Identification of anti-inflammatory compound/compounds in hexane fraction of *Jatropha curcas* root extract

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Abstract. *Jatropha curcas* is a medicinal plant with many therapeutic properties such as anti-inflammatory, anti-malaria, anti-cancer and antioxidant. The root extract has been shown to possess high anti-inflammatory activity. Previously, the compounds responsible for this activity have not been fully elucidated. Two fractions (Fraction 1 and Fraction 2) obtained from a preparative HPLC of the root extract showed significant anti-inflammatory and cytotoxic activities in RAW 264.7 murine macrophage cells with Fraction 1 giving higher nitric oxide (NO) inhibition compared to Fraction 2 and L-NAME. Further purification steps involving column chromatography, thin layer chromatography and analytical HPLC of Fraction 1 produced two fractions labeled as Fraction A and Fraction B. Both fractions showed anti-inflammatory activity without cytotoxic activity in RAW 264.7 cells. Liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis showed that Fraction A contained a group of 18 carbon fatty acid compounds consisting of 2 oxooctadecanoic acids; 15, 16 dihydroxy 9Z, 12Z octadecadienoic acid; octadecadienoic isomer and 15,16 dihydroxy 9Z, 12Z octadecadienoic acid, 15S, 16S. The 18-carbon fatty acid structure was confirmed by nuclear magnetic resonance (NMR) spectral data. The IC₅₀ value of compounds in Fraction A for anti-inflammatory activity in RAW 264.7 cell line was 434.8±0.75 µg/mL. From the analysis, it can be concluded that Fraction A can be classified under 18 carbon long chain fatty acid group based on LC MS/MS and NMR analysis. This active compound shows an inhibition towards NO activity.

Keywords: *Jatropha curcas*, anti-inflammatory, C 18 fatty acids, octadecanoic acid, IC₅₀

INTRODUCTION

Jatropha curcas Linn. belongs to the *Euphorbiaceae* family with about 170 known species (Sabandar *et al.*, 2013). The word *Jatropha* was derived from two

Greek words of “*jatrós*” means “doctor” and “*trophé*” means “food” (Kumar and Sharma, 2008). Although it was a native plant of Mexico

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and Central America, the plant is now widespread in other parts of the world including South-east Asia, India and Africa. It has gained importance in Malaysia as a source of seed oil for biofuel production. In many African and Asian countries, *J. curcas* plant is considered a traditional herb to cure various ailments ranging from simple fever to infectious diseases, including sexually transmitted (Pandey *et al.*, 2012). In the tropics and Central America, *Jatropha* seeds are used as a purgative and supplement in tea drinks. However, this practice was believed to cause strong irritation, poisoning and cancer (Adolf *et al.*, 1984). A research conducted in Sri Lanka showed that *Jatropha* seeds could also be used as a pregnancy abortifacient (Goonasekera *et al.*, 1995). However, in Mexico, the seeds are roasted or boiled before being used as an ingredient in traditional cuisines (Makkar *et al.*, 1998).

The *Jatropha* leaves have been used to treat various illnesses. The Mayan used leaves parts to treat rashes and bone fracture (Comerford, 1996). In Ghana, the leaves were used for anti-malarial treatment by boiling the leaves and using the extract as a drink (Asase *et al.*, 2005). The plant leaves had also been reported to be low in sodium and considered appropriate for a person who practiced low sodium diet (Méndez *et al.*, 2014). The latex from *Jatropha* tree also possesses medicinal property, whereby in Indonesia, the latex was used to treat infected wounds and ulcer (van den Berg *et al.*, 1995). The *Jatropha* root is also useful in the treatment of certain diseases. A report from Brazil claimed that the extract from *Jatropha* rhizome could treat *Schistosomiasis mansoni* disease that is caused by *S. mansoni* worm present in a fresh-water mollusk *Biomphalaria glabrata* which infects the liver (dos Santos and Sant'Ana, 1999). Another property of *Jatropha* root extract is the anti-inflammatory activity on RAW 264.7 macrophage cells (Mujumdar and Misar, 2004; Nayak and Patel, 2010). Our previous report (Othman *et al.*, 2015) showed that the anti-inflammatory activity of *J. curcas* root was due to the presence of fatty acids. However, the nature of the active fatty acids was unknown. Thus, the present study was conducted to elucidate the structure of the active fatty acids involved in the anti-inflammatory activity.

MATERIALS AND METHODS

Purification of compounds by chromatographic methods

Jatropha curcas L. roots were obtained from Farm 2, Faculty of Agriculture, Universiti Putra Malaysia (UPM). The plant was verified by the botanist, Dr. Shamsul Khamis at the Institute of Bioscience, UPM and deposited in the Phytomedicinal Herbarium, Institute of Bioscience, UPM with voucher number SK1764/2010. The root sample was extracted with 80% methanol, followed by liquid partition chromatography (Othman *et al.*, 2015). The hexane fraction which showed high anti-inflammatory activity was subjected to preparative high-performance liquid chromatography (HPLC). Two fractions (Fraction 1 and Fraction 2) obtained showed significant anti-inflammatory and cytotoxic activities in RAW 264.7 murine macrophage cells. The fractions were further purified by a mini open column (1x4 cm), packed with silica gel 60:70-230 Mesh (ASTM) and prewashed with hexane: ethyl acetate (EtOAc) (7:3). A sample of 1 mL (20.0 mg/mL) was loaded and eluted with 3 mL of hexane:EtOAc (7:3). The mobile phase, consisted of 20 mL hexane:EtOAc solution (6:4) was used to collect 1 mL eluates. The eluates were subjected to analytical thin layer chromatography (TLC) on silica gel 60 F₂₄₅ (0.25 mm thickness) coated plates and developed in hexane:EtOAc (6:4). The spots were viewed under UV at the wavelength of 254 nm (short wave) and 360 nm (long wave). The spots with similar profiles were pooled and air dried. The dried samples were first dissolved in hexane and were analyzed by analytical HPLC. A JASCO Liquid Chromatography System consisting of a JASCO Pump PU-2089 Plus and JASCO Absorbance Detector model UV-2077 Plus 4-Intelligent UV/VIS Detector linked by JASCO BORWIN version 1.5 software was used. The analysis was conducted by using an Agilent column, ZORBAX Eclipse XDB-C18 (4.6 x 250 mm: 5 µm). The solvent system was MeOH:H₂O in a ratio that ranged from 2% to 100% of H₂O in a gradient mode at a flow rate of 1.0 ml/min. The gradient was set at 2% of H₂O from 0 to 5 minutes, continuously increasing the water percentage to 5% from 5 min to 20 min. After 20

minutes, the water percentage was increased to 20% for 10 min before 100% MeOH was used to flush the column for 30 min. Two fractions (Fraction A and Fraction B) obtained from this analysis were subjected to anti-inflammatory and cytotoxic assays.

Compound analyses

Fraction A which showed higher anti-inflammatory activity without cytotoxic activity than Fraction B was used for identification of compounds present in the fraction. The fraction was analyzed by Liquid Chromatography Tandem Mass Spectrometry (LCMS/MS) and Nuclear Magnetic Resonance (NMR). LCMS/MS analysis was done using AB Sciex 3200QTrap LCMS with Perkin Elmer FX 15 UHPLC system. An Agilent Zorbax C18 (150 mm x 4.6 mm: 5µm) was used as the column. The mobile phase consisted of A (water) and B (acetonitrile). Both solvents A and B contained 0.1% formic acid and 5 mM ammonium formate. A gradient was programmed at 10% B to 90% B from 0.01 to 8.0 min, hold for 2 min and back to 10% B in 0.1 min with re-equilibration for 5 min. The sample volume was set at 20 µL per injection.

Proton-1 and carbon-13 NMR analyses were carried out on Varian NMR system 500 MHz at 500 and 125 MHz respectively. The signals obtained were described in terms of chemical shifts (δ ppm), multiplicity, coupling constants and a number of protons and carbons where appropriate. Both 1D and 2D NMR were measured in deuterated chloroform (CDCl_3).

Anti-inflammatory, cytotoxic assay and IC_{50} value determination

Murine monocytic macrophage RAW 264.7 cell were cultured in Dulbecco's Modified Eagle Media (DMEM) with 4 mM L-glutamine, 45 g/L glucose, 1 mM sodium pyruvate and 10% of fetal bovine serum (FBS). The cells were incubated in a 50 ml culture flask in a CO_2 incubator with 5% of CO_2 at 37°C. Then the RAW 264.7 cells were seeded in a 96-well microplate (1×10^6 cell/mL) and incubated in 5% CO_2 at 37°C for 2 to 3 hours. Different volumes with final concentration (1.0, 0.5, 0.25, 0.125 mg/mL) of sample were diluted in DMSO were added to the wells by serial dilution for IC_{50} determination. The cells were then stimulated by adding 100 U/mL of IFN- γ and 5

ug/mL of LPS from *Escherichia coli* strain 055:B5. The cells were incubated in 5% CO_2 at 37°C for 18 hours. The nitric oxide (NO) concentration produced by RAW 264.7 cell was determined by Griess assay (Syahida *et al.*, 2006). N-nitro-L-arginine-methyl ester (L-NAME) which acted as iNOS inhibitor was used as a positive control at a concentration of 250 µM. The cytotoxic effect of the sample was determined by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Syahida *et al.*, 2006). All experiments were done in triplicate.

Statistical analysis

All data were subjected to one-way analysis of variance (ANOVA). Treatment means were compared using Tukey's multiple comparison tests. The IC_{50} value was determined by using one-phase decay analysis. Statistic software Graphpad Prism 5.0 (Graphpad Software Inc., San Diego, CA) was used for all statistical analyses.

RESULTS AND DISCUSSION

Anti-inflammatory activity of Fraction A and Fraction B

The analytical HPLC analysis produced two fractions labeled as Fraction A and Fraction B. Fraction A showed a higher nitric oxide (NO) inhibition than Fraction B and L-NAME as shown in Figure 1a. Both fractions were non-toxic towards RAW 264.7 cells growth (Figure 1b). The purification steps conducted with the methanolic extract of *J. curcas* produced an active anti-inflammatory fraction without cytotoxicity activity. The non-toxic property is an important consideration as the anti-inflammatory assay was based on the measurement of NO production and a decreased in NO quantity could also mean cell death. As shown by the MTT assay from previous analysis, the hexane fraction caused cell death, while the Griess assay showed a reduction in NO production (as an indication of anti-inflammatory activity) of the RAW 264.7 macrophage cells (Othman *et al.*, 2015). After a series of purification steps, the fractions obtained (Fraction A and B), showed an anti-inflammatory activity and were non-toxic towards RAW 264.7 macrophage cells. This indicates that the compounds extracted were

actually inhibiting the NO production of the RAW 264.7 macrophage cells through inhibition of NO signaling pathway.

Figure 1a. Anti-inflammatory activity of Fraction A and Fraction B. Fraction A exhibited a higher anti-inflammatory activity compared to Fraction B and L-NAME. L-NAME and induced cells represented the positive and negative control, respectively. Each histogram represents a mean of three replicates with error bar representing the standard deviation. *Indicates significant difference ($P < 0.05$) compared to the experiment control.

Figure 1b. Cytotoxic activity of Fraction A and Fraction B. Both fractions showed no cytotoxic activity towards RAW 264.7 murine macrophage cells growth. Each histogram represents a mean of three replicates with error bar representing the standard deviation. *Indicates significant difference ($P < 0.05$) compared to the experiment control.

Elucidation of active compound by LCMS/MS and NMR

Analysis by LCMS/MS negative mode showed four major peaks in Fraction A that belonged to long chain fatty acid group (Figure 2). The data were then compared with the databases of ACD/Labs advanced chemometrics mass fragmentations predictive software. According to the database analysis, the four compounds with almost similar m/z values belonged to the octadecadienoic acid and an octadecanoic acid group (Figure 2). The compounds nomenclature and their mass values are shown in Table 1. The compounds identified were 12 oxooctadecanoic acid (Figure 2a), a substituted saturated C18 fatty acid; 15, 16 dihydroxy 9Z, 12Z octadecadienoic acid (Figure 2b), a substituted unsaturated C18 fatty acid; octadecadienoic isomer (Figure 2c), an unsaturated C18 fatty acid and octadecanoic acid isomer (Figure 2d), a saturated C18 fatty acid.

The chemical structures of compounds in Fraction A were confirmed by NMR analysis. The NMR data which were analyzed by using ACD/NMR software from ACD/Labs.com are presented in Table 2. The peak signals were assigned at δ_H 0.88 (t, $J = 6.6$ Hz, 2H), 1.26 (br.s., 28H), 1.63 (m, 2H), and 2.34 (t, $J = 7.5$ Hz, 2H). Proton-1 and carbon-13 peak analysis are presented in Figure 3a and 3b, respectively. Although, a fifth peak was observed in the LCMS/MS spectrum, but the structure was not verified by NMR spectral data. Hence, the compound was unidentified.

The LCMS/MS analysis showed the presence of four compounds as members of the C18 fatty acids. The correlations of C-H according to the NMR data were in agreement with the structure belonging to a long chain fatty acid due to the presence of carboxylic group peak at 180 ppm. No aromatic group was observed in the C13 analysis. Hence, the results of both LCMS/MS and NMR analyses were in agreement that the compounds present in the active Fraction A were C18 fatty acids.

However, the results obtained in the present study could not identify the specific compound responsible for the anti-inflammatory without cytotoxic activity. It is possible that all the fatty acids identified possessed anti-inflammatory activity as previous reports have shown that long chain fatty acids such as linolenic acid, linoleic

acid, palmitoleic acid and octadecadienoic acid possessed anti-inflammatory activity (Ballou and Cheung, 1985; Mathavi and Velavan, 2013), these fatty acids was proven to regulate the anti-inflammatory pathway (Aparna *et al.*, 2012). The anti-inflammatory mechanism of fatty acid was shown by using enzyme kinetic study and X-ray crystallography analysis (Kudo and Murakami, 2002). It was reported that the carboxylic group from saturated fatty acid binds to the amino acid Asn 67 of the hydrophobic region of the phospholipase A₂ (PLA₂) active site (Kudo and Murakami, 2002). Based on the fatty acid mechanism explained earlier, it can be assumed that fatty acids competed with PLA₂ substrate,

thus acting as a competitive inhibitor towards PLA₂. This enzyme is involved in catalyzing the release of arachidonic acid, a precursor for the synthesis of inflammatory mediators at the *sn*-2 position of membrane phospholipid (Diaz and Arm, 2003; Calder, 2004).

Table 1. Compounds identified by LC-MS/MS in Fraction A.

Compound name	m/z
12 Oxooctadecanoic acid (Figure 2a)	183.1
15,16-Dihydroxy-9Z,12Z-octadecadienoic acid (Figure 2b)	183.0
Octadecadienoic acid isomer (Figure 2c)	184.0
Octadecanoic acid isomer (Figure 2d)	183.0

Table 2. 2D-NMR spectral data of Fraction A.

δ_H (in ppm)	δ_C (in ppm)	COSY (in ppm)	HMBC (in ppm)
0.88, 2H, <i>t</i> , <i>J</i> = 6.6	14.1	1.63 (s), 1.26	22.6, 31.9
1.26, 28H, <i>br.s</i> (Overlapped)	22.7, 24.7, 29.7, 31.9	1.63	14.1, 22.6, 31.9
1.63, 2H, <i>m</i> , <i>J</i> = 7.5	24.7	2.34	180, 34.1
2.34, 2H, <i>t</i> , <i>J</i> =7.5	34.1	1.26, 1.63	180, 29.7, 24.7

Figure 2. LC-MS/MS spectrum. Compounds 1 to 4 of Fraction A were assigned based on comparison to databases.

Figure 3. NMR spectrum of Fraction A (a) ^1H -NMR spectrum (CDCl_3 ; 500MHz) and (b) ^{13}C -NMR spectrum (CDCl_3 ; 125MHz).

Inhibitory concentration of active compounds in Fraction A

Inhibitory concentration (IC_{50}) indicates the amount of sample concentration that could inhibit 50% of NO production in RAW 264.7 macrophage cells. A series of sample concentrations ranging from 0.0 to 1.0 mg/mL were evaluated. Sample absorbance was measured at 550 nm and IC_{50} graph was plotted using Graphpad Prism 5 software. The IC_{50} value of the active compounds in Fraction A was determined to be 434.8 ± 0.75 $\mu\text{g/mL}$ (Figure 4).

Inhibitory concentration assay of Fraction A showed an IC_{50} value of 434.8 ± 0.75 $\mu\text{g/mL}$ in murine macrophage RAW 264.7 cells. In an earlier study, Oskoueian *et al.* (2011) reported a concentration of 3.1 $\mu\text{g/mL}$ of methanolic extract of *J. curcas* root could inhibit NO production almost completely in RAW 264.7 macrophage cells (Oskoueian *et al.*, 2011). However, the latter authors also observed that the crude extract was toxic to the cells. This raised the question whether NO inhibition was actually the result of cell death or the anti-inflammatory activity per se. On the other hand, it was clearly shown in the present study that the isolated compounds in Fraction A from the *J. curcas* root were non-toxic to the RAW 264.7 macrophage cells. Even though the IC_{50} value for the present study was higher compared to the previous report, it proved that the inhibition of compounds in Fraction A was due to the inhibition of NO signaling pathway and not by the toxicity effect towards RAW 264.7 growth.

The presence of active compounds which showed NO inhibition from other plant sources

has been reported. A crude extract from *Porphyra dentata* (seaweed) showed more than 95% inhibition of NO production at 200 $\mu\text{g/mL}$ with more than 90% of cell viability (Kazłowska *et al.*, 2010), while extract of *Phaleria macrocarpa*, (a tropical herbal plant), showed the highest inhibition of NO production in RAW 264.7 cells at 3.13 $\mu\text{g/mL}$ (Hendra *et al.*, 2011). A hexane fraction prepared from *Orostachys japonicas* at 100 $\mu\text{g/mL}$ showed inhibition of NO production in RAW 264.7 cells with cell viability over 80% (Lee *et al.*, 2013). These inhibitory concentrations were lower than the value observed in the present study. This is not surprising as crude extract might contain a complex mixture of compounds that would possibly work synergistically to give a potent anti-inflammatory activity compared to a single compound.

Figure 4. An IC_{50} analysis of anti-inflammatory activity of Fraction A. The IC_{50} of Fraction A in RAW 264.7 macrophage cells were 434.8 ± 0.75 $\mu\text{g/mL}$. The assay showed that Fraction A was not toxic towards RAW 264.7 cells growth.

CONCLUSION

The nature of compounds presents in *J. curcas* root methanolic extract which possessed anti-inflammatory without cytotoxicity activity in RAW 264.7 macrophage cells in vitro was elucidated. The compounds were identified as long chain fatty acids with 18 carbons based on mass spectrophotometry and NMR analysis. The IC₅₀ value of Fraction A in NO inhibition was 434.8±0.75 µg/mL.

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