and to enhance tolerance to hard work under intense sunlight (Grewal, 1932; Suwanlert, 1975). The plant has long been considered unusual for its dual properties as a stimulant and sedative. In Thailand and Malaysia, the leaves are used for its coca-like stimulant ability to combat fatigue and to enhance tolerance to hard work under intense sunlight (Grewal, 1932; Suwanlert, 1975). The greenish-white veined variety is claimed to possess stronger effect as a stimulant (Suwanlert, 1975). Meanwhile, the sedative opium-like effects were experienced if consumed at large doses and this can be used to treat pain and opium withdrawal (Boyer et al., 2008; Wray, 1907a, 1907b). Till present, scientific research has only communicated mainly on the anaesthetic, antitussive, stimulant and opioid properties of *M. speciosa* (Horie et al., 2005; Matsumoto et al., 2006, 2004, 2005a, 2005b; Takayama, 2004; Takayama et al., 2002, 2006; Watanabe et al., 1997; Yamamoto et al., 1999). Such pharmacological activities were often attributed to the alkaloid contents of the leaf and were obtained through conventional solvent extraction using dichloromethane or methanol.

With the increased concern for the environmental or health hazard through the use of organic solvents for natural product extraction, supercritical fluid extraction is gaining more interest in recent years. Supercritical fluid extraction (SFE) using carbon dioxide (CO₂) in its supercritical state, i.e. pressure > 1070.1 psi (73.8 bar) and temperature > 31.06 °C, permits the process of extraction to be performed at a relatively low pressure and at temperatures close to room temperature. CO₂ is an inert, non-toxic and environmentally friendly solvent. In combination with the fact that CO₂ immediately evaporates when brought to atmospheric conditions, the extracts obtained are free from chemically and thermally degraded compounds. The technique also produces extracts of better quality than those extracted with organic solvents, as the extracts are free from the trace of solvent. Even though present at minute amounts, those organic solvents tend to contaminate the extracts. In supercritical condition, CO₂ possesses unusual properties of high compressibility, liquid-like density, high diffusivity, low viscosity and low surface tension. Therefore, supercritical fluid shows a greater ability to diffuse into the ultrafine matrix compared to the organic solvents, improving yield of extraction of the desired materials from complex matrices.

Therefore, the aim of the present study was to determine the optimize SFE condition of the leaves extract that possesses the most potent anti-inflammatory property with further analysis of the constituents present in this active crude. To the best of our knowledge, this is the first report communicated on the anti-inflammatory effect of the SFE extracts of *M. speciosa* and the chemical constituents of the extract that exhibited the most potent anti-inflammatory activity.

Inflammation is a wellness buzzword these days as it is the culprit behind all diseases. There has been a global trend of increasing incidence of diseases caused by inflammation over time in many countries. In the Arabian region, a significant increase in the trend is evident over time when there has been growing initiative to conduct collaborative multicentre studies for diseases such as arthritis, asthma, inflammatory bowel disease (Crohn’s disease, ulcerative colitis and indeterminate colitis) and other inflammatory diseases (Al-Hussaini et al., 2016; Almoallim and Alharbi, 2014; El Mouzan et al., 2012; Saadah et al., 2016). The magnitude of the inflammatory arthritis problem in the Arabian peninsula has been published for many countries in the region (Saudi Arabia, Oman, Iraq and Kuwait) and the prevalence of rheumatoid arthritis in the Arabian region has been estimated to be 1–2% of the population (Al-Jarallah et al., 2013). Meanwhile, the occurrence rate for Crohn’s disease was 0.27 per 100,000 individuals has been recorded in children aged 0–14 years for the period between 2003 and 2012 (El Mouzan et al., 2014). A large number of steroids and non-steroidal anti-inflammatory drugs (NSAIDS) are available commercially to treat inflammatory diseases. However, despite their great number, their therapeutic efficiency seems to be hampered because they are often associated with severe adverse side effects. This current study has been conducted in hope to search for less toxic anti-inflammatory drug where folk medicinal plants have been established to have advantage in toxicity, due to their time tested safety and efficacy. Findings from this study may bridge up the gaps in inflammation research and could be put into beneficial use for nation worldwide.

2. Materials and methods

2.1. Plant material

Fresh leaves of *M. speciosa* were collected from the forest of Sungai Setong, Kelantan, Malaysia, in November 2006. The plant materials were identified by the herbarium authority at the Department of Chemistry, University of Malaya, where the plant specimen was deposited for future reference (Voucher specimen no. KL5321). The leaves were dried under the shade, assisted by electric light bulbs as a heat source of 35–40 °C for a period of one week. The shed dried leaves were ground by an electrical blender to produce coarse powder of approximately 5 mm size particle.

2.2. Reagents

Carbon dioxide (99.995% purity, SFE grade), contained in a cylinder with an eductor tube, was purchased from MOX (Selangor, Malaysia). Analytical grade solvents, chemical reagents and 95% ethanol were purchased from Merck. The⋯
standard mixture of C7–C24 and other homologous series of \textit{n-}alkanes used in the gas chromatography analysis were obtained from SIGMA Chemical Co., USA. Antibiotic (5000 U/mL penicillin and 5000 \textmu g/mL streptomycin) and Dulbecco’s Modified Eagle’s Media (DMEM) were obtained commercially from Flowlab™, Australia; foetal bovine serum (FBS) was obtained from Mycoplex™ (PAA Lab. GmbH, Austria); recombinant mouse interferon gamma (IFN-\( \gamma \)) was obtained from BD Pharming, USA; lipopolysaccharide (LPS) was from \textit{Escherichia coli} (strain 0111:B4), and sulphanilamide and naphtylethylenediamine were obtained from SIGMA Chemical Co., USA. Dimethyl sulfoxide (DMSO) was purchased from Fisher Scientific, USA, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) from Fluka Chemie GmbH, Switzerland.

2.3. Supercritical fluid extraction (SFE)

Approximately 25 g of the dried ground leaves was loaded into a 100 mL stainless steel extraction vessel of the analytical-scale SFT 100 XW SFE system (illustrated in Fig. 1). An orthogonal test design L9 (3)\(^3\) was employed in order to determine a suitable extraction condition in a wide range with a minimum number of trials, whereby the temperature, pressure and the percentage of modifier (ethanol) were considered to be three major factors for effective extraction. The combinations of three different levels of each factor are listed in Table 1. In each test, two-step extractions were performed. First, the plant material was pre-extracted with pure CO\(_2\) in Step 1 (S1) and afterwards the residue was re-extracted with CO\(_2\) modified with aqueous ethanol solution in Step 2 (S2) as depicted in Table 2. CO\(_2\) was delivered at the rate of 24 mL/min by the SFT-10 Constant Pressure pump. For those studies involving modifier, ethanol solution was added directly to the plant material in the extraction vessel using an additional High Performance Liquid Chromatography (HPLC) pump (Series II Isocratic HPLC pump) prior to the extraction by supercritical CO\(_2\).

In both steps, after 60 minutes of static extraction (no CO\(_2\) flow), the sample material was subjected to dynamic extraction by flowing the CO\(_2\) liquid (depressurized). The extraction process was terminated when it was visually observed that there was no more analyte being trapped in the collection vial (exhaustive extraction). The extracts obtained were evaporated to dryness under reduced pressure at 40 °C (yielding dark green residue). Then their final mass was weighed (Table 2) and later checked by thin layer chromatography (TLC) for their individual profile.

TLC separations were performed using aluminum sheets pre-coated with 0.20 mm thick Silica gel 60 F\(_{254}\) (20 cm × 20 cm) and developed with a mobile phase dichloromethane:methanol (96:4 v/v). The extracts were dissolved in dichloromethane and applied to silica gel plates. TLC plates were then visualized under UV light (254 and 365 nm) and further sprayed with Dragendorff’s reagent to detect the presence of alkaloidal compounds, which was indicated, by the presence of orange colour spots.

Figure 1 The flow sheet of the analytical-scale of SFT 100 XW SFE system.
2.4. Nitric oxide inhibitory activity

2.4.1. Cell cultures and treatment

The murine monocytic macrophages cell line (RAW 264.7 cells) was purchased from American Type Culture Collection (ATCC) and cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin under 5% CO₂ at 37°C. Cells at confluence of 80–90% were scrapped out and centrifuged at 110 g/4°C for 10 min. The cell viability and concentration were determined simultaneously using trypan blue dye exclusion and a hemacytometer. Then, the cells were seeded into 96-well plate at 5 × 10⁴ cells/50 μL per well and incubated for 2 h at 37°C, 5% CO₂ to allow cell attachment. After 2 h, unattached cells were carefully discarded. Attached cells were then induced with 100 U/mL of IFN-γ and 5 μg/mL of LPS in the presence or absence of plant sample tested at a final concentration of 100 μg/mL. The final concentration of the sample vehicle (DMSO) in the reaction mixture was 0.1%. Untreated and drug controls were stimulated with IFN-γ/LPS and also had the same amount of DMSO in culture media. Cells were then incubated at 37°C, 5% CO₂ for 17–20 h. (Syahida et al., 2006).

2.4.2. Griess assay

The production of NO was determined in the supernatants of spent cell culture media by measuring the nitrite ion (NO₂⁻ stable metabolite of NO) formation (Dirsch et al., 1998). Briefly, 50 μL of Griess reagent (1% sulphanilamide and 0.1% naphthylethylenediamine dihydrochloride in 2.5% H₃PO₄) was added to 50 μL of cell culture supernatant and after an incubation time of 10 min, the colour density was measured at 550 nm on a micro plate reader (SpectraMax, Plus 384, Molecular Devices, Inc., USA) at room temperature. Fresh culture medium was used as the blank in all the experiments. The amount of NO₂⁻ in the samples was calculated from a sodium nitrite standard curve (0–100 μM) freshly prepared in deionized water. The percentage of inhibition was calculated against negative control cells (cells that were not treated but induced with IFN-γ/LPS and contained 0.1% DMSO) (Syahida et al., 2006).

\[
\text{NO inhibitory (\%)} = \left(\frac{\text{NO}_2^-_{\text{sample}} - \text{NO}_2^-_{\text{control}}}{\text{NO}_2^-_{\text{control}}}\right) \times 100
\]

2.5. MTT cell viability assay

After the removal of culture media, the wells in micro titre plate were topped up with 100 μL of DMEM followed by the addition of 20 μL of MTT (5 mg/mL). The cells were incubated under 5% CO₂ at 37°C for 4 h. The formed formazan crystals were dissolved in DMSO and the absorbance was recorded at 570 nm on a micro plate reader (SpectraMax, Plus 384, Molecular Devices, Inc., USA) at room temperature.

<table>
<thead>
<tr>
<th>Test no.</th>
<th>M: Matrix Factors</th>
<th>Test no.</th>
<th>M: Matrix Factors</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M1 A1 1300 B1 40 C1</td>
<td>2</td>
<td>M2 A1 1300 B2 60 C2</td>
</tr>
<tr>
<td>3</td>
<td>M3 A1 1300 B3 80 C3</td>
<td>4</td>
<td>M4 A2 3000 B1 40 C2</td>
</tr>
<tr>
<td>5</td>
<td>M5 A2 3000 B2 60 C3</td>
<td>6</td>
<td>M6 A2 3000 B3 80 C1</td>
</tr>
<tr>
<td>7</td>
<td>M7 A3 5000 B1 40 C3</td>
<td>8</td>
<td>M8 A3 5000 B2 60 C1</td>
</tr>
<tr>
<td>9</td>
<td>M9 A3 5000 B3 80 C2</td>
<td>10</td>
<td>M10 A3 5000 B3 80 C2</td>
</tr>
</tbody>
</table>

* Modifier (ethanol, %) = volume of added ethanol (mL)/sample mass (g) × 100.

384. Molecular Devices, Inc., USA). The optical density (OD) of the samples was compared to that of negative control cells (untreated stimulated cells and contained 0.1% DMSO) to obtain the percentage viability using the formula stated below (Syahida et al., 2006):

\[
\text{Cell viability (\%)} = \frac{\text{OD}_{\text{sample}}}{\text{OD}_{\text{control}}} \times 100\%
\]

2.6. Gas chromatographic analyses

The analyses were performed on the SFE extract that possesses the strongest anti-inflammatory activity with no toxicity effect.

2.6.1. Gas chromatography (GC)

GC analysis was carried out using a Shimadzu GC-2010 unit equipped with FID. HP-5 fused silica capillary column of 5\% phenyl 95\% dimethylpolysiloxane (30 m x 0.32 mm i.d., 0.25 \mu m film thickness) was used. The oven temperature was kept at 60 °C for 1 min and programmed to 150 °C at a rate of 4 °C/min. Then it is increased to 280 °C at 3.5 °C/min and finally followed by 20 min under isothermal condition. Detector and injector temperatures were 300 and 250 °C, respectively. The carrier gas was helium (99.999%), adjusted to a linear velocity of 30 cm/s and column flow of 1.21 mL/min. Two \mu L of the diluted M5S1 (4000 ppm) was injected in split less mode.

2.6.2. Gas chromatography–mass spectrometry (GC–MS)

GC–MS analysis was performed using a Shimadzu GC-2010 unit coupled to a quadrupole mass spectrometer, MS-QP2010 Plus with capillary GC conditions consistent with those described above. Mass spectra were taken at 70 eV of ionization energy, 0.5 s of scan time, ion source temperature of 200 °C and a mass range of 40–800 amu.

2.6.3. Identification of constituents

The characterization of components was based on a comparison of their mass spectra with those of NIST08s.LIB mass spectral database. The identifications were further confirmed through comparison of Kovats indices (K1) with those reported in the literature (Adam, 2001; Norsita et al., 2006a, 2006b). The Kovats index was determined with a standard mixture of n-alkanes under the same chromatographic conditions used when analyzing M5S1. Relative amounts of individual components were calculated by the area normalization method without response factor correction. Three replicates of gas chromatography analyses of M5S1 (crude with the strongest anti-inflammatory activity without toxicity effect) were performed. The precision was expressed as the relative standard deviation (RSD) of the peak areas.

2.7. Statistical analysis

Results for NO inhibition and cell viability are expressed as mean ± standard deviation (SD). Data were statistically assessed using Graphpad prism version 5.0 for one-way analysis of variance (ANOVA) with Dunnett’s test. Differences were considered statistically significant at \( p < 0.05 \).

3. Results and discussion

3.1. Supercritical fluid extraction (SFE)

Optimization of the experimental conditions signifies a crucial step in the development of an efficient SFE method through the adjustment of certain parameters that potentially affect the extraction process. Optimization of the method can be performed step-by-step or by using an experimental design. Table 2 exhibits the results of the SFE of M. speciosa carried out under different conditions. Pressure and temperature of the fluid and percentage of the modifier were the selected factors assessed using a three-level orthogonal array design with L\(_9\) (3\(^7\)) matrix. Consequently, a complete evaluation of the three factors will require three level tests, i.e., 27 tests. In order to reduce the number of tests, an L\(_9\) (3\(^3\)) orthogonal design graph was employed, which reduced the total number of tests to 9 (Table 1). A two-step extraction process was designed for this study that included pre-extraction with pure CO\(_2\), makes it a total of 18 tests to be executed.

In general, results depicted in Table 2 indicate the percentage of total yield (S1 + S2) improved with the increment in pressure. At the static pressure of 3000 psi, the yield raised as the temperature was increased from 40 to 60 °C but dropped slightly when the temperature was further increased from 60 to 80 °C. As at 1300 psi and 5000 psi, a rise in temperature resulted in lower yield. The yield of extraction by pure CO\(_2\) also trailed the same trend of increased yield for an increase in the pressure. A rise in the pressure can improve the density of the fluid and thereby resulting in an increased solvating power-giving rise to more yields. At static pressure of 1300 psi, the yield gradually reduced as the temperature increased. Within the pressure range of 3000–5000 psi, the yield increased between the temperatures of 40–60 °C and somehow decreased from 60 to 80 °C. Supercritical CO\(_2\) has a low polarity, making the extraction of polar analytes difficult. This limitation can usually be overcome by adding small amounts of polar entrainer, such as ethanol, to the supercritical CO\(_2\) in order to raise its solubilizing power and thus increase the extraction efficacy. In our experiment, the second step of each test in which the entrainer was added in succession was also meant to be the exhaustive process for completing the recovery of the high-molecular mass components. Anyhow, the results revealed that the addition of entrainer (as modifier) negligibly improved the yield than that of pure CO\(_2\). The increments in the yield were only by 0.01–0.27% except in M7S2, where it was 0.48%.

Basically, in Step-1 (extraction by pure CO\(_2\)) and Step-2 (extraction by CO\(_2\) + modifier) process, when the pressure factor was set to 1300 psi, the yields were low and the total yields were not more than 0.3%. A raise in the pressure from 3000 psi to 5000 psi resulted in a satisfactory improvement in the yield (>0.9%), except for M4S1 (0.69%) for Step-1 process. Overall, the total yields at 3000 psi and 5000 psi, were significant (>0.9%).

3.2. TLC profile of the extracts

Each crude was examined to point out possible interesting differences in the chemical composition obtained by different extraction conditions.
TLC sprayed with Dragendorff’s reagent revealed several orange spots for all crude extracts at 5000 psi but not for those extracted at 1300 and 3000 psi. This indicated that alkaloidal compounds can only be furnished at relatively high pressure and the addition of a polar solvent as modifier did not give any effect in this aspect as shown by crude extracts at 1300 and 3000 psi. Nevertheless, the orange colour spots of the alkaloid were more intense in M7S2, M8S2 and M9S2 compared to M7S1, M8S1 and M9S1 respectively, consequently suggesting that the addition of modifier might enhance the yield of the alkaloid components.

3.3. Nitric oxide inhibitory activity

A total of 15 plant extracts were screened for their inhibitory effect of NO release in RAW 264.7 cells. The overall efficacy of all plant extracts on NO₂ production in IFN-γ/LPS-activated macrophage and cell viability on RAW 264.7 cells are presented in Table 3. The plant extracts were considered as having a strong, moderate or weak activity, if the inhibitory effect of NO was more than 90%, between 50% and 89%, or less than 50%, respectively, as compared to the control. In addition, the viability of RAW 264.7 cells assessed by MTT method must be above 85% to prove that the decrease in cell production was not a result of cell death (Mosmann, 1983). The results revealed that the extracts obtained at 3000 and 5000 psi, using pure CO₂ exhibited stronger NO inhibitory activity (lower production of NO) than those extracted in the presence of modifier (Table 3). Hence suggesting that extraction with 100% CO₂ alone is sufficient enough to enrich extract with compound/s of potent anti-inflammatory property. The results showed that six of the plant extracts demonstrated 51–64%, and nine exhibited 16–48% inhibitory activity towards NO production when tested at a final concentration of 100 µg/mL. Meanwhile, NOS inhibitor, N-nitro-arginine methyl ester (L-NAME), used as the positive control in this assay strongly (86.65%) inhibited the secretion of NO without any cytotoxic effect at the concentration of 250 µM (0.674 µg/mL). Among those samples that moderately reduced NO production, M5S1 showed the highest NO inhibitory activity at 60.08 ± 10.02% without any cytotoxicity effect (cell viability, 91.98 ± 5.58%). Thus, the optimal conditions determined from these tests to furnish an extract that possesses the strongest anti-inflammatory activity with no cytotoxicity effect were extraction conducted using pure CO₂ at 3000 psi of pressure and temperature at 60 °C. The constituents of this extract (M5S1), were identified via GC and GC–MS analyses.

3.4. GC and GC–MS analyses of M5S1

Fig. 2 depicts the GC–MS profile of the SFE extract that possesses the strongest NO inhibitory activity without showing toxicity effect towards the test cells (M5S1). Table 4 lists the compounds identified in M5S1, the relative GC peak areas of these compounds and their experimental KI. RSD values less than 10% were considered good precision for a method. The gas chromatographic profile shows 15 identifiable components that represented 93.53% of the total peak area. The prominent constituents were fatty acids, accumulated to 39.01% of the total yield. The rest of the crude was made up of four hydrocarbons (36.67%), three phytosterols (9.32%), two esters (3.45%), two alcohols (2.30%) and last but not least, a tocopherol (2.78%). Palmitic acid, at 34.90% was the most abundant constituent in the crude matrix followed by heptacosane (18.56%) and nonacosane (11.00%).

Fatty acids have been identified to involve in the formation of healthy cell membranes, proper development and functioning of the brain and nervous system, regulating blood pressure and viscosity as well as immune and inflammatory responses (Eastwood, 1997; Sánchez-Vicente et al., 2009). This may explain the NO inhibition exhibited by M5S1, as it is rich in palmitic acid that has been communicated for its anti-

### Table 3

The effect of *Mitragyna speciosa* SFE extracts on percentage of NO inhibition and cell viability of RAW 264.7 macrophage cells tested at final concentration of 100 µg/mL.

<table>
<thead>
<tr>
<th>Sample</th>
<th>SFE condition</th>
<th>Result</th>
<th>NO inhibition (%)</th>
<th>Cell viability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1S1</td>
<td>1300 psi, 40 °C, 100% CO₂</td>
<td>21.13 ± 4.10</td>
<td>65.41 ± 16.06</td>
<td></td>
</tr>
<tr>
<td>M1S2</td>
<td>1300 psi, 40 °C, 20% EtOH</td>
<td>56.86 ± 8.83</td>
<td>68.51 ± 15.55</td>
<td></td>
</tr>
<tr>
<td>M2S2</td>
<td>1300 psi, 60 °C, 40% EtOH</td>
<td>63.83 ± 4.94</td>
<td>69.40 ± 16.58</td>
<td></td>
</tr>
<tr>
<td>M4S1</td>
<td>3000 psi, 40 °C, 100% CO₂</td>
<td>54.37 ± 7.19</td>
<td>79.39 ± 6.26</td>
<td></td>
</tr>
<tr>
<td>M4S2</td>
<td>3000 psi, 40 °C, 40% EtOH</td>
<td>42.22 ± 4.54</td>
<td>88.46 ± 9.47</td>
<td></td>
</tr>
<tr>
<td>M5S1</td>
<td>3000 psi, 60 °C, 100% CO₂</td>
<td>60.08 ± 10.02</td>
<td>91.98 ± 5.58</td>
<td></td>
</tr>
<tr>
<td>M5S2</td>
<td>3000 psi, 60 °C, 60% EtOH</td>
<td>24.56 ± 9.78</td>
<td>88.10 ± 15.22</td>
<td></td>
</tr>
<tr>
<td>M6S1</td>
<td>3000 psi, 80 °C, 100% CO₂</td>
<td>44.79 ± 1.62</td>
<td>94.45 ± 11.35</td>
<td></td>
</tr>
<tr>
<td>M6S2</td>
<td>3000 psi, 80 °C, 20% EtOH</td>
<td>40.35 ± 11.57</td>
<td>75.61 ± 6.38</td>
<td></td>
</tr>
<tr>
<td>M7S1</td>
<td>5000 psi, 40 °C, 100% CO₂</td>
<td>51.44 ± 15.02</td>
<td>95.28 ± 8.66</td>
<td></td>
</tr>
<tr>
<td>M7S2</td>
<td>5000 psi, 40 °C, 60% EtOH</td>
<td>19.88 ± 6.02</td>
<td>92.92 ± 14.08</td>
<td></td>
</tr>
<tr>
<td>M8S1</td>
<td>5000 psi, 60 °C, 100% CO₂</td>
<td>48.24 ± 0.88</td>
<td>88.33 ± 13.65</td>
<td></td>
</tr>
<tr>
<td>M8S2</td>
<td>5000 psi, 60 °C, 20% EtOH</td>
<td>46.65 ± 2.31</td>
<td>93.95 ± 14.70</td>
<td></td>
</tr>
<tr>
<td>M9S1</td>
<td>5000 psi, 80 °C, 100% CO₂</td>
<td>54.91 ± 7.84</td>
<td>91.32 ± 11.00</td>
<td></td>
</tr>
<tr>
<td>M9S2</td>
<td>5000 psi, 80 °C, 40% EtOH</td>
<td>16.68 ± 1.28</td>
<td>72.24 ± 6.32</td>
<td></td>
</tr>
<tr>
<td>L-NAME</td>
<td>86.65 ± 6.12</td>
<td>95.52 ± 3.50</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are expressed as means ± SD of three independent experiments. NT; not tested.

* Positive control used for Griess assay.
inflammatory activity. Ester bond hydrolysis of membrane phospholipids by phospholipase A2 and consequent release of fatty acids are the initiating steps of inflammation. Therefore, phospholipase A2 inhibitory is one of the ways to control inflammation. The structural and kinetics studies have revealed that palmitic acid is an inhibitor of phospholipase A2, and hence, is an anti-inflammatory compound (Aparna et al., 2012).

Furthermore, the appearance of phytosterols may also enhance the NO inhibition activity exhibited by M5S1 as they have been reported to possess anti-inflammatory (Park et al., 2001), antioxidant (Yoshida and Niki, 2003) and angiogenic (Choi et al., 2002) activities. β-Sitosterol, stigmasterol and campesterol were detected at an appreciable amount, i.e., 6.67%, 0.99% and 1.66%, respectively. Along with these phytosterols, the presence of tocopherol that constituted 2.78% of M5S1, although present at a low concentration, might contribute towards the observed NO inhibition activity through its antioxidant properties, especially against lipid peroxidation in biological membranes (Gast et al., 2005).

Table 4 Constituents identified in the leaves of *M. speciosa* extract obtained by SFE that possesses the strongest nitric oxide inhibitory activity without cytotoxicity effect, M5S1.

<table>
<thead>
<tr>
<th>No.</th>
<th>Identified compounds</th>
<th>KI</th>
<th>Relative content (%)</th>
<th>RSD (%)</th>
<th>ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>α-Bisabolol</td>
<td>1687</td>
<td>1.15</td>
<td>9.1</td>
<td>RI</td>
</tr>
<tr>
<td>2</td>
<td>Z-Nuciferol acetate</td>
<td>1832</td>
<td>2.35</td>
<td>2.3</td>
<td>RI</td>
</tr>
<tr>
<td>3</td>
<td>9 (11,15)-dieno-isopimara</td>
<td>1907</td>
<td>3.54</td>
<td>7.5</td>
<td>RI</td>
</tr>
<tr>
<td>4</td>
<td>Palmitic acid</td>
<td>1984</td>
<td>34.90</td>
<td>1.5</td>
<td>RI, MS, CO</td>
</tr>
<tr>
<td>5</td>
<td>2,3-Dimethoxypropyl tetradecanoate</td>
<td>2073</td>
<td>1.10</td>
<td>1.3</td>
<td>RI</td>
</tr>
<tr>
<td>6</td>
<td>Methyl ester oleic acid</td>
<td>2090</td>
<td>2.20</td>
<td>5.7</td>
<td>RI, MS</td>
</tr>
<tr>
<td>7</td>
<td>Neuzukol</td>
<td>2132</td>
<td>1.15</td>
<td>9.5</td>
<td>RI</td>
</tr>
<tr>
<td>8</td>
<td>Stearic acid</td>
<td>2166</td>
<td>1.91</td>
<td>9.0</td>
<td>RI, MS,</td>
</tr>
<tr>
<td>9</td>
<td>2,10-Dimethyloctacosane</td>
<td>2649</td>
<td>3.57</td>
<td>2.3</td>
<td>RI, MS</td>
</tr>
<tr>
<td>10</td>
<td>Heptacosane</td>
<td>2702</td>
<td>18.56</td>
<td>5.7</td>
<td>RI, MS</td>
</tr>
<tr>
<td>11</td>
<td>Campesterol</td>
<td>2723</td>
<td>1.66</td>
<td>3.7</td>
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</tr>
<tr>
<td>12</td>
<td>β-γ-Stigmasterol*</td>
<td>2731</td>
<td>0.99</td>
<td>6.0</td>
<td>RI, MS</td>
</tr>
<tr>
<td>13</td>
<td>β-γ-Sitosterol*</td>
<td>2739</td>
<td>6.67</td>
<td>3.5</td>
<td>RI, MS</td>
</tr>
<tr>
<td>14</td>
<td>Nonacosane</td>
<td>2904</td>
<td>11.00</td>
<td>2.5</td>
<td>RI, MS</td>
</tr>
<tr>
<td>15</td>
<td>β-γ-Tocopherol*</td>
<td>3036</td>
<td>2.78</td>
<td>9.5</td>
<td>RI, MS</td>
</tr>
</tbody>
</table>

KI; Kovats indices calculated on HP-5 column, RSD; relative standard deviation, ID; means of identification, RI; comparison of Kovats indices with literature values, MS; mass spectral data, CO; co-injection with standard.

* Percentage of total FID peak area obtained on HP-5 column.

* Correct isomer not identified.
Interest has long been focused on the organic solvent extracts of *M. speciosa* obtained through cool percolation or hot extraction (Avula et al., 2015; Beckett et al., 1965; Kitajima et al., 2006; Seaton et al., 1960; Sheppard et al., 1978). Almost all of the previous biological studies have been conducted on the alkaloidal fractions and its major alkaloid constituents (Idayu et al., 2011; Kong et al., 2011; Parthasarathy et al., 2009; Sabetghadam et al., 2013; Shaik Mossadeg et al., 2009; Uthar et al., 2011). There is lack of studies on the non-alkaloidal crude. In this current investigation, studies have been done on the non-organic solvent crude that consist of not only the alkaloids but also the non-alkaloidal fractions. It highlighted the advantage of the SFE technique in affording the non-alkaloid anti-inflammatory compounds such as campesterol, stigmasterol, sitosterol and palmitic acid. This is the first report revealing non-alkaloidal extract of *M. speciosa* exhibited NO inhibition activity, which may be of important potential in anti-inflammatory treatment.

4. Conclusion

In conclusion, this study has identified the nitric oxide inhibitory activity of 15 selected SFE extracts of *M. speciosa* and M5S1 possessed the strongest activity without cytotoxic effect (NO inhibitory activity at 60.08 ± 10.02% and cell viability, 91.98 ± 5.58%). It is noteworthy that M5S1 was constituted largely by fatty acid, in particular palmitic acid (34.90%), which has been claimed as an anti-inflammatory component. Parameters for the extraction of M5S1 are determined as the optimal SFE condition (extraction under pure CO₂, pressure: 3000 psi, temperature: 60 °C) to furnish extract that possesses the highest anti-inflammatory activity without any toxicity. The inference from the observed nitric oxide inhibitory activity supports the traditional use of *M. speciosa* in the treatment of muscle pain and fever.

Authors’ contribution

Conceived and designed the experiments: NT KA SA. Performed the experiments: NT SA. Analyzed the data: NT KA SA. Contributed reagents/materials/analysis tools: KA SA. Wrote the paper: NT JAS YS.

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References


Hall, London


Chemical constituents and nitric oxide inhibitory activity


