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Acute Toxicity and Anti-Inflammatory Activity of the Aqueous and Alcohol Extracts of Philippine Endemic *Pandanus* spp.

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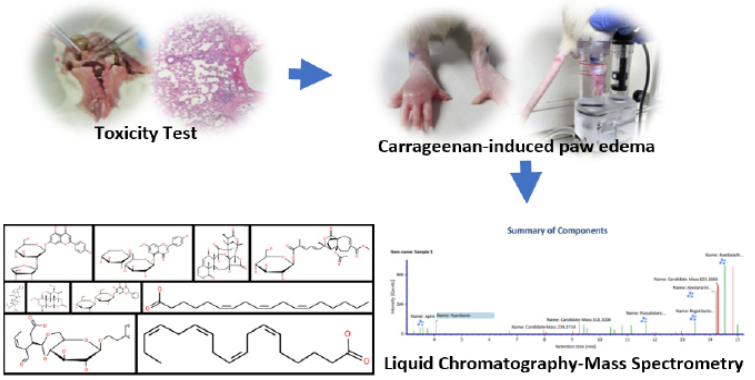
Graphical Abstract



Abstract

The leaf extracts of three endemic species of *Pandanus* namely, *P. luzonensis*, *P. panayensis*, and *P. simplex* were investigated for their safety profile and anti-inflammatory property. The Approximate Lethal Dose (ALD) was established through acute oral toxicity test. Carrageenan-induced rat paw edema assay assessed the anti-inflammatory property. Each treatment group received assigned dose of extract (250, 500, 1000 mg/kg BW), 1% Tween 80 and diclofenac sodium (100 mg/kg BW) for the negative and positive control groups, respectively. The ethanol leaf extract *P. panayensis* at 500 mg/kg BW showed sustained inflammatory inhibition for 6 hours of observation with comparable inhibitory activity with diclofenac sodium (ave p=0.970). Histopathology of the inflamed paw tissues showed mild inflammation. IC_{50} against COX-1 and 2 are 610.69 ug/mL and >1000 ug/mL respectively against <10 ug/mL of Indomethacin as standard. These results suggest that all *Pandanus* leaf extracts have an ALD greater than 2000 mg/kg BW while *P. payanensis* extracts may serve as potential sources of anti-inflammatory agents. This activity may be contributed by flavonoids, steroids, terpenoids and fatty acid which were identified by Liquid Chromatography-Mass Spectrometry.

Keywords: anti-inflammatory; acute toxicity; approximate lethal dose; *Pandanus*

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Introduction

Inflammation is a natural response of the body's immune system to eliminate the invasion of microorganisms resulting to cell damage or tissue injury [1,2]. However, inflammation is the major cause and the frequent key element in the progression of organ disease. Chronic inflammation is associated to the leading causes of mortality in the Philippines and worldwide such as cancer, cardiovascular diseases, diabetes mellitus and chronic kidney disease [3].

Arachidonic acid (AA) pathway is one of the inflammatory processes which is mediated by cyclooxygenase (COX) and lipoxygenase (LOX) enzymes [4] COX, a proinflammatory mediator, is the main enzyme responsible for the conversion of arachidonic acid (AA) and synthesis of prostaglandins (PGs) and thromboxanes (TXs). COX isoforms, COX-1 and COX 2, are involved in cytoprotection and synthesis of inflammatory prostanoids [5]. While COX inhibitors like NSAIDS are widely prescribed and commonly used [6] these drugs are associated with serious side effects like gastrointestinal (GI) ulceration, perforation, hemorrhage and kidney damage [5] This warrants studies on medicinal plants as sources of natural products which can arrest inflammation and prevent chronic diseases.

In the Philippines, variety of *Pandanus* species are widely grown in different regions with high endemism at 82.7% as observed among the fifty-two species of the genus *Pandanus* [7,8]. *Pandanus* are used traditionally as medicinal plants against virus, microbes, hyperglycemia, diarrhea, cancer, hyperlipidemia, and inflammation [9]. According to Ordas et al. (2020), the leaves and terminal shoots of *Pandanus luzonensis* are used ethnomedicinally to alleviate respiratory problem, UTI and kidney stones. Leaves are used for muscle and bone pains [11]. Anti-inflammatory property of *Pandanus tectorius* leaves was established since it contains ethyl caffeate and hydroconiferyl alcohol which inhibit inflammation [12]. Blocking of pro-inflammatory cytokines by *Pandanus fascicularis* was shown by suppressing IL-1β, IL-6, and TNF-α in a lipopolyssacharide-induced RAW 264.7 cell [13].

This study aims to establish the approximated lethal dose (ALD) of three endemic *Pandanus* species and to determine the anti-inflammatory property through in vivo study. Also, this study establishes the possible blocking of COX-1 and COX-2 synthesis of the *P. payanensis* leaf ethanol extract and identifies the putative secondary metabolites responsible for its action through Liquid Chromatography Mass Spectrometry (LC-MS).

Materials and methods

Reagents and materials. COX Inhibitory Screening Assay Kit (no. 701230) was procured from Cayman Chem (Singapore). Indomethacin and diclofenac sodium which were used as standards for in-vitro COX inhibition and in vivo assay were purchased from Cayman Chem (Australia) and Mercury Drug Corp. (Philippines), respectively. Analytical grade of carrageenan, formalin and other reagents were obtained from the University of Santo Tomas Laboratory and Equipment Supplies Office (UST-LESO), Manila, Philippines.

Plant Material. Plant samples of the three (3) *Pandanus* species (*P. panayensis, P. luzonensis* and *P. simplex*) were obtained by the Department of Biological Sciences, College of Science, University of Santo Tomas, Manila, Philippines. *P. luzonensis* leaves (USTH014420), *P. panayensis* leaves (USTH014475) and *P. simplex* leaves (USTH014425) were collected from Orani, Bataan; Lauan, Antique and Luisiana, Laguna, respectively from Jun 2017 to Mar 2018. Specimens were identified and authenticated by the UST Herbarium and kept for future use.

Preparation of plant extracts. Two kilograms (2 kg) each of air-dried leaves of *P. luzonensis*, *P. panayensis* and *P. simplex* were ground separately using Wiley mill and passed through sieve number 60 then percolated with 2.5 L of methanol for *P. luzonensis* while ethanol was used for *P. panayensis* and *P. simplex*. Filtration followed and filtrates were collected in bottles after 24 h. Marc of each *Pandanus* spp. was again soaked for the whole night in their respective solvent of extraction and was filtered. The alcohol extracts were combined and evaporated *in vacuo* using Rotary evaporator (EYELA, Germany) with 45 °C water bath until syrupy in consistency. For aqueous extract, one kilogram (1 kg) of the air-dried ground leaves were soaked in 1.5 L of distilled water and heated at 80 °C for 60 min and were transferred in a percolator while hot for the whole night. Then, the percolate was filtered and lyophilized in a freeze-dryer at -50 °C (HetoPowerdry LL3000, Thermoscientific) until the crude extract appeared syrupy. All extracts were kept in pre-weighed amber bottles, labeled, and stored at -20 °C for pharmacological testing.

Experimental animals. One hundred thirty (130) Sprague Dawley (SD) rats were procured from Department of Science and Technology (DOST) in Taguig City, Philippines and MOTS Animal House in Sta. Rosa, Laguna, Philippines. Female SD rats (35) and male rats (95) aged 7-8 weeks weighing 142-240g were used for acute toxicity test and anti-inflammatory assay, respectively (Supplementary Table 1). Five test animals were randomly assigned per group and acclimatized at the UST-RCNAS Animal Facility for not less than 7 days prior to experimentation. All were kept under standard environmental condition of temperature (25 °C) and light/dark cycles (12/12 h) and with free access to dog pellets (Pedigree, US) and distilled water (Absolute, Asia Brewery, Philippines). Experiments were accomplished according to the guide for the care and use of laboratory animals under the approved protocol of UST Institutional Animal Care and Use Committee (IACUC) with Animal Research Permit No. AR-2018-355.

In vivo Assay

Acute Oral Toxicity Test. Based on existing *Pandanus* research studies, the extracts are assumed to be none toxic, thus, limit test based on the Organization for Economic Cooperation and Development (OECD) Guideline 425 was performed to obtain the approximate lethal dose (ALD). *Pandanus* alcohol extracts were dissolved in 1% Tween 80 in normal saline while lyophilized aqueous extracts were dissolved in distilled water. Extracts were administered orally in a single dose of 2,000 mg/kg body weight to over-night-fasted, healthy female SD rats. Behavioral patterns of animals were observed for 30 min, then every hour up to 4 h and 24 h for mortality and toxidromes.

The animals were euthanized through exposure to carbon dioxide then gross necropsy was done by the veterinarian. Selected organs, liver, kidney, heart, lung and brain were harvested for histopathological studies.

Anti-inflammatory Activity Test. The experimental design was carried out following the method of Patil et al. 2019 with modifications. All test substances were administered orally to randomized and fasted male SD rats consisting of five rats per group. Tween 80 (1%) in normal saline solution (10 mL/kg BW) and diclofenac sodium (100 mg/kg BW) were administered as negative and positive control groups, respectively. Three groups with 5 rats each were assigned for each *Pandanus* leaf extract. Each group (*P. luzonensis, P. payanensis, P.simplex*) received a specific dose of 250 mg/kg BW, 500 mg/kg BW and 1000 mg/kg BW (1/8, 1/4, 1/2 of the ALD) of each crude extract. The paw volume was measured using Ugo Basile Plethysmometer after 1 h of dosing as the baseline. Then, inflammation was induced by subcutaneous (SQ) injection of 0.5 mL of 1% carrageenan (lambda form) suspension. The paw volume was measured again after an hour until the sixth hour with 1-h interval. The increase in paw edema volume (PEV) and percentage inhibition per hour were calculated. The extract with the highest inflammatory inhibition undergone further testing such as COX inhibitory assay and LCMS for identification of putative compounds.

$$Paw\ edema\ value\ (\%) = \frac{Vt - Vo}{Vo}\ x\ 100 \quad (1)$$

Where, Vt is the paw volume at time (after carrageenan injection); and Vo is the paw volume at baseline (before carrageenan injection).

$$Inhibition\ (\%) = \frac{PEV control - PEV\ treated}{PEV\ control}\ x\ 100 \quad (2)$$

Wherein, $PEV_{control}$ is the PEV of the 1% Tween 80 group while $PEV_{treated}$ is the PEV of the group treated with either diclofenac sodium or extracts.

Histopathological Analysis. Six hours after the induction of inflammation using carrageenan, the test animal was euthanized through cervical dislocation and cardiac puncture was performed to collect blood while induced paw was collected and sliced as specimen. A slice of tissue without any bones from the plantar side of right paw was placed separately in a specimen container containing 10% buffered formalin. All specimens were sent to High Precision Laboratory, Manila, Philippines not more than 24 h from the time of harvest for slide embedding. Histopathological studies of the specimen were done by a histopathologist using a scoring system (Supplementary Table 2) to examine the inflammation by identifying neutrophils infiltration in the tissue layers [15,16].

***In vitro* Assay**

COX Inhibition Assay. The assay was performed using COX Inhibitory Screening Assay Kit (Cayman, Item no. 701230). Ethanol extract of *P. panayensis* was dissolved in ethanol at 10, 50, 100 and 1000 ug/ mL of *P. panayensis* solution. The inhibition activity against COX-1 and COX- 2 was carried out by enzyme-linked immunosorbent assay (ELISA) according to the instructions provided by the manufacturer. COX Inhibitory Screening Assay measures the amount of prostaglandin PGF2α by $SnCl_2$ reduction of PGH2. This was determined by spectrophotometry at 412 nm using a Multiskan Go Spectrophotometer (Thermo Scientific, Hudson, NH, USA). The results were expressed as percentage of inhibited COX-1 and COX-2 and compared with Indomethacin (10 and 1,000 µg/mL) as the standard.

Putative Identification of Metabolites by Liquid Chromatography Mass Spectrometry. The LC-MS measurement of the ethanol leaf extract of *P. panayensis* was outsourced from the Department of Chemistry Molecular Biology, College of Medicine, University of the Philippines, Manila. ACQUITY HSS T3 C18, 1.8 µm, 2.1 x 100 mm @ 40 °C was set for chromatographic analysis. The solvent reservoir is composed of mobile phase A (water + 0.1% formic acid) and B (acetonitrile + 0.1% formic acid). MS parameters were set as follows: Waters Xevo G2-XS QTof, MSE mode; capillary voltage: 1.0 kV (ESI+); cone voltage: 40 V; source temperature: 120 °C; cone gas flow: 50; desolvation temperature: 550 °C; desolvation gas flow: 950 L/h; scan range: 100-1,200 m/z; scan time: 0.150 s; collision energy: High energy ramp 15 to 50 eV. Leucine enkephalin was used as a reference for mass correction. Accurate mass screening was carried out using the UNIFI data analysis software. The base peak ions of distinct peaks were subjected to library matching using the Waters Traditional Chinese Medicine (TCM) library. Annotation of the candidate masses was based on the accurate mass match, isotopic ratio match and precursor ion intensity counts.

Statistical Analysis. Data gathered were expressed as mean +/- standard deviation (SD) of replicate analysis (n=2 for COX ELISA, n=5 for toxicity and inflammatory inhibition assays). Single-factor analysis of variance (ANOVA), Games-Howell and Tukey's HSD were used to determine significant differences at p=0.05 using IBM SPSS Statistics version 21. Absolute IC_{50} values for COX ELISA were calculated by plotting the mean % activity over the log of concentration in the non-linear regression curve fit function (viz. log of inhibitor against four parameters variable slope response, and least squares fitting method with interpolation at 95% confidence interval) of the Graph Pad Prism 6 software.

Results and Discussion

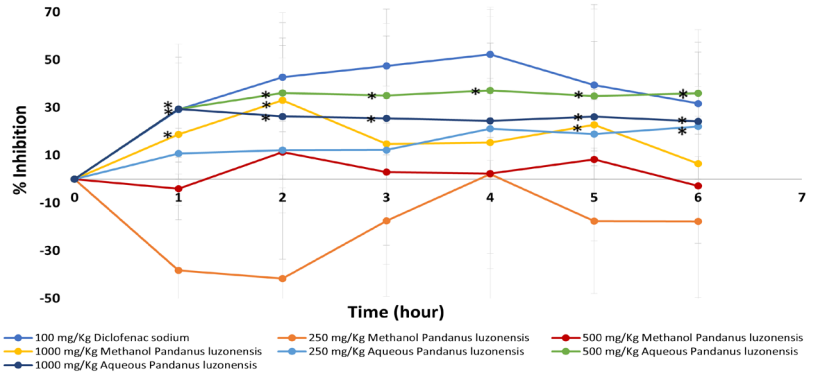
Pandan extracts. Extraction of the leaf by exhaustive percolation, filtration and evaporation in vacuo at 45 °C yielded, 8.35% of *P. luzonensis*, 2.54% of *P. panayensis* and 5.03% of *P. simplex* crude alcohol extracts. All the extracts are dark green in color, thick, viscous, oily in appearance with a pale sweet leafy smell.

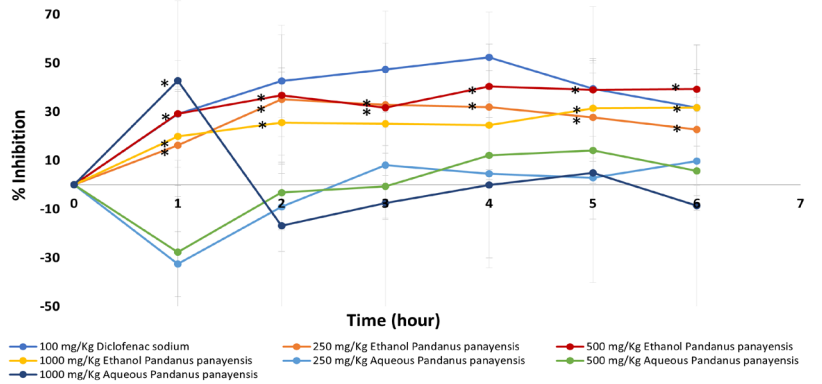
Approximate Lethal Dose Determination through Acute Toxicity test. Natural products like medicinal plants are being used commercially, and it is currently imperative to evaluate their efficacy and safety. Herbal drugs should have no or low toxicities for long term use [17].

All rats showed no abnormalities in weight gain or no signs of toxidromes were recorded such as changes in fur color, convulsion, salivation, respiration, urine color, somatomotor and behavior during the observation period. Food and water intake were normal (Supplementary Table 3). Liver, kidneys, heart and lungs are the major targeted area of metabolic toxicity [17]. These four organs were harvested with the inclusion of brain for histopathological examination. Gross necropsy and histopathologic studies of the organs showed no significant alteration in pathology indicating that the *Pandanus* extracts are nontoxic (Supplementary Figures 1-2). Moreover, all alcohol and aqueous extracts of *P. luzonensis*, *P. panayensis* and *P. simplex* are nontoxic at 2000 mg/kg BW. Monitoring all SD rats from day 1 up to the 14th day given by single dose oral gavage did not show any toxic or untoward effects. This suggests that all extracts have approximate lethal dose higher than 2000 mg/kg BW and are safe to use based on acute toxicity assay.

Carrageenan-Induced Rat Paw Edema Assay. The anti-inflammatory activity of the three *Pandanus* species was evaluated using carrageenan induced rat paw edema assay which is the standard classical method for acute inflammation model [18-19]. Induction of inflammation by subcutaneous injection in the right hind paw using 1% carrageenan (lambda form) covers biphasic state which consists of initial phase characterized by the release of histamines and serotonins, followed by the late phase where inflammatory mediators like prostaglandins, leukotrienes, polymorphonuclear cells and bradykinins are produced. Inflammation peaks at the third hour after induction, which is connected with the release of prostaglandins and expression of the large amount of COX-2 [18,19]. (Supplementary Figure 3). Among the *P. luzonensis* extracts, aqueous extract (AQL) 500 mg/kg BW exhibited the highest inhibitory activity which is comparable to diclofenac sodium having 36.03% and 31.69%, respectively ($p=1.000$) at 6th hour (Figure 1A). It also sustained its inhibitory property from the initial phase up to the late phase. AQL 1000 mg/kg BW had the highest initial inhibition at 29.4%, yet AQL 1000 mg/kg BW slightly decreased its inhibitory activity to 24.2% but still statistically comparable with the standard drug ($p=1.000$). AQL 250 mg/kg BW had a lower inhibitory activity. Nevertheless, it exhibited similarity with diclofenac ($p=0.785$ to $p=1.000$). Although methanol extract of *P. luzonensis* (EPL) 1000 mg/kg BW showed anti-inflammatory activity during the initial phase, it did not sustain its effect. The methanol leaf extract of *P. luzonensis* (EPL) administered at 500 mg and 250 mg/ kg BW did not inhibit the inflammation during the observation period (Figure 1A).

Figure 1B shows that diclofenac sodium (100 mg/kg BW) and 500 mg/kg BW ethanol extract of *P. panayensis* (EPP) had comparable anti-inflammatory effect. EPP 500 mg/kg BW exerted its action up to the late phase even surpassing the standard with inhibitory percentage of 39.30% ($p=1.000$). EPP 1000 mg/kg BW was able to sustain its activity up to the 6th hour with 31.71% inhibition, however, 500 mg/kg BW dose of the same extract still revealed greater activity. EPP 250 mg/kg BW was not able to sustain its action from the 2nd up to the 6th with only 22.73% inhibition but still statistically similar with the diclofenac sodium ($p=1.000$).





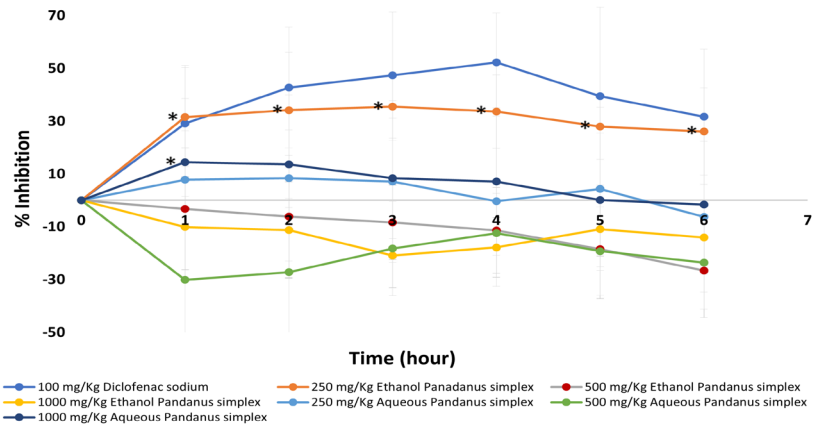


Figure 1. % Inhibitory effect of (1A) Methanol and Aqueous *Pandanus luzonensis*, (1B) Ethanol and Aqueous *Pandanus panayensis* and (1C) Ethanol and Aqueous *Pandanus simplex* Extracts on Carrageenan Induced Inflammation from 0 to 6hr post-sample administration. (Results are expressed as Mean +/- SD of N=5 at α =0.05); *group with optimum similarity at p=1.000 with diclofenac sodium).

In contrast, the aqueous leaf extract of *P. panayensis* (AQP) given at all doses did not exhibit any anti-inflammatory activity.

Among the *P. simplex* extracts (Figure 1C), the ethanol extract of *P. simplex* (EPS) at 250 mg/kg BW exhibited statistical similarities of inhibition with diclofenac sodium from the 1st hour with 31.6% inhibition to the 6th hour 26.1% (p=1.000). The rest of the extracts did not show a promising anti-inflammatory activity.

Based on their inhibitory activity as shown in Figure 2 from the 1st hour up to the 6th hour, 500 mg/kg BW ethanol leaf extract of *P. panayensis* (EPP) and aqueous extract of *P. luzonensis* (AQL) showed constant high similarity with diclofenac sodium with a p-value of 0.971. However, *P. panayensis* (EPP) showed more sustained and higher percent inhibition.

Histopathological Analysis of the Rat Paw tissues. Histopathology of the negative group paw tissues (1% Tween 80 in NSS) showed severe inflammation and edema at the 6th hour after carrageenan induction and was graded with 3 (Figure 3D). Dense inflammatory infiltration particularly of polymorphonuclear leukocytes (PMNs) were seen in the skeletal muscle fibers up to the peri-muscular area. High significant difference between the recorded scores of the negative control and the diclofenac sodium (100 mg/kg BW) as standard control group was observed in acute inflammation and edema at p=0.003 and p=0.021, respectively. This means that the negative control group exhibited significant severe inflammation and edema at a cellular level as compared to the diclofenac-treated group which collectively showed mild inflammation.

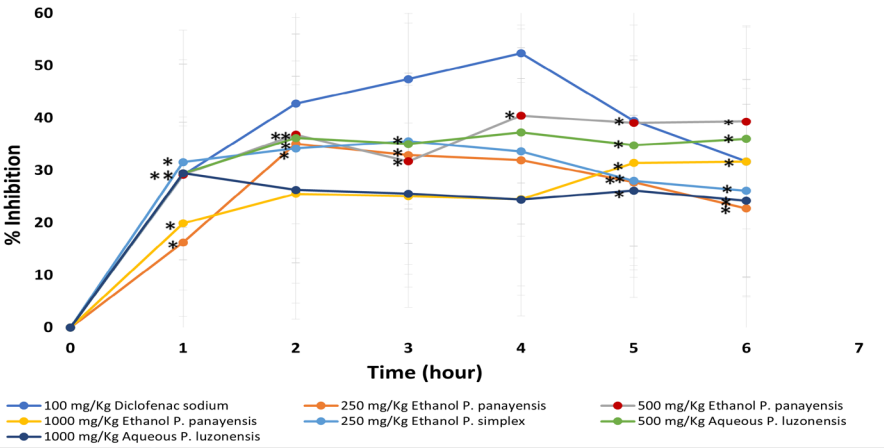


Figure 2. Inhibitory Effect of Alcohol and Aqueous *Pandanus luzonensis*, *Pandanus panayensis* and *Pandanus simplex* Extracts on Carrageenan Induced Inflammation from 0 to 6hr post-sample administration.
(Results are expressed as Mean +/- SD of N=5 at α =0.05).
(*group with optimum similarity at p=0.900 to p=1.000 with diclofenac sodium).

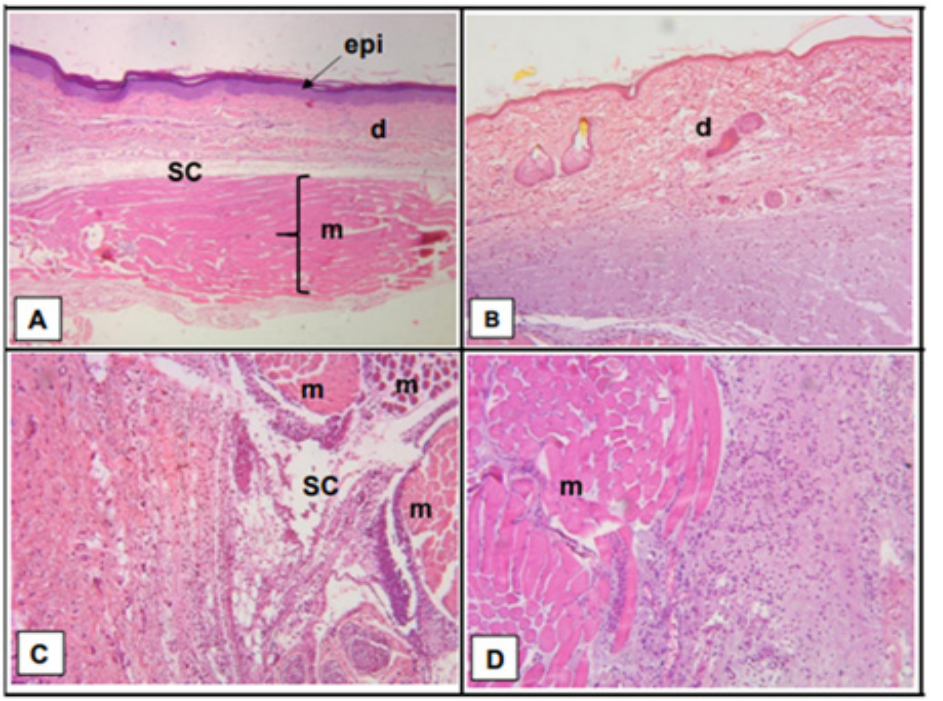


Figure 3. Photomicrograph representing the degree of inflammation of paw tissues: [A] control (score 0) no inflammation seen in the epidermis (epi), subcutaneous tissue (sc), dermis (d) and peri muscular tissue (m), [B] mild (score 1) mild inflammation showing mild edema and neutrophil infiltrates up to the dermis [C] moderate (score 2) inflammatory neutrophil infiltrates in the subcutaneous and peri-muscular tissue [D] severe (score 3) inflammatory neutrophil infiltrates extending within the skeletal muscle layer (Zeiss Primostar® photomicrograph system 100x, except A 40x showing the cross section) (see Supplementary Figure 4).

Methanol extract of *P. luzonensis* (EPL) at 500 mg/kg and 1000 mg/kg BW showed significant difference with negative group in acute inflammation (p=0.059 and p=0.015, respectively) but statistically the same with all doses in terms of edema score. Surprisingly, EPL at 500 mg/ kg and 1000 mg/ kg BW are statistically comparable with diclofenac from p=0.954 to p=1.000 (Figure 4). Edema score of EPL 500 mg/kg showed close comparison with diclofenac (p=0.902) having a mean score of 2, mild edema (Figure 3C). Meanwhile, all doses of the ethanol extract of *P. payanensis* (EPP) 250, 500,1000 mg/kg BW had statistically similar effect to that of diclofenac in acute inflammation, (p=0.998, p=0.438, and p=0.954) and in edema score (p=0.988 to p=1.000) as shown in Figures 4 and 5. However, these doses showed statistically the same with the negative group but the dose, 250 mg/ kg BW had the least similarity in acute inflammation (p=0.184) and in edema (p=0.068). Inflammatory infiltrates mainly in the peri-muscular tissues and subcutaneous tissue layer showing mild inflammation as seen in the photomicrographs (Figure 3). Ethanol extract of *P. simplex* (EPS) at 500 and 1000 mg/kg BW (2.2 and 2.8, respectively) showed severe inflammation than 250 mg/kg (p=0.998) and edema (p=1.000) compared to that of the negative control group. All the aqueous *Pandanus* extracts did not exhibit anti-inflammatory effect (Figure 4) and only *Pandanus simplex* 250 mg/kg BW (AQS) exhibited an anti-edema potential since its edema score is more similar to the diclofenac sodium (p=1.000) than the negative control (p=0.182) (Figure 5).

In general, the isolated paw tissues of rats randomly assigned to *P. luzonensis* and *P. payanensis* alcohol extracts exhibited mild to moderate inflammation and edema which shows reduction of inflammatory cells like PMNs and edema in the dermis, subcutaneous and peri-muscular tissues as depicted in Figures 3B and 3C (Supplementary Figure 4).

COX Inhibition of P. payanensis ethanol (EPP) extract. COX-1 inhibitory activity of indomethacin (standard drug) at different concentrations (10 and 1,000 µg/mL) were at 77.0% and 80.3% with an IC_{50} value of <10 µg/mL. Inhibition of COX-2 by indomethacin was at 89.4% and 92.4% at 10 and 1,000 µg/mL, respectively with an IC_{50} value of <10 µg/mL. The inhibitory activity of 10 ug/mL indomethacin on COX-1 and COX-2 is significantly higher than the inhibitory activities exhibited by the EPP (p=0.000 to p=0.012) at various concentrations. EPP did not exhibit a comparable median inhibitory activity (IC_{50}) with indomethacin against COX-1 and COX-2. However, it exhibited a notable effect on COX-1 with a reported IC_{50} of 610.69 ug/mL. Interestingly, the extract exhibited a significant increase in activity against COX-1 and COX-2 at 1000 µg/mL (p=0.014) (Supplementary Table 4).

Identification of Putative Compounds present in the EPP by LC-MS. Component identification criteria is represented as a good match; mass accuracy error of ≤ 5 mDa (or ≥ -5 mDa), response for precursor ion ≥ 2,000, Isotope match intensity RMS percent ≤ 20, Isotope match Mz RMS PPM ≤ 15. Out of the 27 peaks, seventeen (17) of these have putative identifications based on the TCM library search. Unidentified compounds were detected at retention time 7.97, 8.07, 9.03, 9.24, 11.90, 12.03, 12.76, 12.97, 14.29 and 14.85 (Table 1).

Some *Pandanus* species are recognized as ethnomedicine, as flavoring agents in food, decorative and as handicraft materials [10, 20]. Traditional use of plants has been identified as possible lead in finding new anti-inflammatory agents [19]. Hence, Philippine endemic *Pandanus* such as *P. luzonensis*, *P. panayensis* and *P. simplex* were scientifically evaluated in this study for their toxicity profile and anti-inflammatory activity.

Toxicity profile was conducted using OECD Guideline 425. Plant alcohol extracts were suspended in 1% Tween 80 NSS and aqueous extracts in distilled water given by oral gavage as required under general consideration of toxicity studies. Result showed that all the alcohol and aqueous leaf extracts of the three endemic *Pandanus* species had no signs of toxicity and with approximate lethal dose of more than 2,000 mg/ kg BW. Existing research studies of different *Pandanus* species have shown no toxicities upon oral administration of its extracts which was also observed in most organs of the treated animals in this study. Leaves of *P. odorifer* have been tested in mice of both sexes at 2000 mg/ kg BW and found no significant weight changes throughout the 14-day observation period. Only sleepiness and reduced motor activity were monitored which could be attributed to *P. odorifer* potential CNS depressant action [20]. Similarly, no record of death and abnormalities were observed in the same study done on prop roots of *P. fascicularis* [21]. Also, toxicity study on the fruit oil of *P. conoideus* given orally up to 5000 mg/kg BW also showed no abnormalities in the necropsy of organs [22].

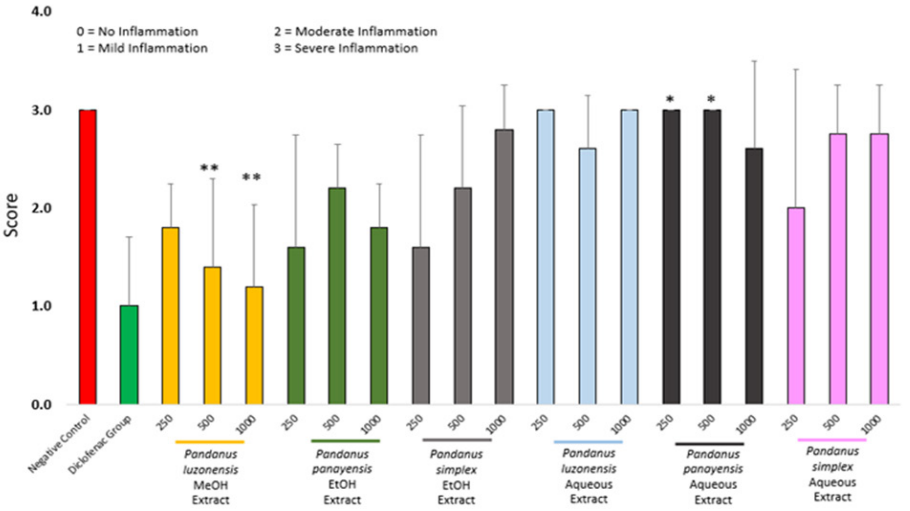


Figure 4. Histopathological score of acute inflammation seen in paw tissues after treatment with *Pandanus extracts* in carrageenan-induced paw edema assay

Results are expressed as Mean +/- SD of N=5 at α =0.05

**p<0.05 significantly different from the negative control but p>0.05 similar with diclofenac sodium group
*p>0.05 significantly similar with negative group but p<0.05 different from diclofenac sodium group

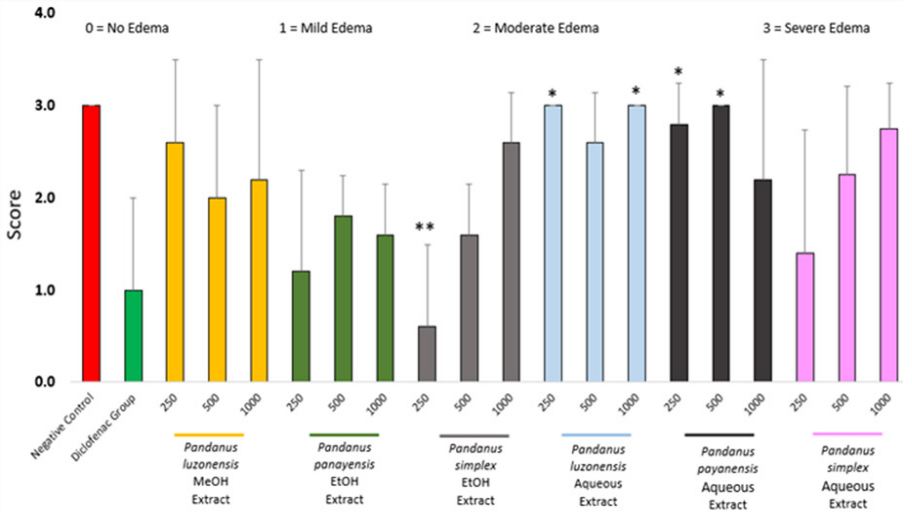


Figure 5. Histopathological score of edema seen in paw tissues after treatment with Pandanus extracts in carrageenan-induced paw edema assay

Results are expressed as Mean +/- SD of N=5 at α =0.05

**p<0.05 significantly different from the negative control but p>0.05 similar with diclofenac sodium group
*p> 0.05 significantly similar with negative group but p<0.05 different from diclofenac sodium group).

Table 1. LC-MS Metabolite Profiling of *P. payanensis* leaf ethanol extract.

RT° (min)	Observed m/z	Calculated m/z	Molecular Formula	Error	Component name Legend
3.26	595.1662	594.15847	$C_{27}H_{30}O_{15}$	0.4821	Oroxin B
3.49	565.1556	564.14791	$C_{26}H_{28}O_{14}$	0.3917	Apiin
3.59	565.1555	564.14791	$C_{26}H_{28}O_{14}$	0.3666	Apiin
3.74	565.1554	564.14791	$C_{26}H_{28}O_{14}$	0.2021	Apiin
4.05	579.1714	578.16356	$C_{27}H_{30}O_{14}$	0.5592	Yuankanin
6.73	331.0811	330.07395	$C_{17}H_{14}O_{7}$	-0.1482	3,5,8-Trihydroxy-3',4'- dimethoxyflavone
7.97	274.2733				Candidate Mass 274.2733
8.07	318.2998				Candidate Mass 318.3000
9.03	300.2893				Candidate Mass 300.2893
9.24	318.3000				Candidate Mass 318.3000
9.44	467.1941	466.1868	$C_{23}H_{30}O_{10}$	2.9433	Ilexin II
9.54	415.2114	392.2222	$C_{22}H_{32}O_{6}$	2.2630	Nigakihemiacetal F
10.41	277.2156	276.2083	$C_{18}H_{28}O_{2}$	-0.6559	Stearidonic acid
10.52	277.2156	276.2083	$C_{18}H_{28}O_{2}$	-0.6553	Stearidonic acid
10.81	529.2101	528.2028	$C_{28}H_{32}O_{10}$	3.2733	Physalin L
11.14	279.2316	278.2243	$C_{18}H_{30}O_{2}$	-0.3028	γ-Linoleic acid
11.68	595.2464	594.2312	$C_{29}H_{38}O_{13}$	7.9244	Pseudolaric acid B O-β-D-glucopyranoside
11.90	306.2058				Candidate Mass 306.2058
12.03	515.3199				Candidate Mass 515.3199
12.76	623.2502				Candidate Mass 623.2502
12.97	639.2457				Candidate Mass 639.2457
13.47	425.2145	424.20972	$C_{22}H_{32}O_{8}$	-2.5083	Nigakilactone H
14.20	609.2715	586.27780	$C_{32}H_{42}O_{10}$	4.4971	Azedarachin C
14.29	625.2666				Candidate Mass 625.2666
14.53	609.2716	586.27780	$C_{32}H_{42}O_{10}$	4.5611	Azedarachin C
14.85	593.2766				Candidate Mass 593.2766
14.99	609.2712	586.27780	$C_{32}H_{42}O_{10}$	4.2295	Azedarachin C

Among all the extracts, ethanol extract *P. payanensis* (EPP) was found to have the highest inflammatory inhibition and could sustain its action up to the 6th hour comparable with the standard, diclofenac sodium. The aqueous extract (AQL) of *P. luzonensis* at 500 mg/ kg BW and ethanol extract of *P. simplex* (EPS) at 250 mg/ kg BW showed comparable inflammation inhibition with diclofenac sodium (Figure 2). However, the COX-1 and COX- 2 inhibitory activity of the EPP at various concentrations is significantly lower and not as potent as that of indomethacin (10 ug/mL) (Supplementary Table 4). The difference between *in vivo* and i*n vitro* analyses could happen as reported in some studies. Different parameters do not always illustrate quantitative or qualitative correlation. In addition, some biological pathways present in *in-vivo* assay cannot be observed in *in-vitro* which confirms the possibility of discrepancies [23]. This could mean that *P. payanensis* extract exhibits anti-inflammatory activity with different mechanism. Similar activity has been studied and showed significant inflammatory reduction in carrageenan-induced rat paw edema treated with an aqueous leaf extract of *P. tectorius* at 500 mg/kg BW [12]. In addition, inhibition at a lower dose of 250 mg/kg BW was also seen in alcohol and aqueous prop roots extract of *P. fascicularis*, which is nearly equivalent to indomethacin at 10 mg/kg BW [21]. Therefore, potential anti-inflammatory activity of the EPP at 500 mg/kg BW is the optimum dose that can sustain until the 6th hour as presented in Figure 2.

Histopathology analysis of induced paw elicited marked reduction of neutrophils infiltration in the carrageenan-treated paws specifically in the diclofenac sodium and the alcohol extract groups while the negative group presented massive inflammation. Comparable decrease in the degree of edema was observed in EPP 250 mg/ kg BW and AQS 250 mg/kg BW. To the researchers' knowledge, this is a pioneering study on the in-vivo anti-inflammatory property of *P. luzonensis, P. panayensis and P. simplex*. Neutrophils and macrophages response to inflammatory mediators by causing tissue inflammation due to pro-inflammatory proteins expression [19,24]. In this study, the alcohol extract group demonstrated anti-inflammatory property by reducing the neutrophil expression based on the histopathological studies as shown on Fig 3.

The putative analysis of the LC-MS revealed oroxin B, a flavonoid isolated from traditional Chinese medicine studied by Li et al. in 2019, which showed that it has antitumor property and acts by downregulating COX-2, Vascular Endothelial Growth Factor (VEGF) and Phosphatidylinositol 3-phosphatase/phosphoinositide 3-kinase/ Protein Kinase B (PTEN/PI3K/ AKT) pathways. The presence of apiin, another natural flavonoid with anti-inflammatory property demonstrated a reduction of inducible nitric oxide synthase (iNOS) enzyme expression both in in-vitro and in-vivo tests [25]. Inducible iNOS is a critical inflammatory modulator that can cause cell damage resulting to several inflammatory diseases in overly expressed nitric oxide (NO) [26]. Moreover, there are presence of Nigakihemiacetal and Nigakilactone H which are quassinoids. A wide range of pharmacological benefits are attributed to quassinoids which include anti-inflammatory and analgesic [27]. Quassinoids function by inhibiting NF-kB signaling pathway leading to reduction of COX-2 and iNOS expression [28]. Like quassinoids, physalins also inhibit NF-kB activation, preventing proinflammatory expression of TNF-α, IL- 6 and IL-12 [29]. Stearidonic acid and pseudolaric acid B were also found to inhibit NF-kB and MAPK pathway [30]. Although linoleic acid (LA) is a precursor of AA and assists the formation of PGs, the epoxides of LA and AA produce epoxyeicosatrienoic acids (EETs) which decrease inflammatory response [26]. In addition, conjugated linoleic acid has been reported to alleviate colitis in inflammatory bowel movement by Peroxisome Proliferator-Activated Receptor-γ (PPAR ϒ) dependent mechanism. PPAR ϒ inactivates and interferes with the function of NF-kB and reduces reactive oxygen species [31]. A flavonol, 3,5,8-Trihydroxy-3',4'-dimethoxyflavone was found in *Blumea balsamifera* (3,5,7-trihydroxy-3'4'-dimethoxyflavone), a non-volatile constituent which was reported to have antitumor activity by acting synergistically with tumor related apoptosis inducing ligand (TRAIL) [32]. Lastly, azedarachin C, a limonoid, which modulates p38 MAP kinase activity, downregulates TLR signalling pathway and reduces NO production, among which explain its anti-inflammatory activity [33]. Although the specific compounds identified have not been reported in other metabolite profiling of *Pandanus* spp., presence of flavonoids, steroids, terpenoids and fatty acid have been attributed to exhibit anti-inflammatory and analgesic activity (Table 1). Interestingly, this is the first report of metabolites found in ethanol extract of *P. panayensis*.

Conclusion

In conclusion, this study has shown that ethanol extracts of *P. panayensis* and *P. simplex* and aqueous extract of *P. luzonensis* possess significant anti-inflammatory activity. Ethanol extract of *P. panayensis* 500 mg/kg exhibited the highest inhibition and showed potential anti-edema property in histopathological studies. Alcohol and aqueous extracts of the three endemic species have an approximate lethal dose of more than 2,000 mg/kg BW and are generally nontoxic. The results established the *Pandanus*' ethnomedicinal use for pain and inflammation reduction. Putative compounds identified can support such biological activities.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Conceptualization, A.C., J.A. and M.N.; methodology, A.C., J.A. and M.N; data collection, A.C., P.D.J. and R.Y.; analysis and interpretation of data, A.C., P.D.J., R.Y., and M.N.; original draft preparation, A.C., P.D.J., and M.N.; review and editing of the draft, A.C., P.D.J., R.Y., and M.N. All authors have read and agreed to the final version of the manuscript.

Institutional Review Board Statement

Not applicable.

Informed Consent Statement

Not applicable.

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Supplementary Table 1A. Weight monitoring of the test animals during Acute Toxicity Test

GROUP	RAT No.	WEIGHT (grams)		*Dose (extract-2000 mg/kg BW and water 10mL/ kg BW)
		1st Day	14th Day	
Control (Water)	1	200	215	2
	2	195	190	1.95
	3	190	190	1.9
	4	190	190	1.9
	5	225	225	2.25
Ethanol leaf extract *Pandanus luzonensis* (OHL)	1	165	190	0.33
	2	170	205	0.34
	3	195	215	0.39
	4	190	210	0.38
	5	185	205	0.37
Ethanol leaf extract *Pandanus panayensis* (OHP)	1	205	220	0.41
	2	165	200	0.33
	3	145	195	0.29
	4	195	205	0.39
	5	175	190	0.35
Ethanol leaf extract *Pandanus simplex* (OHS)	1	170	190	0.34
	2	195	225	0.39
	3	215	200	0.43
	4	220	240	0.44
	5	175	190	0.35
Aqueous leaf extract *Pandanus luzonensis* (AQL)	1	220	226	0.44
	2	203	213	0.41
	3	203	202	0.41
	4	233	243	0.47
	5	208	205	0.42
Aqueous leaf extract *Pandanus panayensis* (AQP)	1	160	175	0.32
	2	160	170	0.32
	3	145	165	0.29
	4	160	160	0.32
	5	185	205	0.37
Aqueous leaf extract *Pandanus simplex* (AQS)	1	240	226	0.48
	2	210	226	0.42
	3	202	218	0.41
	4	176	183	0.35
	5	176	183	0.35

Supplementary Table 1B. Weight of the test animals during Anti-inflammatory Test

Group	Dose	Rat No.	Weight (g)	Calculated Dose (neg Grp-mL; Treated Group-mg)	Stock Soln Conc	No. of mL
Neg Grp (1% Tween 80)	10 mL/kg BW	1	220	2.2	1% Tween 80 in NSS	2.2
		2	220	2.2		2.2
		3	220	2.2		2.2
		4	240	2.4		2.4
		5	240	2.4		2.4
Diclofenac sodium	100 mg/kg BW	1	230	23	20 mg/mL	1.5
		2	290	29		1.45
		3	260	26		1.3
		4	250	25		1.25
		5	220	22		1.1
Methanol leaf extract *P. luzonensis* (OH)	1000 mg/kg BW	1	220	220	167 mg/mL	1.32
		2	170	170		1.02
		3	165	165		0.99
		4	240	240		1.44
		5	220	220		1.32
	500 mg/kg BW	1	210	105	167 mg/mL	0.63
		2	180	90		0.54
		3	240	120		0.72
		4	190	95		0.57
		5	210	105		0.63
	250 mg/kg BW	1	195	48.75	167 mg/mL	0.29
		2	165	41.25		0.25
		3	195	48.75		0.29
		4	210	52.5		0.31
		5	240	60		0.36
Ethanol leaf extract *P. panayensis* (OHP)	1000 mg/kg BW	1	250	250	167 mg/mL	1.5
		2	290	290		1.74
		3	250	250		1.5
		4	225	225		1.35
		5	300	300		1.8
	500 mg/kg BW	1	250	125	167 mg/mL	0.75
		2	365	182.5		1.09
		3	260	130		0.78
		4	275	137.5		0.82
		5	300	150		0.9
	250 mg/kg BW	1	240	60	167 mg/mL	0.36
		2	255	63.75		0.38
		3	290	72.5		0.43
		4	265	66.25		0.4
		5	300	75		0.45

Supplementary Table 1B. Weight of the test animals during Anti-inflammatory Test ***(cont'd.)***

Group	Dose	Rat No.	Weight (g)	Calculated Dose (neg Grp-mL; Treated Group-mg)	Stock Soln Conc	No. of mL
Aqueous leaf extract *P. luzonensis* (AQL)	1000 mg/kg BW	1	283	283	167 mg/mL	1.69
		2	305	305		1.83
		3	266	266		1.59
		4	251	251		1.5
		5	219	219		1.31
	500 mg/kg BW	1	254	127	167 mg/mL	0.76
		2	260	130		0.78
		3	218	109		0.65
		4	223	111.5		0.67
		5	254	127		0.76
	250 mg/kg BW	1	275	68.75	167 mg/mL	0.41
		2	212	53		0.32
		3	230	57.5		0.34
		4	309	77.25		0.46
		5	287	71.75		0.43
Aqueous leaf extract *P. panayensis* (AQP)	1000 mg/kg BW	1	240	240	167 mg/mL	1.44
		2	195	195		1.17
		3	210	210		1.26
		4	225	225		1.35
		5	200	200		1.2
	500 mg/kg BW	1	240	120	167 mg/mL	0.72
		2	160	80		0.48
		3	175	87.5		0.52
		4	215	107.5		0.64
		5	225	112.5		0.97
	250 mg/kg BW	1	230	57.5	167 mg/mL	0.34
		2	140	35		0.21
		3	225	56.25		0.34
		4	205	51.25		0.31
		5	200	50		0.3
Aqueous leaf extract *P. simplex* (AQS)	1000 mg/kg BW	1	288	288	167 mg/mL	1.72
		2	243	243		1.46
		3	228	228		1.37
		4	242	242		1.45
		5	265	265		1.59
	500 mg/kg BW	1	327	163.5	167 mg/mL	0.98
		2	228	114		0.68
		3	338	169		1.01
		4	302	151		0.9
		5	324	162		0.97
	250 mg/kg BW	1	276	69	167 mg/mL	0.41
		2	310	77.5		0.46
		3	238	59.5		0.36
		4	268	67		0.4
		5	333	83.25		0.5

Supplementary Table 2. Scoring Used for the Histopathological Examination of the Paw tissues in Anti-Inflammatory test

Grading	Description
0	absence of any reactive inflammatory infiltrates throughout the skin layers
1	mild, low grade inflammation, presence of inflammatory infiltrates in the dermis and minimal in the subcutaneous tissue layer
2	moderate numbers of inflammatory infiltrates up the subcutaneous tissue layer
3	dense inflammatory infiltrates up to the skeletal muscle

References: Morris, 2003; Gibson-Corley et al., 2013

Supplementary Table 3. Summary of the Observed Behavior of Test Animals and Monitoring Parameters during Acute Toxicity Test

	Parameters								
	Summary of Observation Time (0.5 hr, 1 hr, 4 hrs, 24 hrs, 14 days after treatment)								
Group	changes in skin, fur	eyes	salivation	respiration	feces consistency	somatomotor and behavior	sleep	convulsion	Mortality
Control	Ne	N	N	N	N	N	N	Ne	0/5
Methanol Extract *Pandanus luzonensis*	Ne	N	N	N	N	N	N	Ne	0/5
Ethanol Extract *Pandanus panayensis*	Ne	N	N	N	N	N	N	Ne	0/5
Ethanol Extract *Pandanus simplex*	Ne	N	N	N	N	N	N	Ne	0/5
Aqueous Extract *Pandanus luzonensis*	Ne	N	N	N	N	N	N	Ne	0/5
Aqueous Extract *Pandanus panayensis*	Ne	N	N	N	N	N	N	Ne	0/5
Aqueous Extract *Pandanus simplex*	Ne	N	N	N	N	N	N	Ne	0/5

***Ne**- negative **N**- normal Mortality: No. of deaths/No. of animals tested

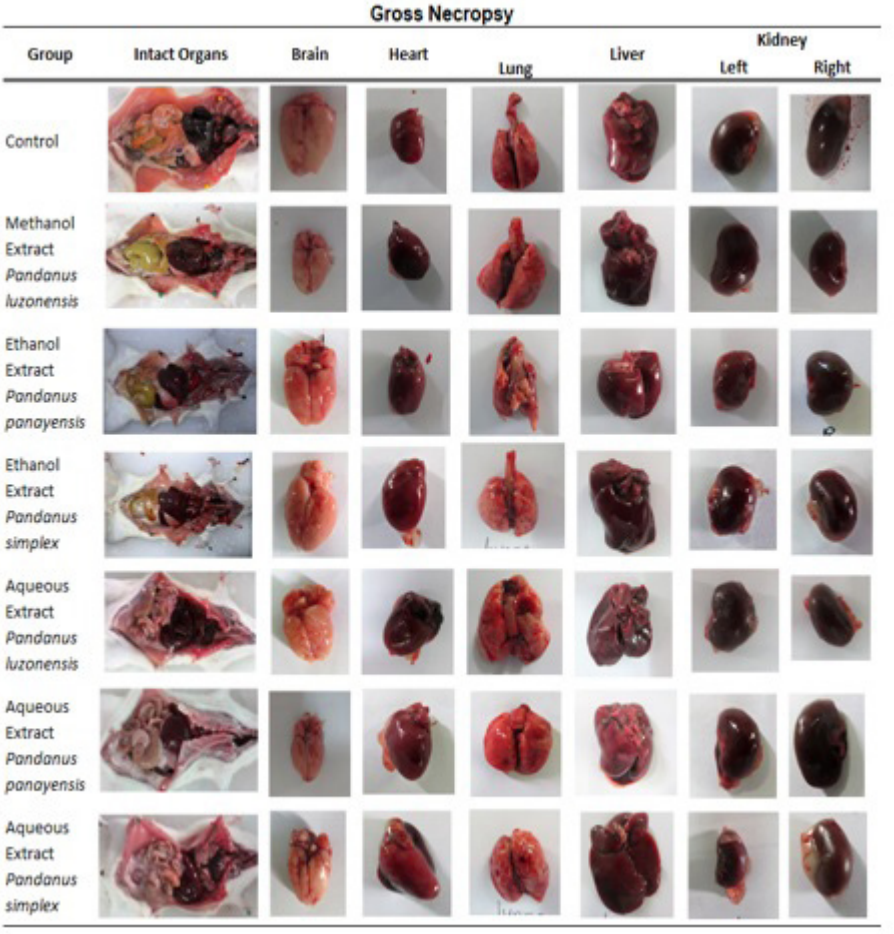
The table summarizes the results of the 14 days cage side observation whereas normal behavior, motor, and neuronal functions were recorded with no mortality.

Supplementary Table 4. COX-1 and COX-2 inhibition (%) of *Pandanus payanensis* ethanol (EPP) extract

Group	Conc (µg/mL)	% COX-1 Inhibition	IC_{50} (µg/mL)	% COX-2 Inhibition	IC_{50} (µg/mL)
Ethanol Extract *Pandanus panayensis*	10	35.1 ± 6.8	610.69	13 ± 3.7	>1000
	50	41.9 ± 1.9		16.9 ± 1.2	
	100	44.5 ± 0.6		20.6 ± 3.6	
	500	48.4 ± 0.5		23.1 ± 3.3	
	1000	55.7 ± 2.7*		27.3 ± 2.2*	
Indomethacin	10	77 ± 6	<10.00	89.4 ± 2.8	<10
	1000	80.3 ± 14.4		94.2 ± 6.7	

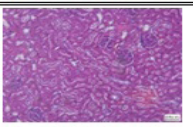
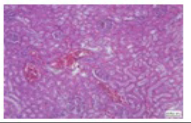
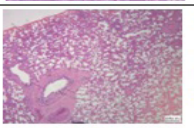
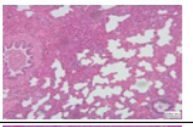
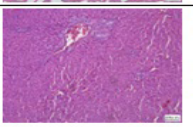
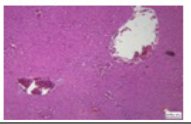
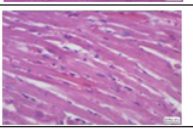
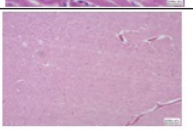
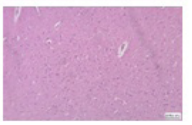
% Inhibition of COX-1 and COX-2 and IC_{50} of the standard drug indomethacin (10 µg/mL) against ethanol extract of *Pandanus panayensis* (10,50,100,500 and 1000 ug/mL) (p=0.000, N=2).(* a significant increase in activity was detected at p=0.014).

Supplementary Figure 1. Gross Necropsy Results for Each Treatment Group after Acute Toxicity Test



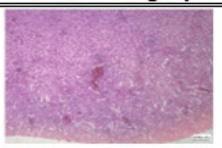
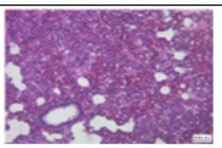
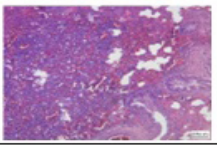
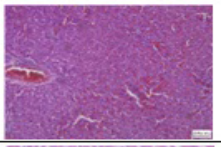
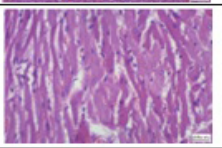
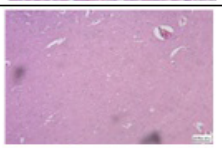
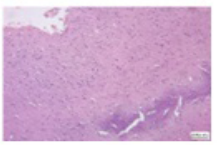
Supplementary Figure 2. Histopathological photomicrographs of representative organs from test animals after acute toxicity study (Control Group)

Control Group

Organ	Magnification	Photomicrograph	Interpretation
kidney	100x		Histology of unremarkable kidney tissue showing intact glomerular apparatuses and renal tubules
	100x		vascular congestion
lungs	40x		Histology of unremarkable lung tissue showing intact alveolar structures and bronchiole. There is mild peribrochiolar lymphoplasmacytic infiltrates which is a normal feature of the mucosa-associated lymphoid tissue (MALT) in the lungs
	100x		severe pneumonia
liver	100x		Histology of unremarkable liver tissue showing intact hepatic lobule. The area of the hepatic triad, centrilobular veins, and arrays of hepatocytes show no cytologic or architectural distortion, haemorrhage or necrosis.
	40x		passive vascular congestion
heart	400x		Histology of unremarkable myocardium. No features of necrosis, inflammation, and haemorrhage is present.
brain	100x		Histology of unremarkable cerebrum showing intact meninges, and cerebral cortex (gray matter). No feature of inflammation, necrosis, or edema is present.
	100x		mild gliosis

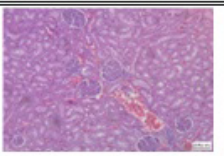
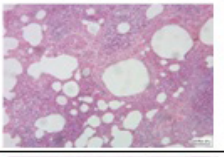
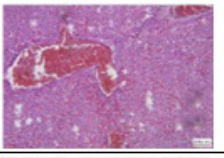
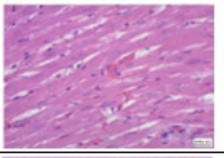
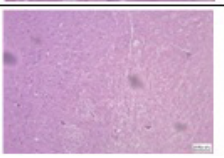
Supplementary Figure 2. Histopathological photomicrographs of representative organs from test animals after acute toxicity study (Methanol Extract of *Pandanus luzonensis*)

Methanol Extract of *Pandanus luzonensis*

Organ	Magnification	Photomicrograph	Interpretation
kidney	40x		vascular congestion
lungs	100x		severe pneumonia
	100x		acute bronchopneumonia
liver	100x		passive vascular congestion
heart	400x		Histology of unremarkable myocardium. No features of necrosis, inflammation, and haemorrhage is present
brain	100x		histology of unremarkable cerebrum showing intact meninges, and cerebral cortex (gray matter). No feature of inflammation, necrosis, or edema is present
	100x		mild gliosis, focal

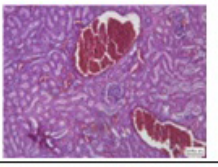
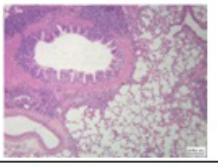
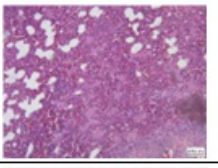
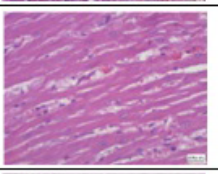
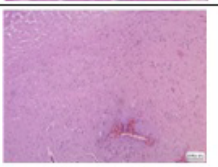
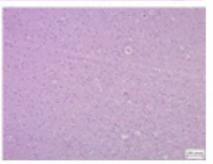
Supplementary Figure 2. Histopathological photomicrographs of representative organs from test animals after acute toxicity study (Ethanol Extract of *Pandanus panayensis*)

Ethanol Extract *Pandanus panayensis*

Organ	Magnification	Photomicrograph	Interpretation
kidney	100x		vascular congestion
lungs	100x		pneumonia, chronic passive congestion
liver	100x		passive vascular congestion
heart	400x		Histology of unremarkable myocardium. No features of necrosis, inflammation, and haemorrhage is present
brain	100x		Histology of unremarkable cerebrum showing intact meninges, and cerebral cortex (gray matter). No feature of inflammation, necrosis, or edema is present.

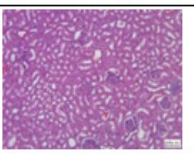
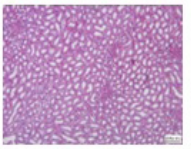
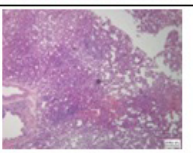
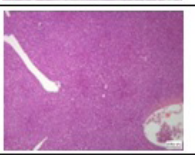
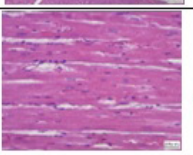
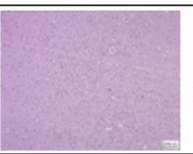
Supplementary Figure 2. Histopathological photomicrographs of representative organs from test animals after acute toxicity study (Ethanol Extract of *Pandanus simplex*)

Ethanol Extract *Pandanus simplex*

Organ	Magnification	Photomicrograph	Interpretation
kidney	100x		vascular congestion
lungs	100x		severe pneumonia
liver	100x		passive vascular congestion
heart	400x		Histology of unremarkable myocardium. No features of necrosis, inflammation, and haemorrhage is present
brain	100x		Histology of unremarkable cerebrum showing intact meninges, and cerebral cortex (gray matter). No feature of inflammation, necrosis, or edema is present.
	100x		mild gliosis

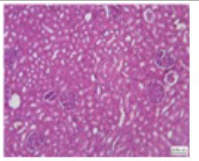
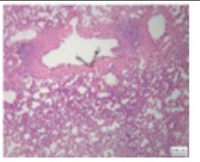
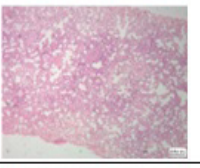
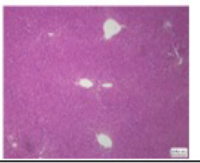
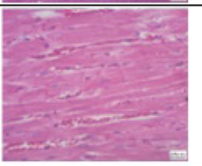
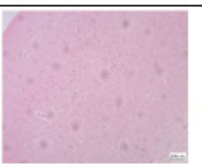
Supplementary Figure 2. Histopathological photomicrographs of representative organs from test animals after acute toxicity study (Aqueous Extract of *Pandanus luzonensis*)

Aqueous Extract *Pandanus luzonensis*

Organ	Magnification	Photomicrograph	Interpretation
kidney	100x		Histology of unremarkable kidney tissue showing intact glomerular apparatuses and renal tubules
	100x		Histology of unremarkable kidney tissue showing intact glomerular apparatuses and renal tubules
lung	40x		diffuse atelectasis
liver	40x		Histology of unremarkable liver tissue showing intact hepatic lobule. The area of the hepatic triad, centrilobular veins, and arrays of hepatocytes show no cytologic or architectural distortion, haemorrhage or necrosis.
heart	400x		Histology of unremarkable cerebrum showing intact meninges, and cerebral cortex (gray matter). No feature of inflammation, necrosis, or edema is present.
brain	100x		Histology of unremarkable cerebrum showing intact meninges, and cerebral cortex (gray matter). No feature of inflammation, necrosis, or edema is present.

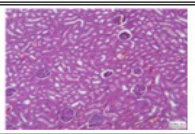
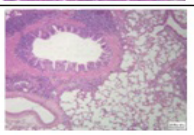
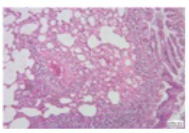
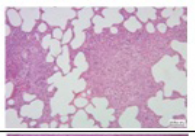
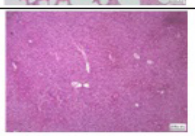
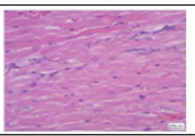
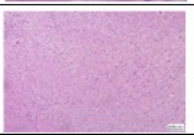
Supplementary Figure 2. Histopathological photomicrographs of representative organs from test animals after acute toxicity study (Aqueous Extract of *Pandanus panayensis*)

Aqueous Extract *Pandanus panayensis*

Organ	Magnification	Photomicrograph	Interpretation
kidney	100x		Histology of unremarkable kidney tissue showing intact glomerular apparatuses and renal tubules
lungs	40x		focal atelectasis
	40x		diffuse atelectasis
liver	40x		Histology of unremarkable liver tissue showing intact hepatic lobule. The area of the hepatic triad, centrilobular veins, and arrays of hepatocytes show no cytologic or architectural distortion, haemorrhage or necrosis.
heart	400x		Histology of unremarkable myocardium. No features of necrosis, inflammation, and haemorrhage is present.
brain	100x		Histology of unremarkable cerebrum showing intact meninges, and cerebral cortex (gray matter). No feature of inflammation, necrosis, or edema is present.

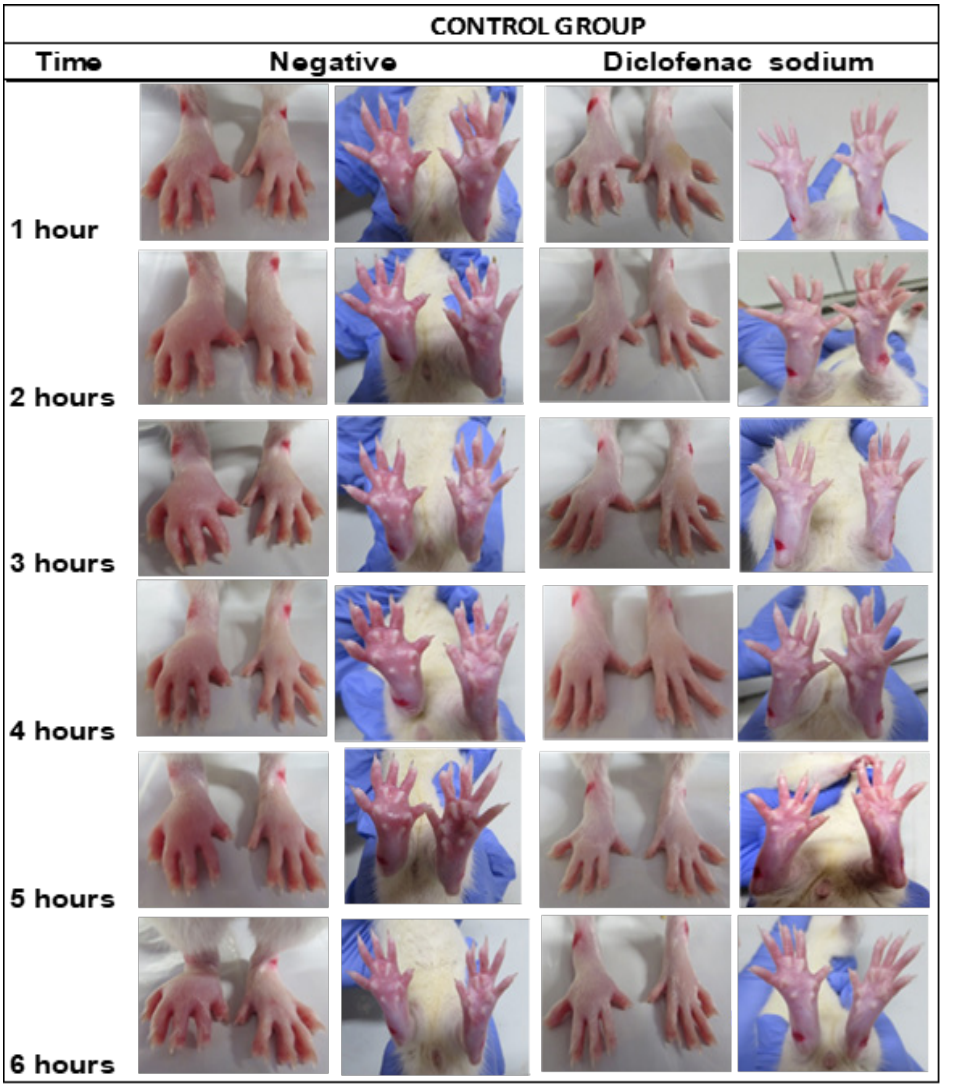
Supplementary Figure 2. Histopathological photomicrographs of representative organs from test animals after acute toxicity study (Aqueous Extract of *Pandanus simplex*)

Aqueous Extract *Pandanus simplex*

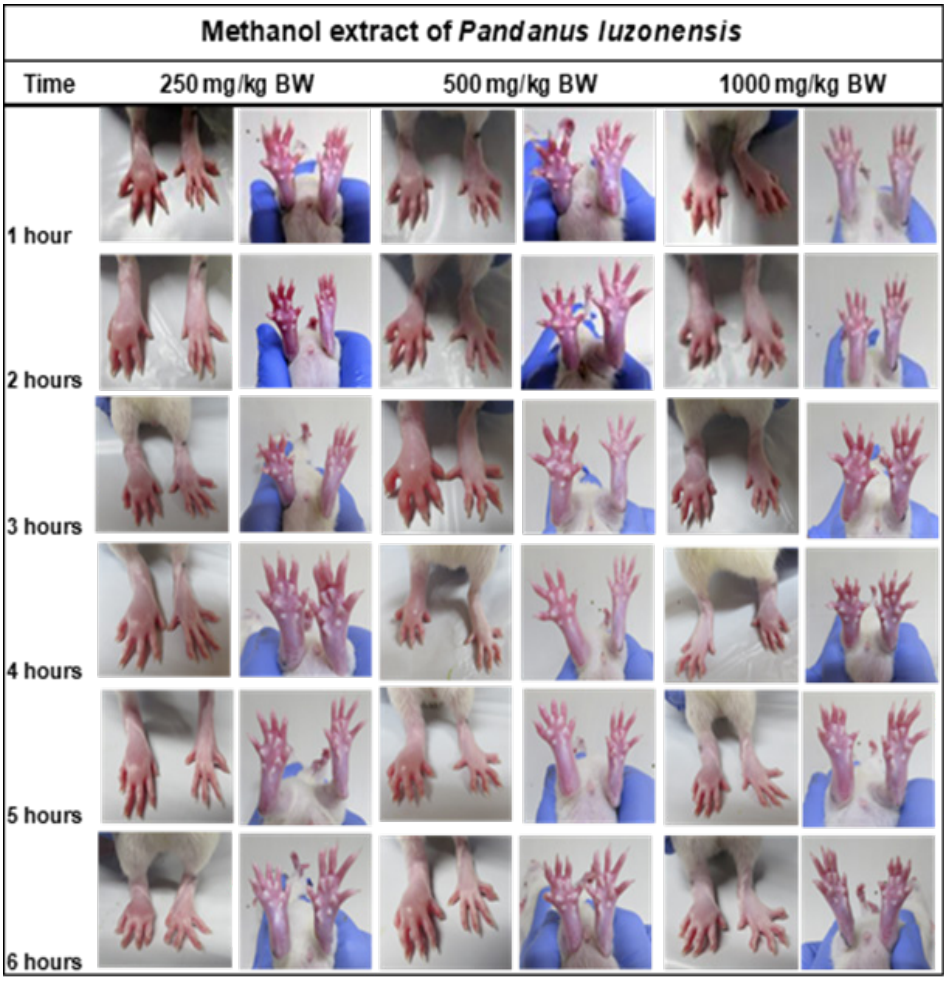
Organ	Magnification	Photomicrograph	Interpretation
kidney	100x		Histology of unremarkable kidney tissue showing intact glomerular apparatuses and renal tubules
lung	40x		Histology of unremarkable lung tissue showing intact alveolar structures and bronchiole. There is mild peribrochiolar lymphoplasmacytic infiltrates which is a normal feature of the mucosa-associated lymphoid tissue (MALT) in the lungs
	100x		focal pulmonary edema
	100x		foci of granulomatous inflammation
liver	40x		Histology of unremarkable liver tissue showing intact hepatic lobule. The area of the hepatic triad, centrilobular veins, and arrays of hepatocytes show no cytologic or architectural distortion, haemorrhage or necrosis.
heart	400x		Histology of unremarkable myocardium. No features of necrosis, inflammation, and haemorrhage is present.
brain	100x		Histology of unremarkable cerebrum showing intact meninges, and cerebral cortex (gray matter). No feature of inflammation, necrosis, or edema is present.

N.B. Overall, postmortem gross examination of selected visceral organs shows no significant pathologic alteration, except for patchy white areas of consolidation in the lungs, presumably inflammatory and infectious in nature, noted in both treated and control groups administered with plain water (See Control Group). These observations suggest that a pre-existing pneumonic pathology was present in some of the test animals, presumably unrelated to the toxic effects of the extracts. This may be the consequences of unforeseen external factors such as stress, diet, environmental conditions, immune resistance, and present state of health as it arrived coming from the distributor of the test animals.

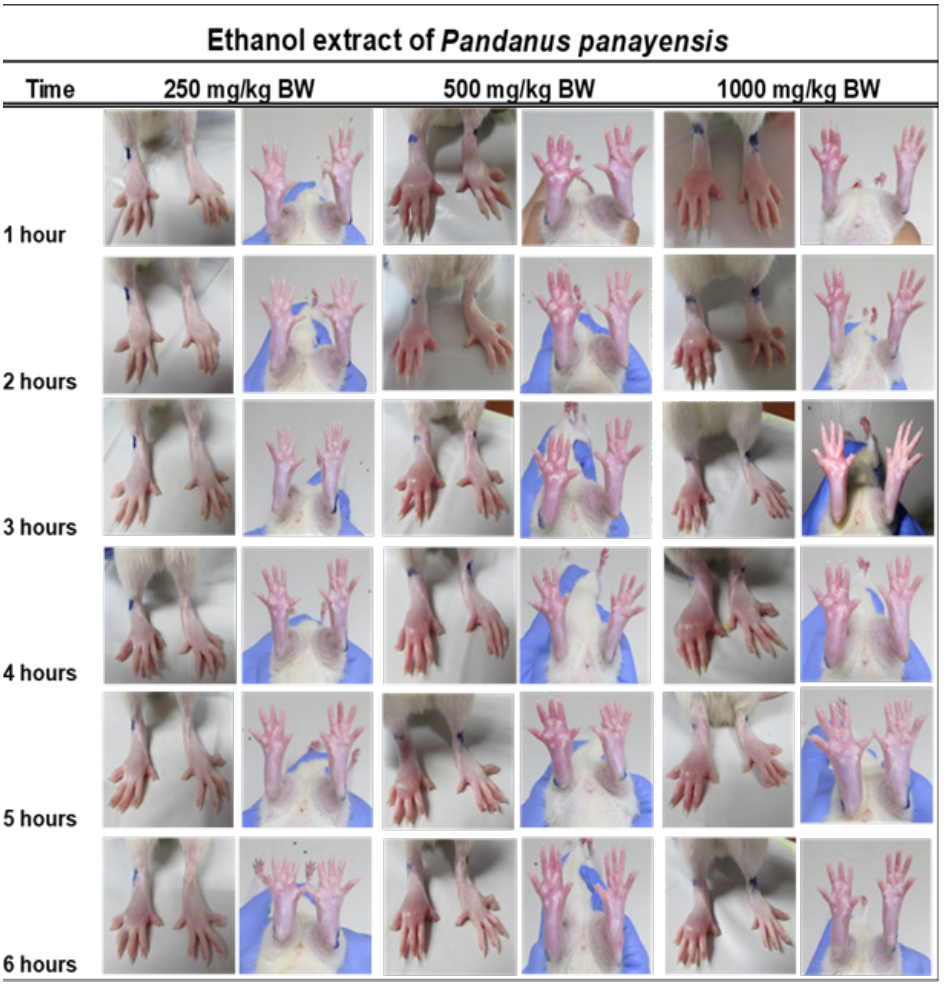
Supplementary Figure 3. The progressive photos of representative rats per treatment group in carrageenan-induced paw edema (1st hour-6th hour). (Control Group)



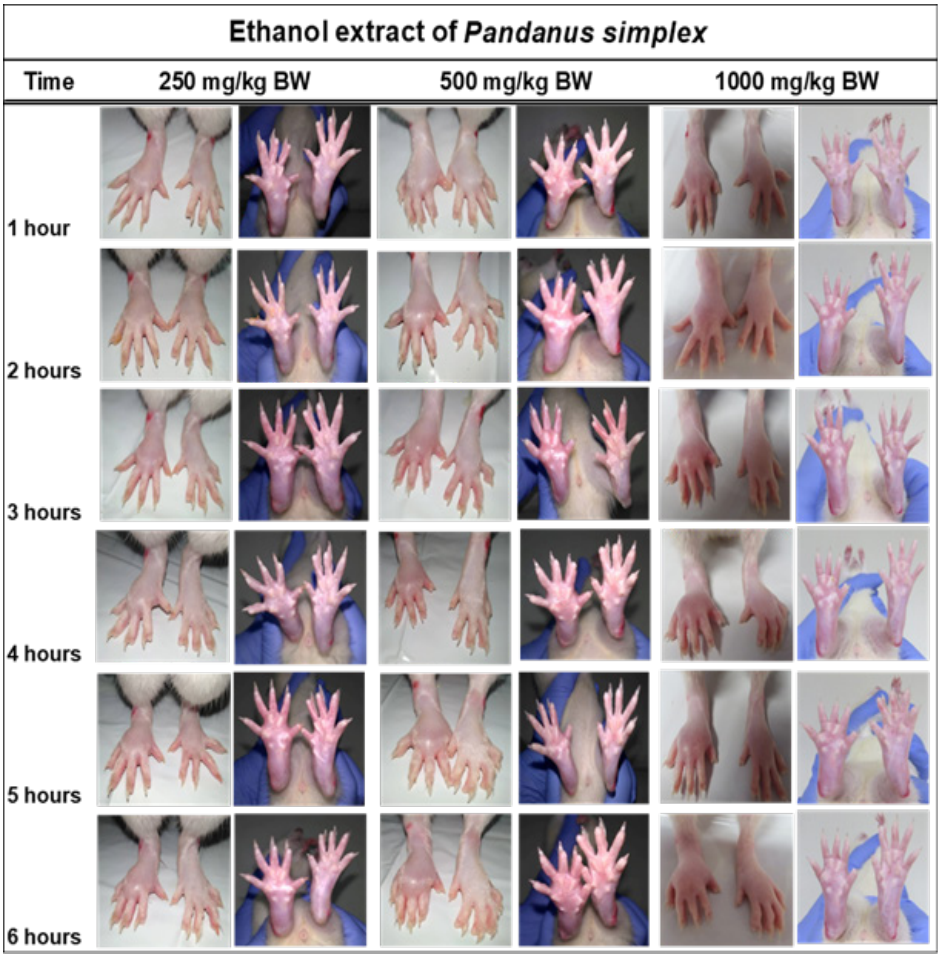
Supplementary Figure 3. The progressive photos of representative rats per treatment group in carrageenan-induced paw edema (1st hour-6th hour). (Methanol Extract of *Pandanus luzonensis*)



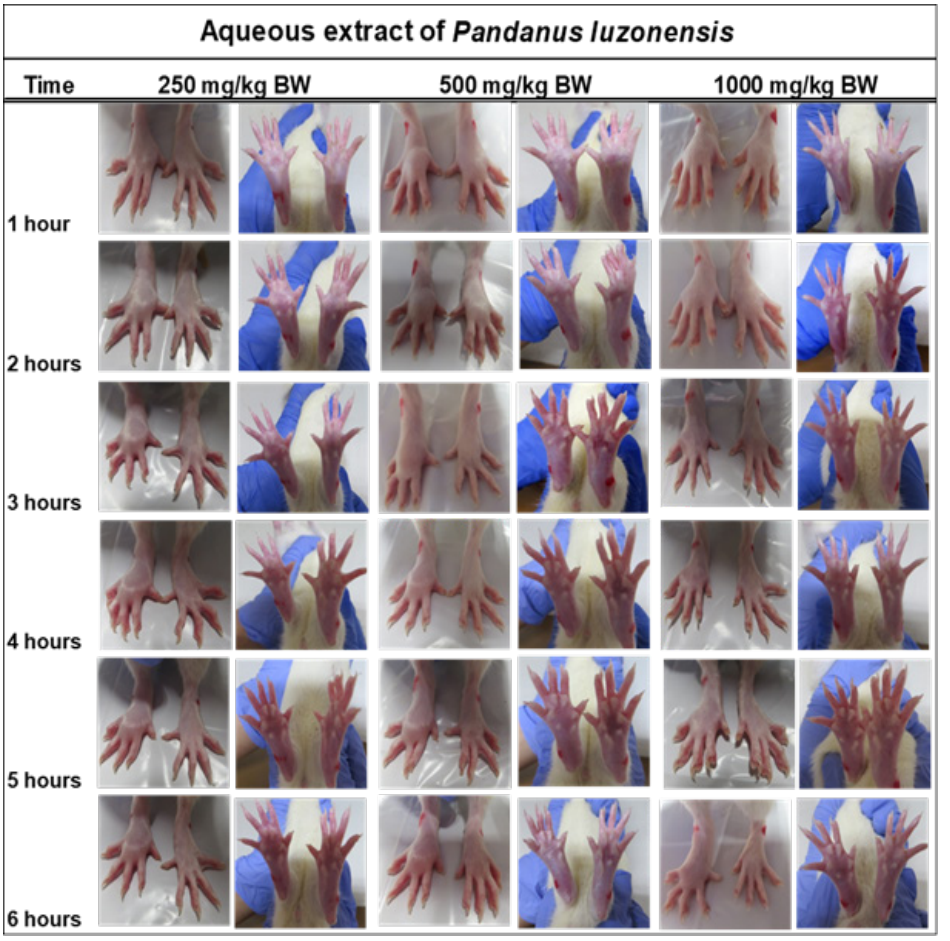
Supplementary Figure 3. The progressive photos of representative rats per treatment group in carrageenan-induced paw edema (1st hour-6th hour). (Ethanol Extract of *Pandanus panayensis*)



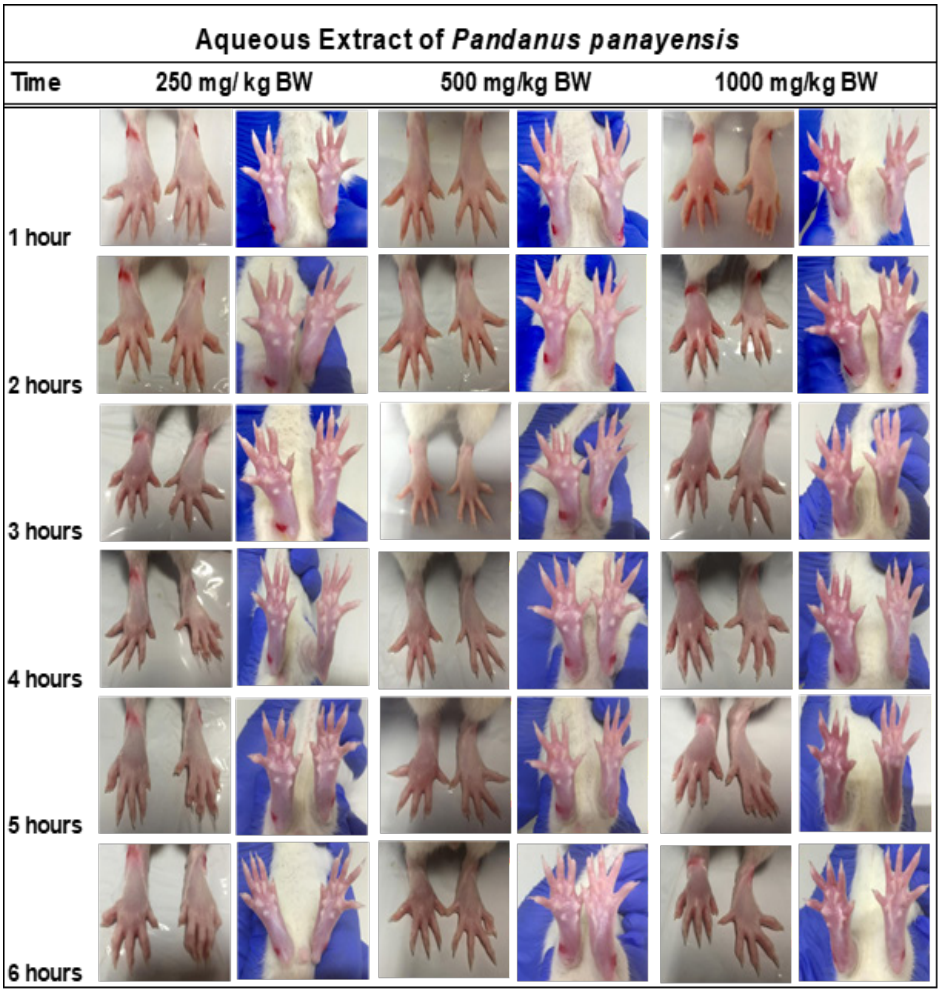
Supplementary Figure 3. The progressive photos of representative rats per treatment group in carrageenan-induced paw edema (1st hour-6th hour). (Ethanol Extract of *Pandanus simplex*)



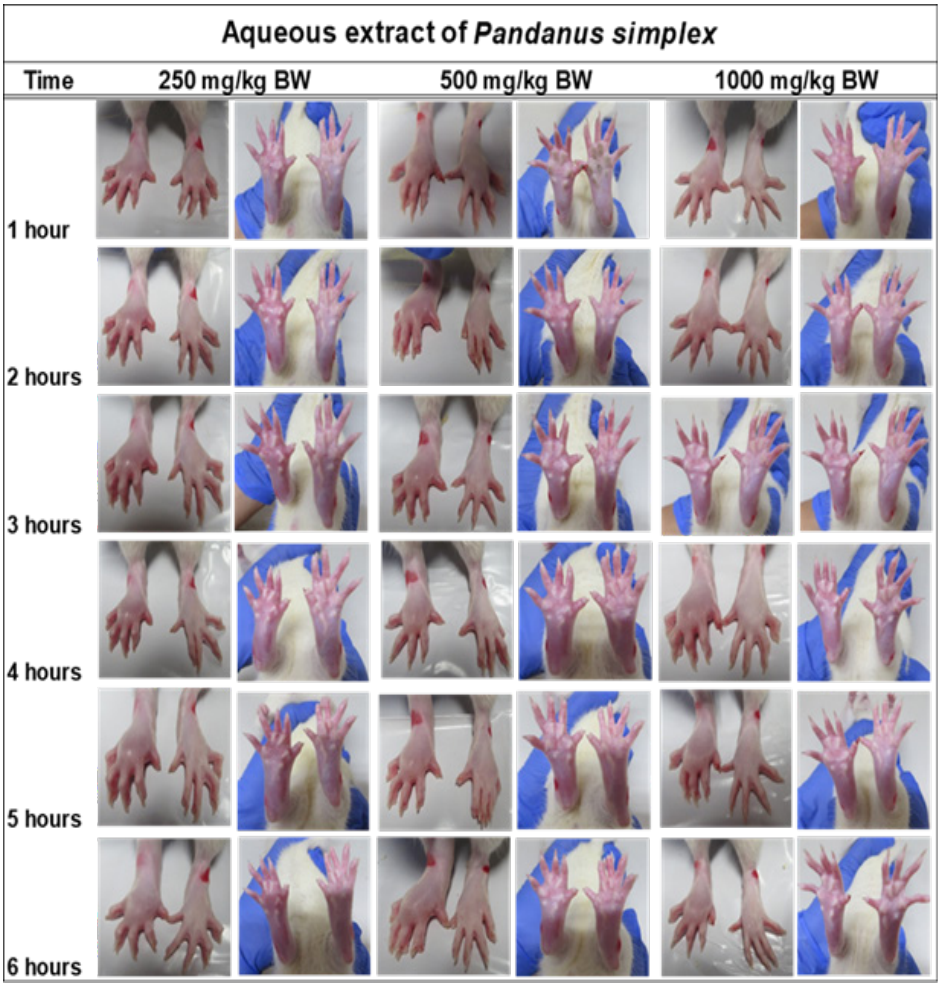
Supplementary Figure 3. The progressive photos of representative rats per treatment group in carrageenan-induced paw edema (1st hour-6th hour). (Aqueous Extract of *Pandanus luzonensis*)



Supplementary Figure 3. The progressive photos of representative rats per treatment group in carrageenan-induced paw edema (1st hour-6th hour). (Aqueous Extract of *Pandanus panayensis*)



Supplementary Figure 3. The progressive photos of representative rats per treatment group in carrageenan-induced paw edema (1st hour-6th hour). (Aqueous Extract of *Pandanus simplex*)



Supplementary Figure 4. Representative photos of the histological studies and scoring of the paw specimen per treatment group after Anti-inflammatory assay

GROUP	Dose (Negative -mL/ kg BW, Treated - mg/kg BW)	Acute Inflammation	Edema	Photomicrograph
Negative (1%Tween 80)	10	3	3	
Diclofenac sodium	100	1	1	
Methanol Extract *P. amplifolia*	1000	1.7	2.2	
	500	1.4	2	
	250	1.8	2.6	
Ethanol Extract *P. pennyeriana*	1000	1.0	1.0	
	500	2.2	1.8	
	250	1.6	1.2	
Ethanol Extract *P. amplifolia*	1000	2.8	2.6	
	500	2.2	1.6	
	250	1.8	0.6	

Supplementary Figure 4. Representative photos of the histological studies and scoring of the paw specimen per treatment group after Anti-inflammatory assay *(cont'd)*

GROUP	Dose (mg/kg BW)	Acute inflammation	Edema	Pictomicrograph
Aqueous Extract *P. luzonensis*	1000	3	3	
	500	2.6	2.6	
	250	3	3	
Aqueous Extract *P. panayensis*	1000	2.6	2.2	
	500	3	3	
	250	3	2.8	
Aqueous Extract *P. simplex*	1000	2.8	2.8	
	500	2.8	2.3	
	250	2	1.4