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Yosie Andriani
Nadia Madihah Ramli
Noor Suryani Musa
Kanwal Kanwal
Habsah Mohamad

See next page for additional authors

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¹Institute of Marine Biotechnology, Universiti Malaysia Terengganu, Mengabang Telipot, Kuala Nerus, Terengganu, Malaysia, ²Educational Chemistry Program, Faculty of Teacher Training and Education, Bengkulu University, Bengkulu, Indonesia, ³Faculty of Science and Marine Environment, Universiti Malaysia Terengganu, Mengabang Telipot, Kuala Nerus, Terengganu, Malaysia

**ABSTRACT**

**Background:** The current study is the first study that investigated the potential of phenolic-rich fraction of *Pandanus tectorius* fruits as an anti-inflammatory agent. The fruit is rich in phenolic compounds, especially from the flavonoids group. Phenolics are known to contribute to an anti-inflammatory activity.

**Objectives:** The first objective of the study was to evaluate the anti-inflammatory potency of phenolic-rich fractions of *P. tectorius* fruits by in vitro and in vivo studies. The second was to investigate the cytotoxicity against RAW264.7 cell lines, antioxidant, and phenolic contents to support the experiment.

**Materials and Methods:** Sample fractionation was done with hexane, ethyl acetate, and methanol successively using column chromatography. Cytotoxicity against RAW264.7 cell lines, phenolic content, and antioxidant property were investigated by MTT, Folin-Ciocalteu, and DPPH assays, respectively. During the *in vitro* study, the anti-inflammatory potency of *P. tectorius* fruit fractions was examined by measuring NO production using Griess reagent in LPS-stimulated RAW264.7 cell lines. During the *in vivo* study, the anti-inflammatory potential of *P. tectorius* fruit fractions was tested on the *Rattus norvegicus* paw edema, with inflammation induced using 2.5% formalin. The fraction was given 30 min before the injection of formalin.

**Results:** All fractions, namely, hexane (PHF), ethyl acetate (PEF), and methanol (PMF), had no cytotoxic activity against RAW264.7 cell lines. PEF fraction showed highest phenolic content, antioxidant activity, and highest ability to reduce NO production. Furthermore, paw edema decreased at 6 h by 16.67% in Group C (PEF fraction treatment) and 29.63% in Group D (drug control, ibuprofen) compared to Group B (inflammatory control and formalin treatment).

**Conclusion:** It is confirmed that phenolic-rich fraction of *P. tectorius* fruits has the potency to reduce inflammation.

**Keywords:** Anti-inflammatory, Antioxidant, Nitric oxide, *Pandanus tectorius*, Paw edema, RAW264.7
INTRODUCTION

Inflammation is a natural and healthy process. It is a physiological immune response of body tissues towards any external stimuli, such as pathogens, irritants, or damaged cells. An inflammatory response begins with the activation of various immune cells, such as macrophages, neutrophils, and lymphocytes. Inflammation can be acute or chronic; acute inflammation is very beneficial when needed, but chronic inflammation gives rise to some problems.[1] In the regulation of inflammation and immune responses, cells that play an important role are macrophages.[2] In general, macrophage cells generate inflammatory mediators such as nitric oxide (NO) and inflammatory cytokines that take an essential role in host survival and tissue repair.[3] A synthesis of NO occurs as a result of oxidation of an amino acid L-arginine through enzymatic action of NO synthase (NOS). The NOS family consists of three isoforms, namely, neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS). Among them, iNOS is known as the prominent factor that induces the production of NO.[4] NO production is controlled by NOS enzyme. The NOS enzyme converts L-arginine into L-citrulline and NO. Three different isozymes of iNOS are INOS, ENOS, and NNOS. Most importantly INOS is highly expressed in macrophages; its activation leads to organ destruction in some inflammatory and autoimmune diseases. Another important inflammatory mediator is PGE, and it is produced from arachidonic acid metabolites by the catalysis of cyclooxygenase-2 (COX-2).[5,6] During inflammation, macrophages play a central role in managing many different immunopathological phenomena, including the overproduction of pro-inflammatory cytokines and inflammatory mediators such as IL-1β, IL-6, NO, iNOS, COX-2, and TNF-α. Indeed, a number of inflammatory stimuli, such as LPS and pro-inflammatory cytokines, activate immune cells to upregulate such inflammatory states, and these are therefore useful targets in the development of new anti-inflammatory drugs and exploration of the molecular anti-inflammatory mechanisms of potential drug.[7,8]

At present, the drugs which are used to treat inflammation are steroidal anti-inflammatory drugs (SAIDs) and non SAIDs (NSAIDs). NSAIDs have been used worldwide; however, studies have shown that these drugs have quite adverse side effects. For example, bromfenac, one of NSAIDs that have been withdrawn by the United State Food and Drug Administration, causes severe liver injury.[9] Therefore, there is a dire need for an effective alternative treatment with lesser or minimum side effects. This alternative treatment may be found in the natural reserves, which have always been a rich and great source of medicines. A number of drugs used nowadays are derived from plants and other natural resources. Plants have been widely used in traditional and folk medicine. Over the last decade, the use of medicinal plants and their products in developing countries has almost doubled, and alternative therapies that are economical and have less side effects have become a present trend worldwide. One of the plants is Pandanus tectorius belonging to Pandanaceae family, and it is an herbal medicinal plant that has been used to treat skin diseases, ulcers, fever, and diabetes.[10] Different parts of P. tectorius contain varieties of biochemical constituents. The bioactive constituents isolated from P. tectorius are mostly flavonoids, phenols, glycosides, tannins, steroids, and triterpenoids. These bioactive compounds are known for their different pharmacological activities, such as anti-diabetic, anticancer, antimicrobial, antioxidant, and anti-inflammatory activities.[11]

From their study of P. tectorius fruits, Zhang et al.[12] has isolated 15 compounds. The most abundant compound present in P. tectorius fruit extract is trans-ethyl caffeate, which is approximately 0.1% of the dry material. Its leaves have also shown a very good antioxidant activity.[13] Anti-inflammatory activity of tangeretin and ethyl caffeate from different plants and its mechanism of action has been investigated.[14] The presence of tangeretin and ethyl caffeate, including as phenolics and flavonoids group compounds, in P. tectorius fruits has also been reported by Andriani et al.[15] Besides tangeretin and ethyl caffeate, the fruits contain caffeoylquinic acids (flavonoid compound) Wong et al.[16] Besides antioxidant properties, this compound displays various bioactivities including anti-inflammatory.[16] Hence, we assume that these three compounds which were reported exist in P. tectorius fruits may have contributed to the its anti-inflammatory potency of P. tectorius fruits extract or fraction. Although several studies on anti-inflammatory potential of P. tectorius have previously been conducted, most of them involved the plant leaves extracts.[17,18] Ethanol leaves extract rich by the chemical constituents of tannins, sterols, triterpenes, and flavonoids. There are currently no data available on in vitro and in vivo anti-inflammatory activity of P. tectorius fruits. Andriani et al.[19] studied the antioxidant activity of different parts of the P. tectorius fruits, but they extracted the keys and core part of the fruit separately. In the current study, the keys and core parts were combined and used to yield fractions, using extraction methods different from those in their study. They also revealed that P. tectorius fruits and their compounds (tangeretin and trans ethyl caffeate) showed anti-atherosclerosis potency.[20,21] As atherosclerosis is regarded as an acute inflammatory disease, the fruits may also have anti-inflammatory potency. Therefore, these plants may serve as an alternative source for treating inflammation.

MATERIALS AND METHODS

The organic solvents and other reagents were of analytical grade and purchased from Sigma-Aldrich. The experimental animals were male Sprague–Dawley rats (species of Rattus norvegicus) and purchased from Takrif Bistari Enterprise, Kuala Lumpur, Malaysia.

Sample Collection and Extraction

P. tectorius fruits were collected from Setiu, Terengganu, Malaysia, in 2015. The hard part of the fruits (seed) was removed and the edible parts (keys and core parts) were cut into smaller pieces. The edible pieces were then kept at −80°C before being dried using a freeze-dryer. The dried P. tectorius fruit pieces (6.8 kg) were ground to powder and extracted with methanol (MeOH). The obtained extract was filtered through cotton in the filter funnel, collected in the flask, and finally evaporated under reduced pressure using a rotary evaporator to yield a methanolic crude extract. The fractionation of the methanolic crude extract was carried out using a method adopted from Andriani et al.[22] with few modifications.
crude extract was fractionated with hexane, ethyl acetate, and methanol, respectively, using column chromatography. The silica gel of 0.04–0.06 nm size was used as a stationary phase. Approximately 627 g MeOH crude extract was fractionated by subjecting it into column chromatography using silica gel (15 cm × 4 cm) as a stationary phase and eluting it with n-hexane, ethyl acetate, and methanol to obtain Pandanus hexane (PHF) (38 g), Pandanus ethyl acetate (PEF) (25 g), and Pandanus methanol (PMF) (498 g) fractions, respectively.

**Total Phenolic Content**

The total phenolic contents in *P. tectorius* crude fractions (PHF, PEF, and PMF) were determined through Folin-Ciocalteu assay;[23] however, a few modifications were made. For this assay, 2.0 M Folin-Ciocalteu phenol reagent, gallic acid (Sigma, Germany), and anhydrous sodium carbonate (Sigma, USA) were used. This assay was divided into standard curve and test sample methods. For the preparation of standard curve, 0.1 ml aliquots of 0.3, 0.105, 0.075, 0.024, and 80 mg/ml ethanolic gallic acid solutions were mixed with 7 ml of distilled water, and 0.5 ml Folin-Ciocalteu reagent was added into the test tube. The mixture was shaken well and incubated for 8 min at room temperature. Then, 1.5 ml of 1.85 M sodium carbonate (Na₂CO₃) solution and 0.9 ml of distilled water were added to the mixture. The mixture was incubated for 2 h in a darkroom at room temperature, and the absorbance was measured using spectrophotometer at 765 nm. The same procedure was repeated for test material, but with gallic acid replaced with 10 mg fraction. The total phenolic content of the fraction was determined from the standard curve of gallic acid and expressed as mg/g gallic acid equivalent (GAE) using standard curve: \( y = 2.002x + 0.067; \) \( R^2 = 0.999, \) where \( y \) is the absorbance at 765 nm and \( x \) is the total phenolic content of fraction.

**Antioxidant Activity by DPPH Radical Scavenging Assay**

The antioxidant activity of the crude fractions (PHF, PEF, and PMF) was obtained by DPPH free radical scavenging assay adopted from Kumaran and Karunakaran. Quercetin and DMSO were used as positive and negative controls, respectively. 10 mg crude fraction was prepared in variable concentrations by two-fold serial dilution in DMSO, with concentrations of 10, 5, 2.5, 1.25, 0.625, 0.313, and 0.156 mg/ml in 96 well plates. 20 µl of DMSO was added to all wells except A line. Then, a crude sample of 10 mg/ml concentration was added to well A and B. Two-fold dilution from B-G was done and 20 µl from G was discarded. Finally, 200 µl of methanolic DPPH solution (6 × 10⁻⁵ M) was added into all wells and the mixture was incubated for 30 min at room temperature. The absorbance was measured at 517 nm using Elisa reader (Multiskan ascent, Thermo Electron Corporation).

**Cytotoxicity Study through MTT Assay**

The cytotoxic effect of samples against RAW264.7 cell line was determined by MTT assay, adopted from Lau et al.[25] 100 µl of the cell suspensions was dispensed in triplicate into 96-well plate with concentration of 2 × 10⁴ cells per well in complete media. The *P. tectorius* crude samples (PHF, PEF, and PMF) were then diluted up to two-fold in 96-well plate to attain different concentrations, which were 100 µg/ml, 50 µg/ml, 25 µg/ml, 12.5 µg/ml, 6.25 µg/ml, 3.125 µg/ml, 1.56 µg/ml, and 0.78 µg/ml. The samples were then incubated for 24 h. 5 mg/ml of MTT was dissolved in PBS. 20 µl of MTT solution was added into each well, and the samples were further incubated for 4 h at 37°C. Medium was discarded, and 100 µl of DMSO was added to the cultures and mixed thoroughly to dissolve the dark-blue crystal formazan. The quantification was then done by spectrophotometer at 570 nm. The optical density OD of the samples was compared with that of the control to obtain the percentage viability, as follows:

\[
\text{Cells viability} \% = \frac{(\text{OD}_{570} \text{ (sample)})}{(\text{OD}_{570} \text{ (control)})} \times 100\%
\]

**Anti-Inflammatory Activity of Phenolic-rich Fractions of *P. tectorius* Fruits**

**Standard nitrite**

The stock solution of sodium nitrite was prepared using DMEM. The concentration of nitrites produced were estimated through using variable concentrations of sodium nitrite ranging from 0 to 100 µM. 100 µl of each standard solution was added to 96-well plate followed by the addition of 100 µl of Griess reagent. The mixture was then incubated for 30 min at room temperature, and the absorbance was measured at 540 nm through microplate reader.[26]

**Measurement of NO production enhanced by LPS stimulation on RAW264.7 cell line**

The RAW264.7 cell line was plated at concentration of 2 × 10⁵ cells/well in 96-well plates and incubated for 24 h. Then, the cells were stimulated with LPS (1 µg/ml), and fractions were added to each well at the different concentration by two-fold dilution and incubated for 24 h. After 24 h, all media were harvested, and nitrite levels were determined using Griess reagent assay and presumed to reflect NO levels. One hundred microliter of cell culture medium was briefly mixed with 100 µl of Griess reagent (equal volumes of 1% (w/v), sulfanilamide in 5% (v/v) phosphoric acid, and 0.1% (w/v) naphthyl ediamine dihydrochloride. The mixture was then incubated at room temperature for 10 min, and the absorbance was measured at 540 nm in plate reader.[25]

**In Vivo Study: Effect of PEF Fraction on Reduction of Paw Edema in *R. norvegicus***

**Paw Edema Induced with 2.5% Formalin and Experimental Design**

Twenty-four Sprague–Dawley male rats from species of *R. norvegicus* aged 6–8 weeks and weighing 220–270 g, obtained from Universiti Putra Malaysia, were used in this study. The animals were housed in cages under a 12 h light and 12 h dark cycle in a temperature-controlled room (25°C–27°C). Before the experiment, the rats were allowed free access to standard laboratory animal diet with water *ad libitum* for a week to make them adapt to the laboratory conditions. The project was approved by the Animal Ethics Committee of university Malaysia Terengganu. The 24 rats were divided into four groups which consisted of six rats per group (n = 6), as
shown in Table 1. The first group served as a negative control which was administered with the saline (Group A). The second group served as an inflammation control and was given distilled water and injected with 2.5% formalin (Group B). The third group was assigned as treatment control and was administered with PEF fraction and 2.5% formalin (Group C), and the last group served as a drug control group and was given ibuprofen and 2.5% formalin (Group D).

Treatments of phenolic-rich fraction on formalin-induced rats (R. norvegicus)

Twenty-four white male rats of R. norvegicus were housed in the Animal Laboratory at the Institute of Marine Biotechnology, Universiti Malaysia Terengganu, under controlled room temperature. The relative humidity of the room was 60-70%, and they were provided with food and water ad libitum. To carry out the study, the animals were divided into four groups, with 6 animals per group. The animals were kept on fast for 24 h before the induction of edema but allowed free access to water throughout by ad libitum. The rats were deprived of water only during the experiment to ensure uniform hydration and minimize variability in oedematous response.

The paws of the rats were induced to edema by 2.5% formalin. The negative control group (A) was injected with 0.9% saline. The inflammatory control group (B) was injected with 0.1 ml of 2.5% formalin. The treatment group (C) and control drug group (D) meanwhile received 100 mg/kg body weight of PEF and 100 mg/kg body weight of ibuprofen, respectively. The PEF fraction and ibuprofen were given 30 min before the injection of 0.1 ml of 2.5% formalin subcutaneously into the plantar surface of each rat’s left hind-paw in Groups C and D, respectively. The measurement of edema inhibition in treated animals was calculated in comparison to that in the negative control group.

Histological Analysis on Paw Edema after Treatment by P. tectorius Fruit Fraction

Protocol for paw skin

Paw edema skin tissues were removed from sacrificed animals using sterile sharp blade. These samples were then treated with 10% of buffered formalin for 24 h and preceded for tissue processing. Tissue processing was carried out using Tissue Processor Machine (LEICA TM, GERMANY) for 24 h. The process involved dehydration through graded alcohol series, clearing in xylene and impregnation. Furthermore, the samples were processed through several steps such as embedding, staining, and Mounting, Hematoxylin, and Eosin Staining (H&E). Finally, histology analysis was conducted under a light microscope, and photomicrograph was taken using an image analyzer microscope (Leica, Nussloch, Germany) at 40x magnifications.

Statistical Analysis

All the experiments were conducted in triplicate, and the data were presented as mean values ± standard deviation. One-way ANOVA was used for analysis of data, followed by Tukey’s multiple comparisons. P < 0.05 was considered as significantly different. All statistical analysis was performed using SPSS software package for Windows (Version 20, Chicago, IL).

RESULTS AND DISCUSSION

Sample Collection, Extraction, and Fractionation of Methanol Extract

It was found that approximately 86% of total weight of P. tectorius fruits was water, and the dry weight of the P. tectorius fruits (core and key parts combined) was 6.8 kg. The samples needed to be dried as dry plant material gives free bonding of solvents with the phytochemicals according to its polarity. Extraction of dried P. tectorius fruits (6.8 kg) using methanol yielded 627 g of crude extract. The methanolic extract obtained was further fractionated using column chromatography to yield three fractions, which were Pandanus hexane (PHF, 38 g), Pandanus ethyl acetate (PEF, 25 g), and Pandanus methanol (PMF, 498 g) fractions.

Total Phenolic Content

Phenols are classified as one of the phytochemical class of compounds synthesized during plant growth and development and in response to certain conditions, such as infection, wounds, and exposure to UV radiations. Phenols play various diverse roles and functions within the plant body; they help in nutrient uptake, protein synthesis, enzyme activity, allelopathy, and photosynthesis. They also provide the plants with structural integrity and support. In the current study, the total phenolic content of P. tectorius extract was determined using Folin-Ciocalteu colorimetric method, and gallic acid was used as standard. This method has been widely used for the estimation of total phenolic content in herbs and natural products. To calculate the phenolic content of the samples, a linear calibration curve of gallic acid (Y = 1.115X + 0.087) with R² value of 0.998 was plotted. The phenols extracted from biological plants could be composed of different classes of phenols depending on the solvents used for extraction. Total phenolic contents of P. tectorius fruit crude fractions (PHF, PEF, and PMF) were calculated and are shown in Table 2. The ethyl acetate fraction (PEF) had the highest total phenolic content.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Inflammation substance</th>
<th>Group Code</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>None</td>
<td>A</td>
</tr>
<tr>
<td>Distilled water</td>
<td>Formalin</td>
<td>B</td>
</tr>
<tr>
<td>PEF</td>
<td>Formalin</td>
<td>C</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>Formalin</td>
<td>D</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sample</th>
<th>µg GAE/g sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHF</td>
<td>10.38±0.22</td>
</tr>
<tr>
<td>PEF</td>
<td>90.12±1.00</td>
</tr>
<tr>
<td>PMF</td>
<td>44.27±0.42</td>
</tr>
</tbody>
</table>
Antioxidant Activities of *P. tectorius* Fractions

The antioxidant ability of *P. tectorius* fruits might be attributed to the presence of phenols and flavonoids, which have been discovered during phytochemical screening of the plant. Ethyl caffeate, a natural phenolic compound, has been isolated from fruits belonging to genus *Pandanus*. All fractions of *P. tectorius* fruits (PHF, PEF, and PMF) were screened for their antioxidant activity using DPPH radical scavenging assay. As presented in Table 3, the percentage of inhibition for DPPH assay was found to be dose dependent; the inhibition increased with the increased concentrations of fractions and positive control (Quercetin). The IC$_{50}$ values were also calculated for the samples and the standard showed 50% or more inhibition. The calculated values are listed in Table 4.

The results showed that, among all *P. tectorius* fruit fractions (PHF, PEF, and PMF), only the PHF fraction exhibited less than 50% antioxidant activity. The ethyl acetate (PEF) and methanol (PMF) fractions displayed more than 50% inhibition and were found to possess good antioxidant activities with IC$_{50}$ values of 2.43 ± 0.09 and 5.86 ± 0.15 mg/ml, respectively. Both samples also showed higher phenolic content compared to that of the PHF [Table 2]. Nevertheless, these fruit fractions displayed lower antioxidant activities in comparison with the standard quercetin (IC$_{50}$ = 0.2 mg/ml). There is a correlation between antioxidant property and phenolic content in this plant. In the current study, the antioxidant content of the *P. tectorius* fruit fractions was proven to be in line with their phenolic content. The higher the phenolic content of the sample, the higher its antioxidant property would be. Hence, we can see that the PEF fraction showed both the highest phenolic content and the highest antioxidant property.

<table>
<thead>
<tr>
<th>Concentration of extract (mg/ml)</th>
<th>Quercetin*</th>
<th>PHF</th>
<th>PEF</th>
<th>PMF</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>90.93 ± 1.62</td>
<td>23.20 ± 2.01</td>
<td>85.30 ± 0.78</td>
<td>77.21 ± 0.11</td>
</tr>
<tr>
<td>5</td>
<td>90.78 ± 1.45</td>
<td>12.31 ± 0.88</td>
<td>68.75 ± 1.27</td>
<td>44.81 ± 0.86</td>
</tr>
<tr>
<td>2.5</td>
<td>90.78 ± 1.45</td>
<td>6.32 ± 0.08</td>
<td>69.02 ± 1.32</td>
<td>21.70 ± 3.07</td>
</tr>
<tr>
<td>1.25</td>
<td>91.97 ± 0.97</td>
<td>3.30 ± 0.65</td>
<td>27.39 ± 1.02</td>
<td>9.92 ± 1.03</td>
</tr>
<tr>
<td>0.625</td>
<td>81.41 ± 8.13</td>
<td>4.90 ± 0.82</td>
<td>12.70 ± 0.27</td>
<td>6.50 ± 2.88</td>
</tr>
<tr>
<td>0.313</td>
<td>74.01 ± 15.37</td>
<td>2.64 ± 1.02</td>
<td>10.46 ± 1.30</td>
<td>3.25 ± 0.92</td>
</tr>
<tr>
<td>0.156</td>
<td>64.23 ± 6.77</td>
<td>1.23 ± 0.36</td>
<td>7.95 ± 1.08</td>
<td>1.55 ± 0.71</td>
</tr>
</tbody>
</table>

*Significantly different in comparison between % antioxidant values of samples compared to control (quercetin) and at $P<0.05$
Figure 1: Cytotoxicity property of Pandanus tectorius fruit fractions against RAW 264.7 cell lines. The values are presented as mean±SD. There is a significant difference in comparison between cytotoxicity value of PEF with other fractions (*) at P<0.05.

Table 4: The values of IC₅₀ of DPPH free-radical scavenging activity of P. tectorius fruit fractions (PHF PEF PMF)

<table>
<thead>
<tr>
<th>Sample</th>
<th>IC₅₀ (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHF</td>
<td>22.66±0.51</td>
</tr>
<tr>
<td>PEF</td>
<td>2.43±0.09</td>
</tr>
<tr>
<td>PMF</td>
<td>5.86±0.15</td>
</tr>
<tr>
<td>Quercetin*</td>
<td>0.20±0.08</td>
</tr>
</tbody>
</table>

*Significantly different in comparison between IC₅₀ values of samples compared to control (quercetin) and at P<0.05.

Anti-inflammatory Activity by NO Assay on LPS-induced Macrophage Cell Lines (RAW264.7)

A standard curve of sodium nitrate with coefficient correlation (R²) of 0.988 and linear regression y = 0.0118x (Figure 2) was used for for quantification of NO produced by RAW264.7 cells treated with different samples. Since the level of nitrite can be obtained from the standard curve, the level of nitrites produced in the cells can be calculated using the regression plot obtained. Primary data collected from an anti-inflammatory assay represents the production of NO from the cell supernatant. An inflammatory stimulator such as LPS, a major component of Gram-negative bacteria cell wall, is a nitrite synthase inducer, and when it is used to activate macrophages, there is an increase in the nitrite production by the independent NOS enzyme (iNOS). Besides that, Yoon et al. stated that LPS stimulation in murine macrophage RAW264.7 cells can induce iNOS transcription, protein synthesis, and subsequent NO production. iNOS catalyzes the oxidative deamination of L-arginine to produce NO. In general, NO plays an important role as vasodilator, neurotransmitter, and in the immunological system as a defense against tumor cells, parasites, and bacteria. However, under pathological condition like inflammation, NO production is increased by the iNOS, which subsequently causes cytotoxicity and tissue damage. Therefore, the inhibition or suppression of NO production by iNOS can be a very important therapeutic target in the development of drugs for treating inflammation. In the current study, the effects of P. tectorius fruit fractions on NO production in LPS-stimulated RAW 264.7 cells were investigated. The effects of the fractions on NO production in LPS-stimulated RAW264.7 are shown in Table 5.

All fractions significantly inhibited nitrite accumulation in LPS-stimulated RAW 264.7 cells compared to the control, N⁰-monomethyl-L Arginine (NMMA) at (P < 0.05), with the percentage of NO production at 3.56% and 72.80%, respectively. The range of NO production for PHF, PEF, and PMF fractions were 3.41-5.6%, 3.32-4.23%, and 3.21-4.37%, respectively. However, PEF fraction was chosen for in vivo study since this fraction was rich in phenolic content and had higher antioxidant properties than PHF and PMF. According to Chohan et al. there could be a correlation between antioxidant and phenolic content with the anti-inflammatory potency. Lee et al. have reported the plant-derived inhibitors of NO production in the ethyl acetate extract of Broussonetia kazinoki (Paper mulberry) extract. Seven polyphenols isolated from the EtOAc fractions demonstrated a concentration-dependent inhibition of NO production in LPS-stimulated RAW 264.7 macrophages (0–20 µM). This finding is in agreement with Joo et al. which reported that, among the different fractions, ethyl acetate fraction of Ulmus pumila L. has reduced the highest nitrite level at concentration of 32.09 µM.

In Vivo Study: Effect of PEF Fraction of P. tectorius Fruits as Anti-inflammatory Agent on Reduction of Edema on Rats’ Paws (R. norvegicus)

Figures 3 and 4 shows the representative paws of animals from all groups for the last 6 h. All paws were compared to the control, which did not receive any treatment and also was not injected with any chemical solutions. Picture (A) represents the negative control group, which was administered 0.9% saline, injected subcutaneously. This group showed lowest paw edema, followed by the drug control group (Group C), the treatment control group (Group D), and the inflammatory control group (Group B). Formalin-induced paw edema in rats is one of the most suitable procedures to screen the acute inflammation, and the behavior response of the rats due to the formalin-induction.
Table 5: Inhibitory effects of *P. tectorius* fruit fractions (PHF, PEF, and PMF) with different concentrations on NO production compared to the drug control (NMMA) and LPS.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Percentage of NO production (% of samples)</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PHF</td>
<td>PEF</td>
</tr>
<tr>
<td>100</td>
<td>5.60±0.27</td>
<td>3.98±0.11</td>
</tr>
<tr>
<td>50</td>
<td>4.41±0.05</td>
<td>3.66±0.08</td>
</tr>
<tr>
<td>25</td>
<td>3.80±0.11</td>
<td>3.89±0.19</td>
</tr>
<tr>
<td>12.5</td>
<td>3.53±0.22</td>
<td>3.53±0.22</td>
</tr>
<tr>
<td>6.25</td>
<td>3.44±0.19</td>
<td>3.46±0.19</td>
</tr>
<tr>
<td>3.125</td>
<td>3.41±0.07</td>
<td>3.32±0.07</td>
</tr>
<tr>
<td>1.5625</td>
<td>3.57±0.09</td>
<td>3.35±0.15</td>
</tr>
<tr>
<td>0.78125</td>
<td>3.57±0.11</td>
<td>3.21±0.07</td>
</tr>
</tbody>
</table>

*Significantly different in comparison between % NO production values of control (LPS) and samples at *P*<0.05.

Figure 3: Effect of treatment on rat's paw edema diameter among groups. Group A: negative control (Saline), Group B: inflammatory control (2.5% Formalin + distilled water), Group C: treatment control (2.5% Formalin + 100 mg/kg body weight PEF fraction), and Group D: drug control (2.5% Formalin + 100 mg/kg body weight Ibuprofen).

The inhibitory effect of PEF fraction on formalin-induced paw edema can be attributed to the phenoic compounds present in the fraction. Phenolic compounds are known to possess antioxidant and anti-inflammatory properties. However, the actual mechanism of action correlated to the phenolic compounds in the *P. tectorius* fruit is still unknown. This fruit has been reported to contain tangeretin and trans ethyl caffeate, which are known as flavonoids compounds group. According to Ambriz-Pérez et al., the mode of action of phenolic compounds (especially for flavonanes group) as anti-inflammatory agents can be observed through many ways depending on the types of phenolic compounds. Mostly they perform as anti-inflammatory agents through inhibition of NF-κB, iNOS expression, NO production, COX-2 expression, cytokines TNF-α, IL-6, and PGE2 production. Hence, one or more of these actions could be correlated to the anti-inflammatory action of the *P. tectorius* fruits which are rich in flavonoids. However, this suggestion needs to be further proven in future investigations.

**Histological Analysis for Anti-inflammatory Activity of *P. tectorius* Fruit Fractions**

The histopathological effects were also studied to further evaluate the development and effect of treatment on inflammation. Analysis showed that the normal paw tissue had...
no infiltration of immune cells, indicating that no inflammation occurred due to the injection of saline [Figure 4a]. Meanwhile, the hind paws that received 2.5% formalin injection showed the accumulation of infiltrated inflammatory cells [red arrows in the picture, Figure 4b] compared to the control group. However, with 100 mg/kg body weight of PEF fraction given as treatment (Group C) and ibuprofen as drug control (Group D), the infiltrated inflammatory cells were found to be reduced 6 h after being injected with 2.5% formalin [Figure 4c and d]. Histopathological analysis of paw tissue of rat shows the inhibition of edema formation and inflammation in PEF fraction treated group as compared to the formalin-treated group. The paw tissue of the normal rats showed no signs of inflammation [Figure 4a] with normal keratin and sub epidermal layer. In the rats treated with formalin shows massive influx of inflammatory cell infiltration, sub epidermal edema, and hyper keratotic skin [Figure 4b]. Treatment with PEF fraction showed improvement in the reduction of inflammatory signs. Keratinization was decreased, dermis and hypodermis seemed to be mild [Figure 4c]. Mild sub epidermal edema, and mild inflammation were also detected in ibuprofen treated group [Figure 4d]. Histopathological indicated that inflammatory cell infiltration, hyperkeratinization, and edema formation were slightly suppressed in the rats treated with the PEF fraction. Higher concentration of PEF and ibuprofen may be needed to increase the anti-inflammatory effect in vivo. However, this result shows the usefulness of PEF fraction of Pandanus tectorius fruits in reducing the inflammatory signs.

Figure 4: Histopathological analysis on anti-inflammatory potency of PEF fraction (Hematoxylin and Eosin with 40× magnification). (a) Negative control (Saline), (b) Inflammatory control (2.5% Formalin + Distilled water), (c) Treatment control (2.5% Formalin + 100 mg/kg body weight PEF fraction), and (d) Drug control (2.5% Formalin + 100 mg/kg body weight Ibuprofen). Red arrows indicate as KR (keratin), SEL (sub epidermal layer), SEO (sub epidermal edema), MSEO (mild sub epidermal edema), HK5 (Hyper keratotic skin), ICI (inflammatory cell infiltration).

Formalin-induced paw edema can produce an elevated level of lipid peroxide (LPO). Tissue glutathione depletion seems to be responsible for the induction of LPO[48]. The increasing of its levels may due to the free radicals which responsible for damaging cell membranes and further inflammatory damage.[51] Antioxidant compound such as flavonoid and phenolic compounds exert an effective role as anti-inflammatory factors by blocking two major signaling pathways such as mitogen-activated protein kinases and NF-κB which have the key role in the production of numerous pro-inflammatory mediators.[52] Since, Pandanus tectorius fruits are rich by phenolic compounds, the reduction of inflammation by its fraction may be occur through the same pathway.

CONCLUSION

The current study has confirmed the antioxidant and anti-inflammatory potentials of Pandanus tectorius fruits. The fruits were able to reduce NO production in vitro study; and also reduce inflammation in in vivo study. Among all fractions, the PEF displayed the most promising anti-inflammatory activity, which could be due to its higher phenolic content, especially from flavonoids compound group. Nevertheless, this fraction must be further studied; their mechanisms of action may be explored in more detail through molecular studies, in vitro and in vivo.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.
REFERENCES

Andriani, et al.: Antiinflammatory activity of phenolic-rich fraction of Pandanus tectorius fruits


