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ORIGINAL PAPER

Antioxidants, Toxicity, and Nitric Oxide Inhibition Properties of Pyroligneous Acid from Palm Kernel Shell Biomass

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Abstract

Huge volumes of lignocellulosic biomass residues generated from agricultural activities such as oil palm biomass pose great environmental threats if improperly treated. In this study, pyroligneous acid (PA) obtained from slow pyrolysis of palm kernel shell (PKS) were evaluated for its antioxidant activity via chemical assays, toxicity, and potential as anti-inflammatory agent based on nitric oxide (NO) inhibition activity. The PA was extracted using ethyl acetate (EA) and fractionated using column chromatography. Fractions 13–17 that contain highest total phenolic contents (866.84 ± 54.28 µg GAE/mg) were chosen for subsequent studies. Results obtained were as follows; DPPH—75.34 ± 3.40%, TEAC—1346.48 ± 5.29 µg Trolox/mg, FRAP—11.80 ± 0.41 mmol Fe(II)/mg, hydroxyl radical scavenging—IC_{50} 270.34 ± 4.88 µg/mL, superoxide radical scavenging—IC_{50} 472.32 ± 1.87 µg/mL, cytotoxicity after 24 h at less than 50 µg/mL—cell viability of ≥ 93.08% for RAW 264.7 macrophage cell and anti-inflammatory activity with NO production of 6.55% after 24 h at sample concentration of 25 µg/mL. From the GC–MS analysis, phenols and derivatives were identified as major compound (83.24%) followed by esters (11.23%), and ketones (5.53%). The present of phenolic compounds namely benzene-1,2-diol, catechol (35.01%), 1,3-dimethoxy-2-hydroxybenzene, syringol (23.81%), and other catechol derivatives can be attributed to the antioxidant and anti-inflammatory activities determined. This study has successfully demonstrated the potential use of PA obtained from PKS as alternative antioxidant and anti-inflammatory agent.

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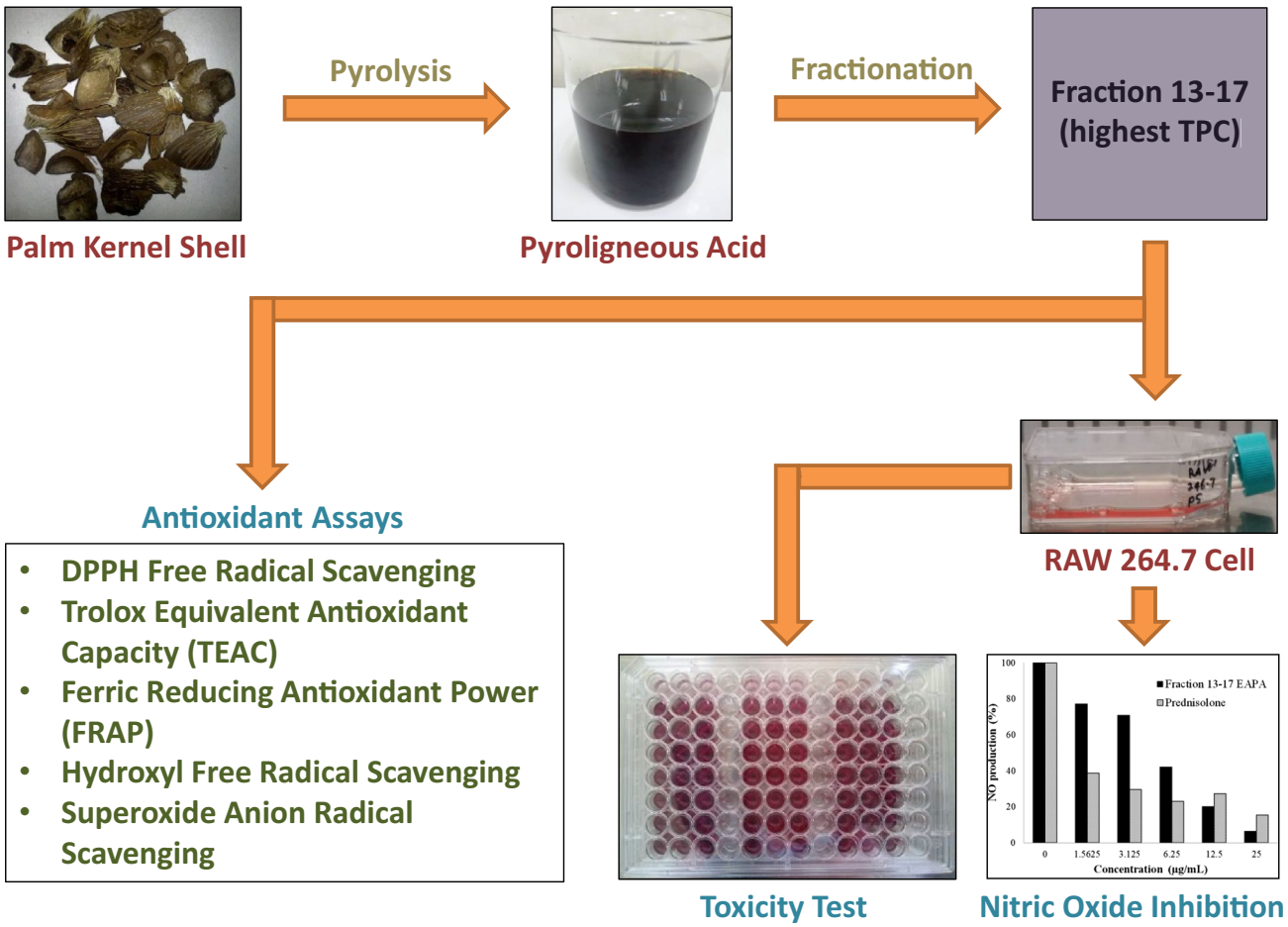
Graphic Abstract



Keywords Pyroligneous acid · Palm kernel shell · Phenolic · Antioxidants · Anti-inflammatory

Statement of Novelty

Evaluation on the efficiency of phenolic compounds in pyroligneous acid (PA) obtained from palm kernel shell (PKS) as anti-inflammatory agent and application of PA from PKS as anti-inflammatory agent.

Introduction

In recent years there has been a growing attention on plant-derived bioactive compounds for application in the pharmaceutical and nutraceutical areas [1]. However, the depletion of natural growth area and the long period needed for the plant to mature makes it imperative to find alternative sources for bioactive compounds [2]. Plants biomass or by-products derived from agricultural activity have been proven to be effective sources of bioactive compounds, of which pyrolysis is one of the techniques that can be employed for the treatment processes [3–5]. Pyrolysis is thermal degradation process of the organic components in biomass wastes in the absence of oxygen. Pyrolysis process offers efficient utilization of biomass to produce solid (char), liquid (bio-oil, tar and pyroligneous acid) and gaseous product [6].

Pyroligneous acid (PA) or wood vinegar is highly oxygenated organic aqueous by-product resulting from smoke condensate during slow pyrolysis for charcoal production [5]. During slow pyrolysis process, the plant biomass is slowly heated and the smoke released is channeled through a pipe to allow condensation and cooling of the smoke. The sedimentation process of the aqueous condensed vapor gives three layers of liquid, the top layer will be the light oil; the middle layer will be crude brown pyroligneous acid and wood tar will be at the bottom. Pyroligneous acid consists of more than 200 different compounds including phenolics,

ketones, organic acids, furan and pyran derivatives, esters, alcohols, aldehydes, alkyl aryl ether and benzene derivatives, and sugar derivatives, in variable concentrations [7]. With the presence of multiple compounds, pyroligneous acid has shown various potential benefits in agriculture as insecticide [8], antimicrobial [9], fertilizer [10], soil enhancer [11], and for plant growth and development [12]. Pyroligneous acid has also been used as a supplement in animals feeding and to improve digestibility and nutrient absorption [13, 14]. Various sources of pyroligneous acid including mangrove [15], walnut shell [16, 17], rosemary leaves [4], and pineapple biomass [18] have been reported to have antioxidant activity. Studies showed that the phenolic compounds are major compound responsible for the antioxidant activity of pyroligneous acid [4, 15, 16]. Three compounds namely 2,6-dimethoxyphenol, 1,2-benzenediol, and 3-methoxycatechol appeared to be the main compound that contribute to the antioxidant activity of pyroligneous acid from mangrove [15]. Phenolic compounds of pyroligneous acid from walnut shell namely 2,6-dimethoxyphenol [16, 17] and 1,2-benzenediol [16] was identified as major components that showed best antioxidant activity. Additionally, alongside with phenolic and its derivatives compounds which are phenol and eucalyptol, alkene compounds namely 1S-α-Pinene also acted as major functional components which responsible for the antioxidant activity of pyroligneous acid from rosemary leaves [4]. Anti-inflammatory activities of pyroligneous acid from oak wood [19] and bamboo [5] also have been reported. The pyroligneous acid from oak wood contains phenolic acid and was claimed to possess both antioxidant and anti-inflammatory properties. The phenolic fraction and purified phenolic compound namely 2-methoxy-4-methylphenol of pyroligneous acid from bamboo showed inhibition of inflammatory mediator.

In Malaysia, oil palm trees are widely cultivated for palm oil production. Their residuals are likely burnt to produce charcoal and activated carbon, where the pyroligneous acid as by-product. In the current study, we investigate the antioxidant, toxicity, and nitric oxide inhibition properties of the pyroligneous acid obtained from the slow pyrolysis of palm kernel shell (PKS). The crude extracts and different fractions obtained were evaluated and major compounds present were also identified.

Materials and Methods

Characterization of Palm Kernel Shell (PKS)

The PKS sample was collected from oil palm mill in Kulai, Johor, Malaysia. The PKS is a fibrous material, brownish in colour and it has size range of 0.5–2.5 cm. PKS was grounded using commercial blender (38BL41, Waring Commercial, USA) and sieved to 50 micron size prior to analysis. Methods as highlighted in the American Society for Testing and Materials (ASTM) were used to determine moisture content (ASTM D4442-07) [20], ash (ASTM E1755-01) [21] and volatile matter (ASTM D5832-98) [22]. While the Standard Laboratory Analytical Procedure (LAP) [23] was used to determine lignin content (LAP-003 and LAP-004), cellulose (LAP-002) and extractives (LAP-010). Ultimate analysis (C, H, N, S) were performed on Macro Elemental Analyzer (Elementar, Germany).

Production of Pyroligneous Acid (PA)

The PA was obtained from the carbonization process of PKS using pilot-scale pyrolysis microwave reactor from the Malaysian Palm Oil Board (MPOB) Research Station located at Pekan Bangi Lama, Kajang, Malaysia. The pyrolysis system (Fig. 1) consisted of microwave reactor (3 ft. × 3 ft. × 3 ft.) equipped with magnetron and stirrer. PKS (25 kg) was loaded into the microwave and heated with gradient temperature elevation until 350 to 400 °C for 12 h for complete carbonization where the condensate was collected at 17 °C. The condensate (PA) has a clear reddish brown colour, with pH value of 2.94 ± 0.02 (Eutech Instrument, Singapore), density of 1.03 ± 0.01 g/mL and water content of 64.35 ± 0.23% (870 KF Titrino plus, Metrohm, Swiss).

Extraction of Pyroligneous Acid (PA)

The solid particle and debris from PA was removed using the Whatman No. 1 filter paper. The PA was then extracted using ethyl acetate ($C_4H_8O_2$, 88.11 g/mol, 99.5%, QRec) at 1:1 ratio. The collected organic phase solution was concentrated in a rotary vacuum evaporator (Heidolph, Germany) at 40 °C to remove the solvent and was further dried in a vacuum desiccator for 1 week to obtain ethyl acetate extract of pyroligneous acid (EAPA). Ethyl acetate showed high extraction efficacy for phenolic compound of pyroligneous acid from *Eucalyptus* wood, followed by dichloromethane and diethyl ether without significant differences between them [24]. The use of ethyl acetate as extraction solvent has advantages due to its high degradability and considered safe as opposed to the halogenated solvents dichloromethane and chloroform [25].

Fractionation of Ethyl Acetate Extract of Pyroligneous Acid (EAPA)

The aliquot of EAPA was spotted using a microcapillary on the TLC plate baseline (about 0.5 cm from one end of the plate) and placed into the TLC chamber containing single (*n*-hexane/dichloromethane/chloroform/ethyl acetate) or combination of two

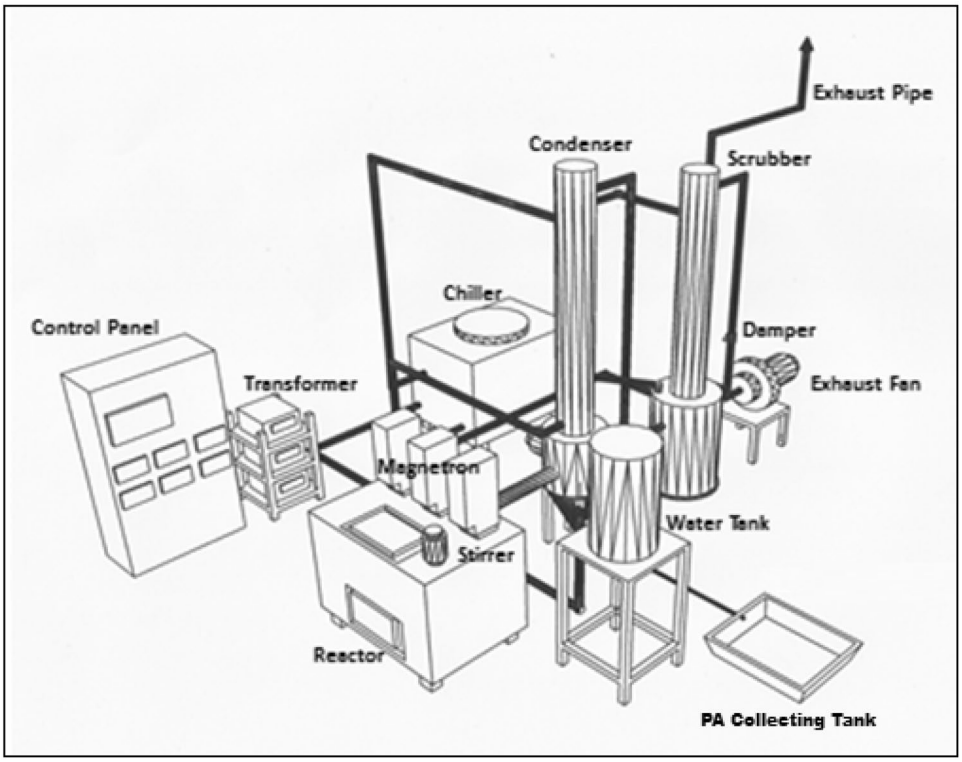


Fig. 1 Pilot-scale pyrolysis microwave reactor

solvent (*n*-hexane:dichloromethane/*n*-hexane:chloroform/*n*-hexane:ethyl acetate) at different ratio of increasing polarity. The TLC plate was visualized under 254 and 365 nm UV light. The EAPA (23.60 g) was coated with silica gel 60 (0.063–0.200 mm) (Merck, Germany) and fractionated by gravity column chromatography. It was then eluted using 100% of *n*-hexane, *n*-hexane: ethyl acetate, ethyl acetate: methanol and 100% methanol. A total of 137 fractions of 200 mL each was collected in a conical flask and then concentrated using rotary evaporator (Heidolph, Germany). Each concentrated fractions of EAPA was further spotted on TLC silica gel plate. Fractions showing similar profiles observed on TLC silica gel plate were combined (6–8, 9–12, 13–17, 18–20, 21–23, 24–27, 28–38, 39–46, 47–61, 62–92, and 93–137) to yield the corresponding 11 subfractions.

Total Phenolic Content (TPC) Determination

The total phenolic content (TPC) in the EAPA fractions were determined using the Folin–Ciocalteu's assay as follows [26]; 1 mL of sample was transferred into a 15 mL falcon tube containing 1 mL of 50% (v/v) Folin–Ciocalteu phenol reagent. The mixture was vortexed (IKA, German) for 30 s and left to stand for 2 min at room temperature. Then, 1 mL of 10% (w/v) Na_2CO_3 solution was added into the mixture and was immediately vortexed for 10 s and allowed to stand in the dark for 2 h at room temperature. The mixture was then measured for absorbance at 765 nm using UV–Vis spectrophotometer (Shimadzu, Japan) where the TPC content was determined from the calibration curve of gallic acid, as standard ($y = 0.03686x - 0.112$; $R^2 = 0.9992$) and reported as mean values expressed as microgram of gallic acid equivalents per milligram of sample (μg GAE/mg sample). Fraction with the highest amount of total phenolic content was chosen for subsequent studies. It was determined for chemical composition using GC–MS analysis (Agilent 6890 N-5973 N, CA, USA) as follows; the stationary phase used was HP-5 MS (5% phenyl and 95% dimethyl polysiloxane) coated fused silica capillary column (30 m × 0.25 mm × 0.25 μm) (J&W Scientific, USA). The oven temperature was programmed at 70 °C for 0 min, heated at 10 °C/min to 250 °C, and then heated again at 5 °C/min to 300 °C and held for 10 min. The injector and detector temperature were maintained at 250 °C. A sample volume of 1 μL with a 20:1 split ratio was injected. Helium was used as carrier gas with a constant flow rate of 2.0 mL/min. The electron impact ionization mode was set at 70 eV and the scan range was between 50 and 550 *m/z*. The compounds were identified by the comparison of mass spectra with the library data (NIST Search Library 2.0).

Antioxidant Activity Assays

The antioxidant activity assays were evaluated for DPPH free radical scavenging activity, Trolox Equivalent Antioxidant Capacity (TEAC), Ferric Reducing Antioxidant Power

(FRAP) Assay, Hydroxyl Free Radical Scavenging Capability and Superoxide Anion Radical Scavenging Capability.

The scavenging capability of EAPA fraction towards 1,1-diphenyl-2-picrylhydrazyl (DPPH.) free radical was determined using a slightly modified procedure [27]; 200 µL of sample or standard L(+)-Ascorbic acid ($C_6H_8O_6$) (QReC, New Zealand) and butylated hydroxyanisole, 96% (BHA) ($C_{11}H_{16}O_2$) (Acros Organics, New Jersey, USA) at different concentration of 10, 15, 20, 25, 30 µg/mL respectively, was mixed with 1.8 mL of the methanolic DPPH. solution (60 µM). The control was prepared by mixing 1.8 mL of the methanolic DPPH. solution and 200 µL of methanol, while methanol alone was used as blank. The mixture was shaken and allowed to stand at room temperature in the dark for 30 min and absorbance was monitored at 517 nm. The scavenging capability of DPPH free radical was calculated according to Eq. (1). To determine the quality of the antioxidant activity, IC_{50} value was calculated from the graph plotted between DPPH radical scavenging capabilities (%) against samples concentration.

$$\text{DPPH radical scavenging (\%)} = \left[\frac{(Abs\ Control - Abs\ Sample\ or\ Standard)}{AAbs\ Control} \times 100\right] \quad (1)$$

Method suggested by [28] was used to determine TEAC of the EAPA fraction. 200 µL of 20 µg/mL of PA or standard L(+)-Ascorbic acid ($C_6H_8O_6$) (QReC, New Zealand) and butylated hydroxyanisole, 96% (BHA) ($C_{11}H_{16}O_2$) (Acros Organics, New Jersey, USA) was mixed with 1.8 mL of the $ABTS^{\cdot+}$ solution. The mixture was incubated at 37 °C for 7 min and the absorbance was monitored at 734 nm. The result was determined from the calibration curve of Trolox ($y = -0.0104x + 0.6324$; $R^2 = 0.9934$) and reported as mean values expressed as microgram of Trolox equivalents (TE) per milligram of sample (µg TE/mg sample).

FRAP assay was performed to determine the reducing antioxidant capacity using the modified method [26]. 70 µL of samples or standard L(+)-Ascorbic acid ($C_6H_8O_6$) (QReC, New Zealand) and butylated hydroxyanisole, 96% (BHA) ($C_{11}H_{16}O_2$) (Acros Organics, New Jersey, USA), respectively, was added into 2.1 mL of freshly mixed FRAP reagent and was allowed to react for 90 min at 37 °C in the dark before analyzed at absorbance of 593 nm. The standard curve was constructed using $FeSO_4$ solution ($y = 0.7285x - 0.0044$; $R^2 = 0.9999$) and the results are expressed as mmol Fe(II) being reduced by per milligram of sample (mmol Fe(II)/mg sample).

The hydroxyl free radical scavenging capability was measured based on method described previously [16]. Briefly, 0.5 mL sample was added to a tube that contained a mixed solution of 2.0 mL phosphate buffer (150 mmol/L, pH 7.4), 2.0 mL 1,10-phenanthroline (0.75 mmol/L), and 2.0 mL $FeSO_4$ (0.75 mmol/L). 1.0 mL H_2O_2 (0.01%, v/v) was added immediately, and the absorbance (A_0) was measured at 536 nm. Absorbance A_1 was measured by repeating the same procedure except that the sample was replaced by 0.5 mL distilled water. Similarly, absorbance A_2 was measured by the same procedure except that the sample and H_2O_2 was replaced by 1.5 mL distilled water. Ascorbic acid was used as a reference antioxidant compounds. The rate of hydroxyl radical scavenging was calculated using Eq. (2):

$$\text{Hydroxyl free radical scavenging (\%)} = \left[\frac{A0 - A1}{A1 - A2} \times 100\right] \quad (2)$$

The superoxide anion radical scavenging capability was measured based on a pyrogallol autooxidation method [16]. 2.95 mL phosphate buffer (50 mmol/L, pH 8.34) and 200 µL distilled water was mixed and homogenized at 25 °C water bath for 20 min, then 50 µL pyrogallol (0.2 mmol/L) that preheated at 25 °C water bath was added with a total volume of 3.2 mL. The mixture was shaken up quickly, and the absorbance was measured at 325 nm every 30 s, the difference of absorbance within per minute was calculated (ΔA_0). Ascorbic acid was used as reference antioxidant reagent. The same procedure was conducted except that the distilled water was replaced by the pyroligneous acid sample to calculate ΔA_1. Superoxide anion radical scavenging capability was calculated using Eq. (3):

$$\text{Superoxide anion radical scavenging capability (\%)} = \left[\frac{\Delta A0 - \Delta A1}{\Delta A0} \times 100\right] \quad (3)$$

Cell Viability and Anti-inflammatory Assay

Cell Culture and Subculture

A murine macrophage RAW 264.7 cell lines was provided by the Department of Pharmacy, Faculty of Medicine, Universiti Malaya, Kuala Lumpur, Malaysia. The stock cell culture of RAW 264.7 in dimethyl sulfoxide (DMSO) was previously stored at − 80 °C and was in Passage 4. The stock cell culture was rapidly thawed in waterbath (Memmert) at

37 °C. Then, 1 mL of thawed cells was cultured in 25 cm^2 tissue culture flasks containing 5 mL culture medium composed of Rosewell Park Memorial Institute 1640 medium (RPMI 1640) (Gibco, Thermo Fisher Scientific, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Thermo Fisher Scientific, USA), 100 units/mL penicillin and 100 µg/mL streptomycin and incubated overnight at 37 °C in a HEPA Filtered incubator containing 5% CO_2 (Forma Steri-Cycle CO_2 Incubator, Thermo Scientific). Then, the residual culture medium was discarded and replenished with 5 mL of fresh culture medium followed by overnight incubation prior to subculturing.

For subculturing, residual culture medium was removed and the cell was washed with sterilized phosphate buffer saline (PBS). The cell was then de-attached from the flask using 2 mL of accutase (Gibco, Thermo Fisher Scientific, USA). The flask was placed inside the incubator for 5 min and observed for cell detachment under light microscope. Then, 2 mL of fresh RPMI 1640 medium was added and the mixture (4 mL) was transferred into 15 mL of falcon tubes. The mixture was centrifuged at 1500 rpm for 5 min and the supernatant was discarded. 3 mL of fresh RPMI 1640 medium was added to the cells pellet and mixed well. The mixture was transferred into three 75 cm^2 flask with 1 mL each and top-up with 9 mL of fresh media in each flask. The culture was incubated for 3 days at 37 °C in a HEPA Filtered incubator containing 5% CO_2 (Forma Steri-Cycle CO_2 Incubator, Thermo Scientific). The growth of cell was further observed under microscope after incubation.

Cell Viability Assay

Cell viability assay as suggested by [29] was carried out with slight modification. The RAW 264.7 cells at a density of 5×10^3 cells per well in 96-well plates (Costar, Corning Inc.) in 100 µL of culture medium was incubated for 24 h at 37 °C in a humidified atmosphere containing 5% CO_2. After incubation, residual culture medium was discarded and treated with different concentration (100, 50, 25, 12.5, 6.25, 3.125, and 1.5625 µg/mL) of EAPA fraction in 100 µL fresh medium and incubated for 24 h, 48 h and 72 h at 37 °C in a humidified atmosphere containing 5% CO_2. After incubation of cell at indicated treatment times, 20 µL of MTT (3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide) (Life Technologies, Oregon, USA) was added and incubate at 37 °C for 4 h. The medium was removed and 100 µL of DMSO was added to each well to solubilize the crystallized formazan product. The absorbance was recorded using ELISA at 570 nm to quantify MTT reduction. The relative cell viability in percent relative to control containing culture medium without samples was calculated using Eq. (4):

$$\text{Cell viability } (\%) = \left[\frac{Abs\ sample}{Abs\ control} \times 100\right] \quad (4)$$

Anti-inflammatory Assay

Nitric oxide inhibition assay [30] was conducted to evaluate the anti-inflammatory activity of EAPA fraction. RAW 264.7 macrophage was activated using lipopolysaccharides (LPS) from *E. coli* 0111:B4 strain (Sigma, USA). RAW 264.7 cells (3×10^4 cells/200 µL medium) was seeded onto 96-well plates and incubated for 2 h for adherence at 37 °C in humidified atmosphere (5% CO_2). The cell then was treated with 1 µg/mL of lipopolysaccharide (LPS) in the absence or presence of different concentration (25, 12.5, 6.25, 3.125, and 1.5625 µg/mL) of EAPA fraction. Following incubation for 24 h, 100 µL of culture supernatant was mixed with 100 µL of Griess reagent kit for nitrite determination (Molecular Probes, USA) and was incubated for 30 min in the dark. Nitrite concentration was determined using ELISA by measuring the absorbance at 540 nm with a plate reader. Sodium nitrite ($NaNO_2$) was used to generate a standard curve ($y = 0.009x + 0.0223$; $R^2 = 0.996$). Prednisolone was used as reference compounds.

Statistical Analysis

All assays were carried out in triplicate and results obtained were expressed as mean ± standard deviation (SD). GraphPad prism was used for growth curve analysis in dose-dependent experiments and to determine the IC_{50} values. The significance of differences ($p < 0.05$) among groups of data was determined with one-way analysis of variance (ANOVA) using statistical software SPSS 16.0 for Window and Duncan's multiple range test.

Table 1 Properties of the palm kernel shell raw materials

Component analysis (wt%)[a]		Ultimate analysis (wt%)[a]		Proximate analysis (wt%)[a]	
Lignin	42.97	C	47.70	Moisture	9.5
Cellulose	35.64	H	5.75	Ash	2.5
Hemicellulose[b]	21.39	N	0.90	Volatiles	84.5
Extractive (ethanol)	3.19	S	0.04	Fixed carbon[b]	3.5
		O[b]	45.61		

[a]As-received basis

[b]Calculated by difference in summative

Results and Discussion

Characterization of Palm Kernel Shell (PKS) and Pyroligneous Acid (PA)

Table 1 profiles the component, proximate and ultimate analyses for PKS. The contents of lignin, cellulose, hemicellulose and the ethanol soluble extractive were 42.97%, 35.64%, 21.39%, and 3.19%, respectively. The determination of ethanol soluble extractives was conducted to determine the non-structural components of biomass [23]. For ultimate analysis, carbon was determined as the highest element present in PKS with value of 47.70%, followed by oxygen (45.61%), hydrogen (5.75%), nitrogen (0.90%), and sulphur (0.04%). High carbon content is general characteristic of woody biomass [31]. The results for proximate analysis was 9.5% for moisture content, 2.5% for ash yield, 84.5% for volatile matter, and 3.5% for fixed carbon. The PA has the following characteristics; pH of 2.94 ± 0.02 at room temperature, density of 1.03 ± 0.01 g/mL and water content of 64.35 ± 0.23%. The acidity of PA was contributed by the presence of organic acids such as acetic acids derived from the acetyl groups linked to the xylose unit of hemicellulose [32].

Total Phenolic Content of EAPA Fractions

For the EAPA fractions, highest total phenolic content (TPC) was determined for fraction 13–17 with a value of 866.84 ± 54.28 µg GAE/followed by fraction 9–12 (794.39 ± 21.52 µg GAE/mg) and fraction 6–8 (640.50 ± 14.03 µg GAE/mg), which were significantly different ($p < 0.05$). Lowest TPC was determined for fraction 93–137 with a value of 4.49 ± 1.17 µg GAE/mg). From Table 2, it was shown that fractions obtained using least polar solvent (mixture of *n*-hexane: ethyl acetate (9 to 6: 1 to 4) as mobile phase in column chromatography) contain higher TPC values. The suitability of using least polar solvent to extract phenol and its derivatives was supported from report by other researcher [33]. Phenolic compounds are large class of plant secondary metabolites that play a major role in delaying oxidation process due to its potent antioxidant properties [34]. Previous study has shown the importance of phenols and its derivatives compounds in antioxidant activity [4, 15, 18, 22]. Several reports also have highlighted the close relationship between TPC and antioxidative activity of the plant extracts. It has been reported that phenolic compounds with ortho and para-dihydroxylation or a hydroxyl and a methoxy group are more effective antioxidant than simple phenolics [35]. Fraction 13–17 was subjected to further characterization using Gas Chromatography–Mass Spectrometry (GC–MS) analysis to determine the structure of the compounds responsible as antioxidant agents.

Table 2 The total phenolic content of EAPA fractions

Sample	Fraction	TPC (µg GAE/mg sample)
EA-IPA-PKS	6–8	640.50 ± 14.03^{c}
	9–12	794.39 ± 21.52^{b}
	13–17	866.84 ± 54.28^{a}
	18–20	445.85 ± 6.52^{d}
	21–23	347.15 ± 3.14^{e}
	24–27	313.39 ± 7.33^{f}
	28–38	$158.03 \pm 1.61^{g,h}$
	39–46	179.97 ± 1.76^{g}
	47–61	131.95 ± 0.65^{h}
	62–92	50.35 ± 1.74^{i}
	93–137	4.49 ± 1.17^{j}

Each value is expressed as mean ± SD; different superscript alphabets show significant difference at $p < 0.05$ by Duncan's multiple range test

Chemical Profiles of EAPA Fraction

Fraction 13–17 of EAPA was further analysed using GC–MS to determine the compounds presents in the fraction. Compounds were identified by their mass spectra with those in commercial library data (NIST Search Library 2.0). Results were accepted when a matching percentage ≥ 80% was obtained. From the GC–MS analysis, 9 compounds were identified from fraction 13–17 EAPA with phenols and derivatives as major compound (83.24%) followed by esters (11.23%) and ketones (5.53%) (Table 3). The main phenols and derivatives compounds included benzene-1,2-diol (catechol) (35.01%), 1,3-dimethoxy-2-hydroxybenzene (syringol) (23.81%), 3-methoxy-1,2-benzenediol (12.55%), 4-methylbenzene-1,2-diol (8.09%), 4-ethyl-1,2-benzenediol (2.23%), and 5-(2-methyl-2-propanyl)-1,2,3-benzenetriol (1.55%). Other groups were esters (methyl 4-hydroxy-3-methoxybenzoate, 9.22% and methyl 4-hydroxy-3-methoxybenzaldehyde, 2.01%) and ketones (2-hydroxy-3-methyl-2-cyclopenten-1-on, 5.53%).

Figure 2 shows the structure of the compounds present in fraction 13–17 EAPA. Benzene-1,2-diol, 3-methoxy-1,2-benzenediol, 4-methylbenzene-1,2-diol, and 4-ethyl-1,2-benzenediol has two hydroxyl group (–OH) structure compared to 1,3-dimethoxy-2-hydroxybenzene with one hydroxyl group structure. Meanwhile, 5-(2-Methyl-2-propanyl)-1,2,3-benzenetriol has three hydroxyl group structure. Other compounds present i.e. 2-hydroxy-3-methyl-2-cyclopenten-1-on, 4-hydroxy-3-methoxybenzaldehyde, and methyl 4-hydroxy-3-methoxybenzoate, each contain one hydroxyl group structure. The presence of aromatic ring and hydroxyl groups can act as electron or hydrogen

Table 3 Chemical composition of fraction 13–17 EAPA

No	Retention time (min)	Compounds	Molecular formula	Relative contents (%)
Phenols and derivatives				83.24
1	6.08	Benzene-1,2-diol (Catechol)	$C_6H_6O_2$	35.01
2	6.97	3-Methoxy-1,2-benzenediol	$C_7H_8O_3$	12.55
3	7.25	4-Methylbenzene-1,2-diol	$C_7H_8O_2$	8.09
4	8.15	1,3-Dimethoxy-2-hydroxybenzene (Syringol)	$C_8H_{10}O_3$	23.81
5	8.46	4-Ethyl-1,2-benzenediol	$C_8H_{10}O_2$	2.23
6	10.29	5-(2-Methyl-2-propanyl)-1,2,3-benzenetriol	$C_{10}H_{14}O_3$	1.55
Ketones				5.53
7	3.94	2-Hydroxy-3-methyl-2-cyclopenten-1-one	$C_6H_8O_2$	5.53
Esters				11.23
8	8.76	4-Hydroxy-3-methoxybenzaldehyde	$C_8H_8O_3$	2.01
9	9.33	Methyl 4-hydroxy-3-methoxybenzoate	$C_9H_{10}O_4$	9.22

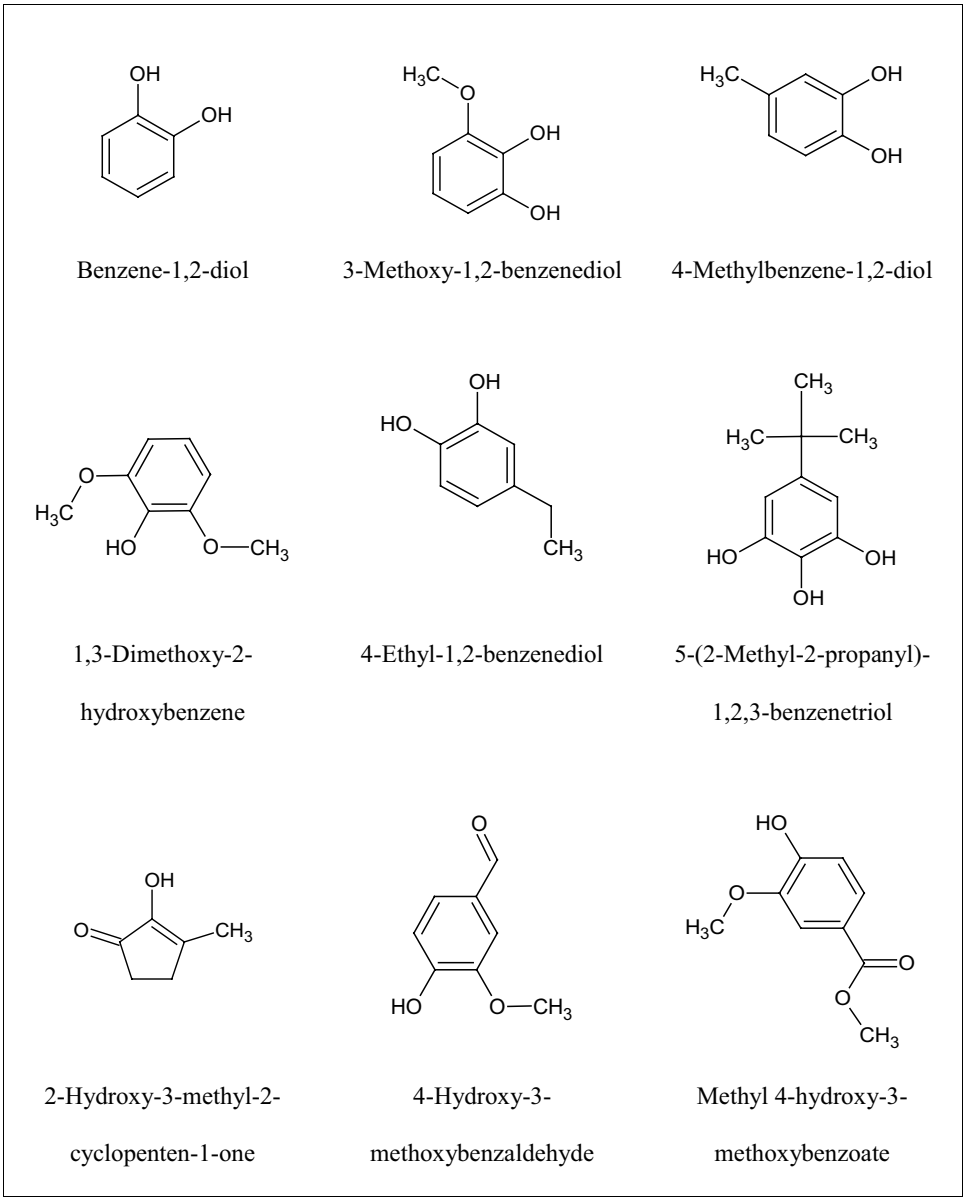


Fig. 2 Structure of the compounds presented in fraction 13–17 EAPA. The basic structure consists of the aromatic rings with hydroxyl group (–OH)

donators and neutralize the free radicals and reactive oxygen species [36]. High phenols and its derivatives content in PA originated from the pyrolytic degradation of lignin [37]. Degradation of lignin during thermal reaction release aromatics compounds of guaiacyl and syringyl due to cleavage of α-ether and β-ether weak bonds [18]. Lignin would generally be converted into phenol, syringol, catechol and other derivatives [26]. While chemical compounds such as alcohols, aldehydes, ketones, carboxylic acids and ethers were mainly derived from the decomposition of cellulose and hemicelluloses [38]. PA produced from other types of lignocellulosic biomass were also reported to contain high phenolic contents that indicates its potential application as antioxidant agent. Some examples are PA from biomass of *Rosmarinus officinalis* with phenolic contents between 6.08 and 22.2% [4], *Rhizophora apiculata* (1,3-dimethoxy-2-hydroxybenzene, 49.34%) [15], walnut shell (1,3-dimethoxy-2-hydroxybenzene, 8.38–13.80%) [16] and *Schisandra chinensis* Baill (4-methylphenol (p-cresol), 16.24–22.84%) [26]. Phenolic compounds play an important role as a free radical scavenger, hydrogen donator, metal chelator and singlet oxygen quenchers [39].

Antioxidant Activity

Highest DPPH free radical scavenging activity for fraction 13–17 EAPA was obtained at 30 µg/mL with inhibition value of 75.34 ± 3.40%, followed by ascorbic acid (62.81 ± 0.49%), and BHA (38.62 ± 2.59%). Similar trend ($p < 0.05$) was obtained for IC_{50} value with EAPA fraction showing most potent activity with value of 18.58 ± 0.57 µg/mL, followed by ascorbic acid (24.37 ± 0.43 µg/mL), and BHA (38.60 ± 1.97 µg/mL). From the results obtained, the TEAC values decreases in the following order (in µg equivalents/mg sample); fraction 13–17 EAPA (1346.48 ± 5.29), BHA (1231.80 ± 5.29), and ascorbic acid (956.57 ± 5.29). These findings were clearly significant as it signifies the suitability of using EAPA fraction from PKS as antioxidant. Previous study on phenolic compounds namely syringol, catechol, and 3-methoxycatechol isolated from PA of *Rhizophora apiculata* shows the TEAC value of 956 ± 40, 1022 ± 53, and 1039 ± 51 µg equivalents/mg of the sample, respectively [15]. For the FRAP assay, fraction 13–17 EAPA showed almost similar capabilities with ascorbic acid with values of (in mmol Fe(II)/mg sample) 14.37 ± 0.26 and 14.24 ± 0.10 respectively ($p < 0.50$). At 0.01 mg/mL, PA from walnut shell showed FRAP values of 10–33 mg/L of $FeSO_4$ compared to ascorbic acid (13 mg/mL) [16]. The FRAP properties for purified fractions of *Rhizophora apiculata* was also reported as follows (in mg ascorbic acid equivalents/g of the sample) [15]; benzene-1,2-diol, catechol (2283 ± 168), 3-methoxy-1,2-benzenediol, 3-methoxycatechol (1560 ± 155) and 1,3-dimethoxy-2-hydroxybenzene, syringol (635 ± 35). However, fraction 13–17 EAPA showed much lower capacity of scavenging hydroxyl free radical (IC_{50} of 270.34 ± 4.88 µg/mL) as opposed to ascorbic acid (IC_{50} of 3.12 ± 0.00 µg/mL). Hydroxyl radicals (.OH) are endogenously produced in cells by Fenton type-driven reactions and they react efficiently with most molecules at the site of generation [40]. Any compounds has to be present at a concentration equal to or higher than other compounds (proteins, lipids, nucleic acids, amino acids, numerous metabolites, etc.) that already present in any biological system to be even 50% effective in solution [41]. Based on GC–MS profiling for fraction 13-17 EAPA, two phenolic compounds (benzene-1,2-diol (catechol) and 1,3-dimethoxy-2-hydroxybenzene (syringol), with known antioxidative properties were present. This explains why .OH cannot be scavenged effectively, in contrast to numerous incorrect claims that examine the addition of antioxidants to the cellular medium [41]. Ascorbic acid shows highest antioxidant properties against superoxide anion radical with inhibition value of 97.48 ± 0.28% followed by fraction 13–17 EAPA (51.62 ± 0.10%) at concentration of 500 µg/mL. However BHA did not exhibit any radical scavenging effect at these concentrations (result not shown). The present study shows that fraction 13–17 EAPA can effectively scavenge reactive oxygen species such as superoxide radicals in place of synthetic antioxidant, such as BHA. Ascorbic acid was the most potent superoxide anion radical scavenger with IC_{50} of 283.80 ± 1.00 µg/mL, followed by fraction 13–17 EAPA (472.32 ± 1.87 µg/mL). Even though PA from PKS exhibits superoxide scavenging capability of twofolds lower than ascorbic acid, it is still worthy to be considered as a potential alternative antioxidant agent.

Cell Viability and Anti-inflammatory Assay

Cell Viability Assay

The cytotoxicity of fraction was rated based on the percentage of viable cell; 90%—non cytotoxic, 60 to 90%—slightly cytotoxic, 30 to 59%—moderately cytotoxic, and < 30%—strongly cytotoxic [42–44]. At 100 µg/mL, fraction 13–17 EAPA exhibit moderate cytotoxicity towards the RAW 264.7 macrophages cells with 41.33% cell viability after 24 h of treatment. When the contact time was increased to 48 and 72 h, the percentage of cell viability was reduced to 27.83% and 25.40% respectively. The cell showed morphological alteration with cells shape becoming irregular and exhibited shrinkage, indicating strong toxicity of EAPA at high concentration (Fig. 3). When the EAPA was reduced to 50 µg/mL, the cell viability increased to 71.03% after 48 h of treatment which indicates slight cytotoxic properties towards cells. However, prolonging the contact time to 72 h, cells viability decreased to 24.83% (indicative of strong

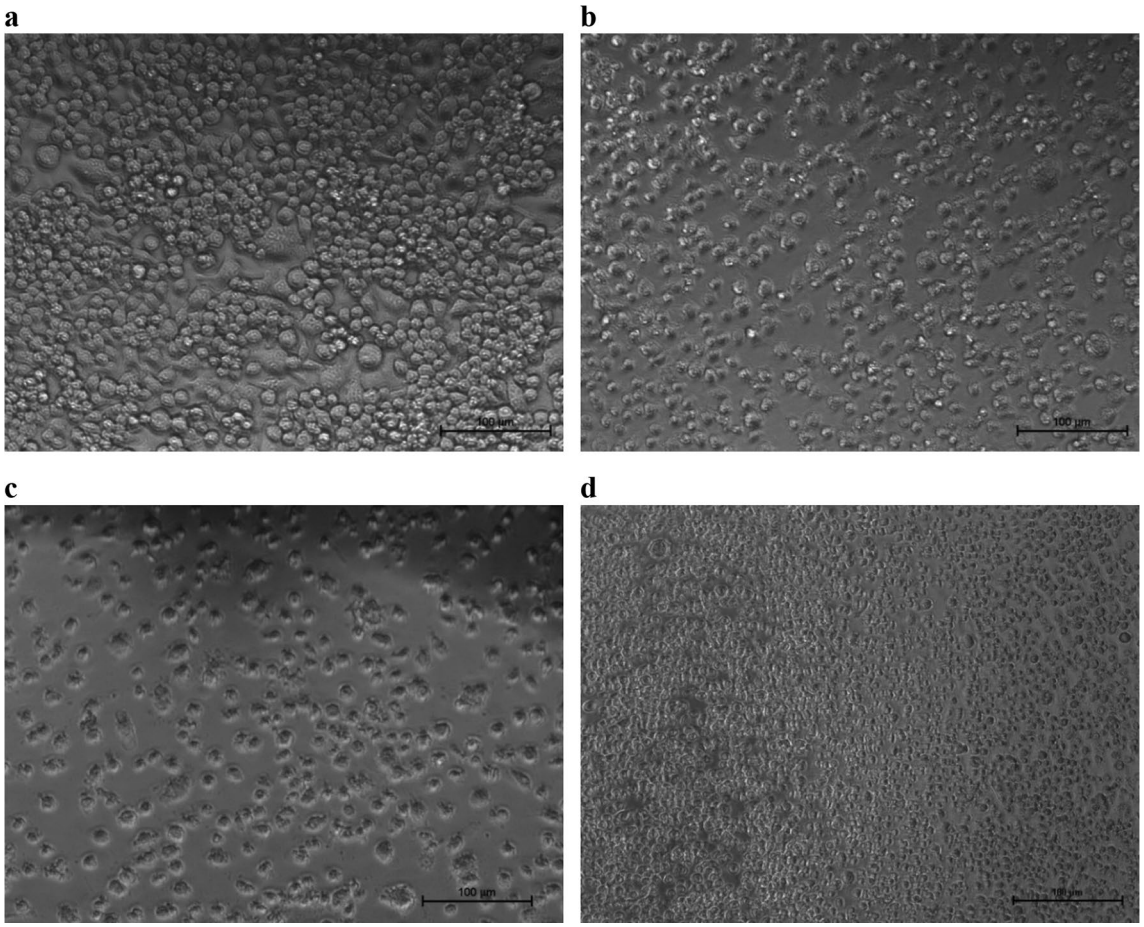


Fig. 3 Morphology of RAW 264.7 macrophage cell visualised under inverted light microscope (×10) after incubation with 100 µg/mL of fraction 13–17 EAPA: **a** Control, **b** 24 h, **c** 48 h, **d** 72 h. Scale bar 100 µm

cytotoxicity towards cells). At concentration of ≥ 50 µg/mL, cell viability was ≥ 93.08% (after 24 h of contact time) and ≥ 97.84% (6.25 µg/mL, 48 h) and 85.90% (25 µg/mL, 72 h) indicating its non-cytotoxicity and slightly cytotoxic towards cells. At this concentration, morphology of the cell macrophages remain intact relative to the normal RAW 264.7 macrophage cell with round and smooth appearance [45]. All concentrations evaluated below than 25 µg/mL did not show toxic effects to the cells with viability of ≥ 90.82%. Slight reduction of cells viability observed for 3.125 µg/mL after 72 h can be attributed to cell apoptosis due to nutrient depletion rather than cytotoxic effect from fraction 13–17 EAPA. RAW 264.7 cells has the highest growth rate, thus the nutrients in the medium might be rapidly depleted during the log growth phase of the cells [46].

Other researchers also reported on the cytotoxicity effects of phenolic content in PA towards cells macrophages. Cell viability of RAW 264.7 cells was not significantly reduced by 50 µg/mL of phenolic fraction present in PA from bamboo after 24 h of contact time [5]. The phenolic compounds are able to stimulate two signalling events in the mammalian cell lines namely mitogen-activated protein kinases (MAPKs), which regulate cells function including proliferation, gene expression, differentiation, mitosis, cell survival, and apoptosis, and ICE/Ced-3 proteases (caspases), which play a key role in apoptotic cell death [47]. At low concentrations, phenolics activated the MAPKs, leading to expression of genes provoking protective mechanisms (homeostasis response). However, on increasing phenolics concentrations, these compounds additionally activated the ICE/Ced-3 proteases pathway, leading to apoptosis (potential cytotoxicity). Other causes that contribute to the toxicity of the pyroligneous acid, may originate from the presence of wood tar derivatives [48].

Nitric Oxide Inhibition Assay

The anti-inflammatory activity of fraction 13-17 EAPA and steroid drug prednisolone as positive standard was investigated using LPS-activated RAW 264.7 macrophages. As shown in Fig. 4, nitiric oxide (NO) generation was inhibited by all samples and standard in a dose-dependent manner.

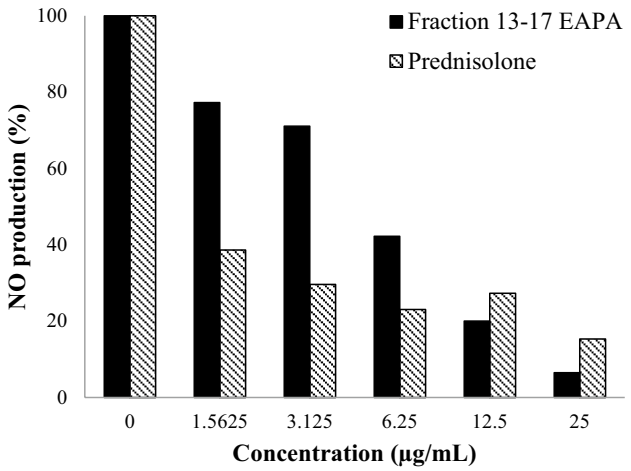


Fig. 4 NO production (%) of LPS-activated RAW 264.7 macrophage treated with different concentration (µg/mL) of fraction 13–17 EAPA and prednisolone for 24 h

For RAW 264.7 macrophages treated with fraction 13-17 EAPA, the production of NO was 77.24%, 71.09%, 42.22%, and 20.01% at concentration of 1.5625, 3.125, 6.25, and 12.5 µg/mL, respectively. The prednisolone showed high inhibition of NO with NO production of 38.62%, 29.61%, 23.06% and 27.31% at concentration of of 1.5625, 3.125, 6.25, and 12.5 µg/mL, respectively. To examine whether the effect of NO generation was due to reduced cell viability, the toxicity of fraction 13–17 EAPA and prednisolone for LPS-activated RAW 264.7 macrophage was examined using MTT assay and were found to have no effect on cell viability at concentrations 1.5625 to 25 µg/mL when compared to control (without LPS activation, 0 µg/mL of test sample) after 24 h. The reduction of NO production of the LPS-activated RAW 264.7 macrophage treated with PA from bamboo was reported not due to reduction in cell viability [5]. Thus, it can be deduced that fraction 13–17 EAPA able to inhibit the NO generation without reducing the cell viability. Other researcher has also reported the phenolic fraction of PA and its active compound namely 2-methoxy-4-methylphenol (creosol) from bamboo which was capable reduce the NO levels in LPS-activated RAW 264.7 macrophages in a dose-dependent manner [5]. Similar finding was reported for PA from oak wood [49].

NO is an endogenous free radical species and has identified as a potent pro-inflammatory mediator and the production is catalyzed by iNOS (inducible Nitric Oxide Synthase) enzyme [50]. iNOS generally exist in macrophages and the expression of iNOS is stimulated by endotoxins, lipopolysaccharide-stimulated macrophage or TNFs [5, 51]. NO regulates various physiological activities, including smooth muscle contractility, platelet reactivity, neurotransmission, and cytotoxic activity of immune cells. Overproduction of NO caused various illness conditions, including septic shock, neurodegenerative disorders, and inflammation [52].

Conclusion

The present study suggests that the ethyl acetate extract of PA from PKS biomass may be a novel source for the development of potential natural antioxidant as well as anti-inflammatory agents. The phenol and derivatives compounds recovered from the PA of PKS may be applied in various applications such as in chemicals, food, pharmaceutical, and agriculture industries.

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