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RESEARCH ARTICLE

Anti-inflammatory activity of *Piper umbellatum* Linn. leaf extracts

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ABSTRACT

Background: Earlier studies reported the anti-inflammatory activity in several species of Piper, and *Piper umbellatum* Linn. leaves containing some phytochemicals that are potent anti-inflammatory agents. However, there was no thorough investigation on the anti-inflammatory activity of the locally grown *P. umbellatum* in the Philippines.

Objective: The study aimed to determine the anti-inflammatory activity of *Piper umbellatum* leaves using *in vitro* and *in vivo* assays.

Methodology: Crude extracts were obtained from *P. umbellatum* leaves using polar and non-polar solvents. The anti-inflammatory activities of all crude extracts were determined using the carrageenan-induced paw edema test in mice and phytochemical analysis. The crude extract with the highest activity was partially purified using column chromatography. The fractions with similar TLC profile were pooled and tested for anti-inflammatory activity. COX-1 and COX-2 enzyme inhibitory activity were determined in pooled fractions that showed initial activity in animal model.

Results: Among the crude extracts of *P.umbellatum*, the crude ethyl acetate extract exhibited a significant dose-dependent inhibition on paw edema test with doses of 500 mg/kg bw, 1,000 mg/kg bw and 1,500 mg/kg bw ($p<0.05$). Among the 20 pooled fractions (PF) collected from the ethyl acetate extract, PF58, PF60 and PF64 had the highest COX-2 enzyme inhibitions of 83.12 %, 84.78% and 77.47%, respectively ($p<0.05$). PF60 also exhibited the highest anti-inflammatory activity on paw edema with inhibitions of 62.45% at low dose (250 mg/kg bw) and 76.10 % at high dose (1,000 mg/kg bw) in mice.

Conclusion: The ethyl acetate extract of *P. umbellatum* leaves and its fraction-PF60 exhibited a significant anti-inflammatory activity in *in vitro* and *in vivo* assays and contained high amounts of total phenolic and total flavonoid.

Keywords: *Piper umbellatum, cyclooxygenase, carrageenan, inflammation, inhibition*

Introduction

Inflammation is the immune system's response to an infection and injury. It has been associated with the pathogenesis of cancer, arthritis, cardiovascular, and neurodegenerative diseases. Intrinsically, inflammation is beneficial in the removal of offending factors and restoration of tissue structure and physiological function after injury [1]. In the inflammatory process, arachidonic acid is the precursor of inflammatory mediators. These arachidonic acids are converted by the cyclooxygenase (COX) pathway into prostaglandins which play a key role in the generation of inflammatory response [2]. However, the prostaglandins produced depend on the activity of cyclooxygenases. Excess activation of arachidonic acid results into a massive production of prostaglandins and is considered the underlying mechanism associated with cancer and other diseases. Their biosynthesis is significantly increased in inflamed tissue, and they contribute to the development of acute inflammation [3].

Non-steroidal anti-inflammatory drugs (NSAIDs) are over-the-counter medications used to treat pain and inflammation by the reduction of prostaglandin production in the body [4]. However, these NSAIDs pose some adverse effects in the gastrointestinal tract that cause gastric mucosal damage with ulcer formation, bleeding, and

perforation [5,6]. Some cyclooxygenase-2 (COX-2)-selective NSAIDs are also associated with an increased risk of cardiovascular diseases [7].

Hence, medicinal plants with anti-inflammatory activity are, nowadays, becoming popular because of their minimal side effects [8]. One of the medicinal plants used for various purposes is *Piper umbellatum*, popularly known as “Kamamba.” *P. umbellatum* possesses antioxidant, antibacterial, antifungal, anti-atherogenic, antispasmodic, antiprotozoal, analgesic, and cytotoxic properties. Its popularity as an effective therapy for diarrhea, malaria, toothache, diabetes, skin diseases dysentery, and colitis has been documented [9] In addition, other species of Piper such as *Piper guineense*, *Piper chaba*, *Piper nigrum*, *Piper sarmentosum*, *Piper longum* and *Piper betle* are known to exhibit anti-inflammatory effect [10].

Previous chemical analyses showed that the leaves of *P. umbellatum* contain very high amounts of steroids, moderate amounts of phenols, flavonoids and saponins with little traces of tannins and alkaloids [11]. The concentrations of phenols, flavonoids and saponins present in *P. umbellatum* leaves suggest its potential for anti-inflammatory activityy. Thus, this study investigated the anti-inflammatory activity of *P. umbellatum*.

Methodology

Chemicals and Reagents

The materials and reagents used in this study were the following: analytical grade solvents (Merck, ACS, Germany): hexane, ethyl acetate, methanol and ethanol and dimethyl sulfoxide (DMSO); freshly prepared phytochemical reagents; Dragendorff's reagent, Mayer's reagent, Hager's reagent, Wagner's reagent, acetic anhydride, sulphuric acid, chloroform, ferric chloride solution, gelatin-salt solution, ammonia, ninhydrin reagent, hydrocholoric acid, gallic acid, folin ciocalteu reagent, sodium carbonate, quercetin and aluminum chloride; saline solution, mineral oil, carrageenan lambda (Marcel, Philippines), COX Assay Kit (No. 760111, Cayman Chemical, USA), aspirin (Cortal, Pharma-Rex, Philippines) and celecoxib (Celebrex, Pfizer Pharmaceuticals LCC, USA).

Plant Sample

The plant leaves were collected from a farmland in Brgy. Kulapi, Lucban, Quezon near Southern Luzon State University in October 2017, 14.1127° N, 121.5612° E. A voucher specimen of the *P. umbellatum* was deposited and authenticated at the Jose Vera Santos Herbarium of the Institute of Biology, University of the Philippines, Diliman.

Plant Sample Extraction

Air-dried leaves (150 g) of *P. umbellatum* were pulverized using a laboratory blender and underwent a serial extraction with hexane (3 x 500mL), ethyl acetate (3 x 500mL), methanol (3 x 500mL) and lastly ethanol (3x 500mL) and were concentrated using a rotatory evaporator until a gel like crude mass was obtained. The remaining volume (5-10 mL) of each extract were air dried. Another batch (150g) of pulverized leaves were extracted with distilled water separately three times. The extract was boiled with water for three minutes, and the decoction of aqueous extract was freeze-dried. All plant extracts were properly labeled and stored at a temperature between 0-5 °C until further use. Percentage yield was computed using the weight of crude extract divided by the weight of the dried leaves.

Thin Layer Chromatography

The plant extracts (hexane, ethyl acetate, methanol, ethanol and aqueous) were spotted in a pre-coated silica gel 60 F254 thin layer chromatography plates with a diameter of two-five mm. The plate was developed with hexane-ethyl acetate-glacial acetic acid solution (9:5:0.1) and was inspected under ultraviolet (UV) light; short wave (240 nm) and long wave (365 nm) prior to the spraying of the visualizing agent (5% sulfuric acid-methanol solution) for general visualizing agent for secondary metabolites. Retardation Factor (**R***f*) values were computed by the distance of the solute over the distance of the solvent from the point of application.

Phytochemical Screening [12]

The hexane, ethyl acetate, methanol, ethanol, and aqueous extracts of *P. umbellatum* leaves were tested for the presence of alkaloids, amino acids, steroids, tannins, phenols, flavonoids, saponins, and glycosides following the protocol used by Bhandary *et al.* (2012) with some modifications. The results were expressed as positive (+) for the presence and negative (−) for the absence of the phytochemical. The presence of the alkaloids was detected using Mayer's, Wagner's, Hager's and dragendorff's tests; steroids using Liebermann-Burchard test; terpenes and terpenoids using Salkowski's test; tannins using ferric chloride and gelatin tests; saponins using froth tests; cardiac glycosides using keller-killiani test; reducing sugars using

Molisch; glycosides using modified borntrager's test; amino acids using ninhydrin test; flavonoids using Shinoda test, phenols using ferric chloride test; betacyanin and coumarins using alkaline reagent test and free athraquinone test.

The phenolic and flavonoid content of all extracts of *P. umbellatum* were determined using Folin-Ciocalteu and aluminum chloride methods, respectively. The total phenolic content of the five extracts was expressed in milligrams of gallic acid/grams of sample, while the total flavonoid content was expressed in milligrams of quercetin/grams of sample. Amounts were derived from a standard curve.

Animal Experiments

Preparation of the Test Animal

Animal experiments were conducted using ICR mice (20–30g, 90 days old) procured from the Research Institute for Tropical Medicine (RITM) and housed at the UP College of Medicine Multidisciplinary Laboratory Animal Room with controlled temperature at 22 ± 3 °C and under a 12h light/dark cycle, with free access to food and water. Animal care and research protocols were approved by the Institutional Animal Care and Use Committee (IACUC), UP Manila with IACUC Protocol No. AR-2018-252 issued on May 25, 2018. All animals were acclimatized for one week prior to anti-inflammatory assay using carrageenan-induced paw edema method.

Safety Profile of Ethyl Acetate Extract

The safety profile testing of the extracts followed the IACUC recommendation of 2,000 mg/kg dosage. Thirty mice were not given food but had ad libitum access to water four hours prior to treatment. Five mice per treatment were used for the negative and extract groups. These groups were: 1) negative control (NSS and mineral oil), 2) extracts (hexane, ethyl acetate, methanol, ethanol and aqueous. NSS was used to dissolved polar extracts and mineral oil was used to dissolved nonpolar extracts. The extracts were prepared with a volume not exceeding one mL/100g of body weight (bw). A fixed dose level of 2,000 mg/kg bw was used and administered to animals. Mice were observed for toxic symptoms and behavioral changes after 30 minutes, periodically and daily for 14 days. Mortality after 14 days was observed and recorded.

Carrageenan-Induced paw edema assay of extracts

Inflammation was induced on the right hind paw with a 100 µL of 1% lambda carrageenan in normal saline and 100 µL of normal saline solution on the left hind paw as sham-control. The basal diameter of both paws (1.8-2.0 mm) was determined prior to administration of any treatments and paw edema (2.4-2.7mm) was measured with a digital Vernier caliper. All the crude extracts (hexane, ethyl acetate, methanol, ethanol and aqueous) at 300 mg/kg bw concentration were subjected to paw edema assay. The extract which exhibited significant anti-inflammatory activity was further evaluated.

The ethyl acetate extract found with significant activity was further tested using 500mg/kg bw as low dose, 1000mg/kg bw for medium dose and 1500mg/kg bw for high dose which were formulated from its safety profile result. The mice were randomly divided to seven groups with six mice per group (n=42) and all the studies used negative control (NSS and mineral oil) and positive control [aspirin (25 mg/kg bw) and celecoxib (20 mg/kg bw). NSS was used to dissolved polar extracts and mineral oil was used to dissolved nonpolar extracts. The right hind paw volume was evaluated every hour after carrageenan injection for a maximum of 4-6 hours [13]. The percentage of inhibition was calculated using the final paw volume subtracted to the initial paw volume divided by the initial paw volume then multiplied by 100 % using the formula below:

$$\% \text{ inhibition} = \frac{C_o - C_t}{C_o} \times 100$$

where Ct= Final Paw Volume and C_0=Initial Paw Volume.

Partial Purification

The partial purification of ethyl acetate crude extract was carried out using gradient elution column chromatography, packed with 30 g of silica (0.2-0.5 µm size). One gram of crude extract was dissolved in hexane and added in the column. Elution of the compounds started with 100% hexane, followed by mixtures containing 90% hexane-10% ethyl acetate and successive mixtures of 80-20%, 70-30% up to 100% ethyl acetate [14]. The fractions were also subjected to thin-layer chromatography and 5 % sulfuric acid-methanol was used as a general visualizing agent for secondary metabolites.

COX-1 and COX-2 Inhibition Assay [15]

Both ethyl acetate crude extract and its bioactive fractions were evaluated using COX colorimetric inhibitor screening assay kit (No. 76011; Cayman Chemicals). The peroxidase activity was assayed by monitoring the appearance of oxidized N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD) at 590 nm using plate reader. Both isomers COX-1 and COX-2 enzymes were used to screen isozyme-specific inhibitors.

Aspirin and celecoxib served as the positive controls for comparison. The standard reference drug, ethyl acetate extract and fractions (1mg/mL) were dissolved in 1% dimethyl sulfoxide (DMSO). The assays were done in triplicates. The mean absorbance for each blank, sample, and inhibitor treated wells were determined. The percentage of COX activity inhibition was calculated using the absorbance of the inhibitor wells subtracted from the absorbance of the 100% initial activity divided by the absorbance of the 100 % initial activity then multiplied by one hundred.

Carrageenan-induced Paw Edema on Selected Fractions

The pooled fractions with the highest COX-2 inhibition from ethyl acetate extract, were also subjected to the carrageenan-induced paw edema test. The mice were randomly divided into ten groups with six mice per group (n=60). Negative control (NSS and oil), positive control [aspirin (25 mg/kg bw) and celecoxib (20 mg/kg bw)], and the treatment group [fractions-PF58, PF60, PF64 (250 mg/kg bw and 1,000 mg/kg bw)] were introduced via oral gavage one hour after administration of carrageenan in the paws. The right hind paw volume was evaluated every hour for six hours after carrageenan injection.

Statistical Analysis

The results were expressed as mean ± Standard Error Mean (SEM) and the data obtained from the negative control group were used as a baseline value. Means, SEM, and standard deviations were calculated from triplicates within the experiments. Statistical analysis was done using one-way analysis of variance (ANOVA) and mixed ANOVA with repeated measures followed by the Tukey's post hoc test for multiple comparisons. The statistical significance was set at $P < 0.05$.

Results

Extraction of Plant Samples

The crude extracts of *P. umbellatum* leaves (150 g) were obtained from serial extraction using hexane, ethyl acetate, ethanol, methanol and a decoction extraction for aqueous extract. Table 1 showed that the aqueous extract gave the highest percentage yield of 40.47%, while the hexane and ethyl acetate extracts had the lowest percentage yield. Polar extracts (ethanol, methanol and aqueous) had a higher percentage yield than nonpolar extracts (hexane and ethyl acetate).

Phytochemical Analysis

Phytochemical screening was performed on the five extracts of *P. umbellatum*. The screening confirmed the presence of constituents which are known to exhibit medicinal and physiological activities as reported in the literature. The results (Table 2) revealed the presence of steroids, triterpenes, general carbohydrates, cardiac glycosides and flavonoids in all extract types, while alkaloidal and saponin components were absent in this plant. Variations between extracts of *P. umbellatum* leaf were observed. Phenols, coumarins and tannins were all present in ethyl acetate, ethanol and aqueous extracts but were absent in hexane and methanol extracts. Triterpenes was only found in methanol, while betacyanin was found in both methanol and ethanol extracts only.

Among the extracts of *P. umbellatum* (Table 3), ethyl acetate showed the highest total phenolic content (TPC) with 164.98± 17.99 mg GAE/g followed by hexane, ethanol, methanol, and aqueous extracts. Hexane extract exhibited the highest total flavonoid content (TFC) of 4.30 ± 0.06 mg QE/g while Aqueous extract showed the lowest content of both phenolic (29.33 ± 2.43 mg GAE/g) and flavonoid (1.8 ± 0.03mg QE/g).

Safety Profile

Table 4 showed the toxic effects of the crude extracts (hexane, ethyl acetate, ethanol, methanol and aqueous) on the general appearance and the general behavioral pattern on mice in a span of 14 days. A single dose level of 2,000 mg/kg bw of the extracts were administered and there were no immediate toxic symptoms or mortality observed on mice within the day. Animals in both vehicle-treated and

Table 1. *Percent yield of Piper umbellatum leaf extracts.*

Extracts	Color	Texture	Weight (grams) recovered crude	Percentage %
Hexane	dark green	sticky	10.20	6.81
Ethyl acetate	dark green	sticky	10.31	6.87
Ethanol	dark green	sticky	17.24	11.49
Methanol	dark green	sticky	15.14	10.09
Aqueous	ashy green	dry, porous	60.71	40.47

Table 2. *Phytochemical screening of Piper umbellatum leaf extracts.*

Test	Hexane	Ethyl acetate	Ethanol	Methanol	Aqueous
Alkaloids	–	–	–	–	–
Steroids	+	+	+	+	+
Terpenoids	+	+	–	–	+
Flavonoids	+	+	+	+	+
Triterpenes	–	–	+	–	–
Tannins	–	+	+	–	+
Saponins	–	–	–	–	–
Phenols	+	+	+	+	+
Carb-Glycosides					
*Anthraquinone	–	–	–	–	–
*Cardiac	+	+	+	+	+
Gen. Carb.	+	+	+	+	+
Amino Acids	–	–	–	–	–
Quinones	–	–	–	–	–
Anthraquinones	–	–	–	–	–
Betacyanin	–	–	+	+	–
Coumarins	–	+	+	–	–

(+) represents presence of the phytoconstituent; (-) represents absence of the phytoconstituent

Table 3. *Total phenolic and flavonoid contents of Piper umbellatum leaf extracts.*

Plant Extracts	Total Phenolic Content (mg GAE/g)	Total Flavonoid Content (mg GAE/g)
Hexane	149.79 ± 13.12	4.30 ± 0.06
Ethyl acetate	164.98 ± 17.99	2.72 ± 0.02
Ethanol	57.39 ± 4.72	2.10 ± 0.02
Methanol	40.51 ± 1.10	1.90 ± 0.02
Aqueous	29.33 ± 2.43	1.80 ± 0.03

Values were expressed as "mean ± SD". GAE: Gallic Acid equivalent, QE: Quercetin equivalent.

extract-treated were normal and did not display significant changes in appearance and behavior. This safety profile test determined that the extracts were fit for consumption in mice models.

Carageenan-induced Paw Edema on Extracts

The pilot study showed that among the extracts, ethyl acetate exhibited significant anti-inflammatory activity at 300mg/kg bw. Thus, the ethyl acetate extract was subjected to the main paw edema test. Figure 1 shows that all dosages of ethyl acetate extracts showed significant inhibition of paw edema in mice, with maximum inhibition of 67.64% (low dose-500 mg/kg bw), 77.33 % (medium dose-1,000 mg/kg bw) and 80.62% (high dose-1,500 mg/kg bw) ($p<0.05$). Aspirin treatment produced a 78.20 % inhibition while celecoxib treatment showed 90.58% inhibition. A dose-dependent inhibition was observed, as the dosages of the ethyl acetate extract increased, the inhibition also increased compared with the negative control ($p<0.05$).

The EtOAc medium dose (1,000 mg/kg bw) was comparable with (25 mg/kg bw) aspirin having difference in inhibition of 0.87 % only ($p>0.05$). A difference of 10.56 % and 2.42 % was also seen with a low (500 mg/kg bw) and high dose (1,500 mg/kg bw) of ethyl acetate extracts when

Table 4. *General appearance and behavioral observations of control and treated groups*

Observations		Salivation	Tail Erection	Pilomotor	Micturition	Colpectasia	Robichaud	Circling motion	Abdominal Gripping	Tail Lashing	Mean Weight (g)
Control: NSS	Day 1	0	0	0	0	0	0	0	0	0	31.00 ± 0.58
	Day 14	0	0	0	0	0	0	0	0	0	31.67 ± 0.88
Control: Oil	Day 1	0	0	0	0	0	0	0	0	0	31.33 ± 0.88
	Day 14	0	0	0	0	0	0	0	0	0	33.67 ± 1.45
Hexane	Day 1	0	0	0	0	0	0	0	0	0	33.20 ± 0.49
	Day 14	0	0	0	0	0	0	0	0	0	32.60 ± 0.93
Ethyl acetate	Day 1	0	0	0	0	0	0	0	0	0	31.40 ± 0.68
	Day 14	0	0	0	0	0	0	0	0	0	31.20 ± 0.58
Ethanol	Day 1	0	0	0	0	0	0	0	0	0	31.80 ± 0.20
	Day 14	0	0	0	0	0	0	0	0	0	32.00 ± 0.24
Methanol	Day 1	0	0	0	0	0	0	0	0	0	30.20 ± 0.37
	Day 14	0	0	0	0	0	0	0	0	0	30.40 ± 0.40
Aqueous	Day 1	0	0	0	0	0	0	0	0	0	25.00 ± 0.89
	Day 14	0	0	0	0	0	0	0	0	0	24.20 ± 0.68

Observations are scores as “0” for normal, “1” mild change and “2” severe change. Values in weight are expressed as mean ± SEM

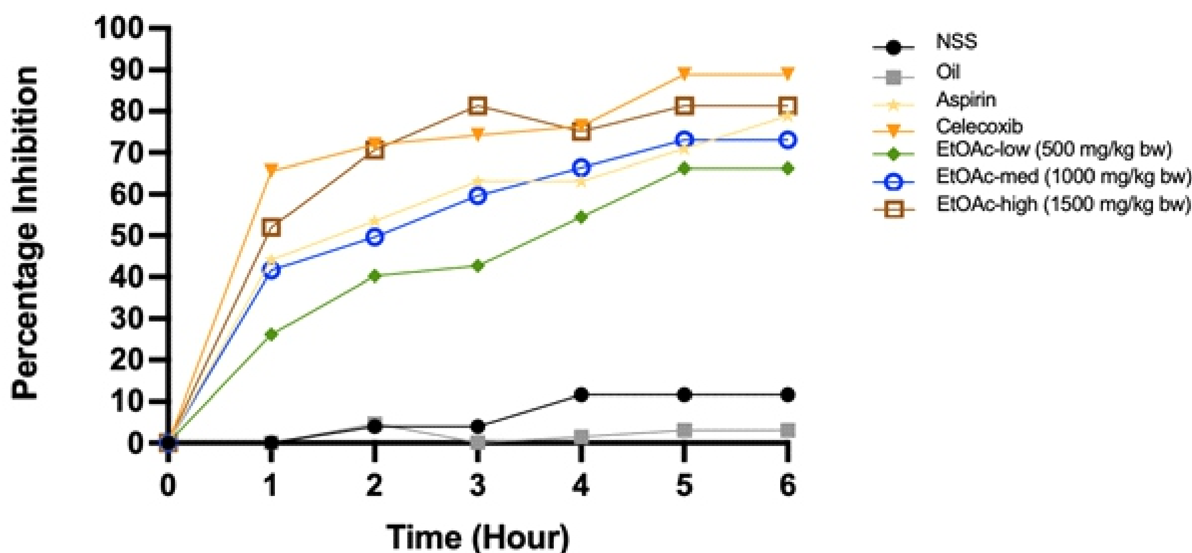

Figure 1. *Inhibitory effect of P. umbellatum ethyl acetate extract in low, medium and high dosages in carrageenan induced paw edema in mice; NSS and oil were used as a negative control while Aspirin and Celecoxib were used as positive controls.*

compared with Aspirin. All dosages of the ethyl acetate extract showed no significant differences with Aspirin ($p>0.05$). Comparing the inhibitions of EtOAc low, EtOAc medium and EtOAc high with celecoxib, the differences were 22.94 %, 13.25 % and 9.96 %, respectively, ($p>0.05$).

The inhibition of low and medium doses (500 mg/kg bw and 1,000mg/kg bw) of ethyl acetate extract plateaued but there was a decrease in activity at the 6th hour. However, the high dose (1,500 mg/kg bw) of ethyl acetate extract maintained its activity even beyond the 6th hour. The high dose plateaued at the 4th hour until the 6th hour.

Thin-layer Chromatography and Purifications

The solvent system and its corresponding ratio were obtained via trial and error method to dissolve extract and isolate its components for further study. The TLC profile results of ethyl acetate extract showed significant indication of the presence of a variety of secondary metabolites. Blue, violet, brown and green spots were observed from the TLC plate. The result indicated that hexane-ethyl acetate solvent is a good solvent system for further separation of compounds using column chromatography. There were 71 fractions collected from the ethyl acetate extract via column chromatography by gravity. These fractions were subjected to thin layer chromatography and a similar profile was pooled. There were 20 pooled fractions tested for *in vitro* COX-1 and COX-2 enzyme inhibitory assays.

COX-1 and COX-2 Enzyme Inhibition Assay

The inhibitory activity of the ethyl acetate extract and the bioactive pooled fractions against COX-1 and COX-2 were determined. As shown in Figure 2, the ethyl acetate extract inhibited the COX-1 and COX-2 enzymes by 52.12 % and 34.96 %, respectively. The inhibition of COX-1 and COX-2 enzymes of ethyl acetate extract was comparable to aspirin, which inhibited the activity by 47.30 % and 41.49 %, respectively ($p>0.05$). The ethyl acetate extract was also comparable with the COX-1 inhibition of celecoxib, which inhibited by 38.00 %. However, celecoxib inhibited the COX-2 enzymes largely at 95.17 % than the ethyl acetate extract ($p<0.05$).

Among the 20 pooled fractions, PF58, PF60, and PF64 fractions showed the highest COX-2 inhibition of 83.12%, 84.78%, and 77.47%, respectively. Celecoxib (1000 g/mL) inhibited the COX-2 enzyme by 95.17% which was higher than the said fractions by 12.05%, 10.39 %, and 17.71%, respectively. On the other hand, both celecoxib (1000 g/mL) and the three fractions showed a lower inhibition on the COX-1 enzyme by 38%, 19.68%, 29.52% and 26.84 %, respectively. The difference in the inhibitory activity between Celecoxib and the fractions was not significant. These pooled fractions were further tested for their activity using *in vivo* anti-inflammatory assay.

Carrageenan-induced Paw Edema on Selected Fractions

Figure 3 shows that the PF58, PF60 and PF64 fractions significantly inhibited paw edema in mice with PF60 having the highest inhibition of 62.45% (low dose) and 76.10% (high

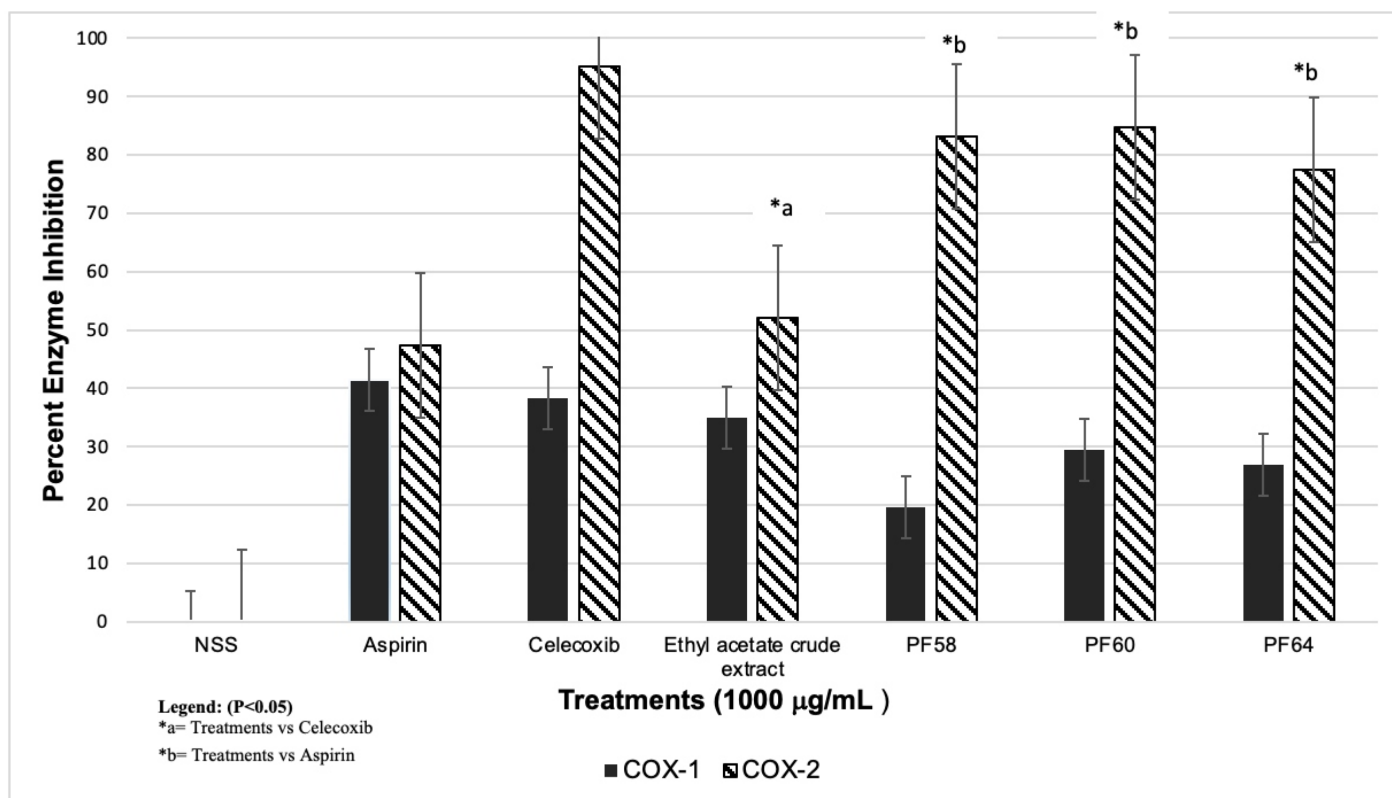

Figure 2. *COX-1 and COX-2 inhibitory activity of Piper umbellatum ethyl acetate extract and pooled fractions. *a denotes P<0.5 comparison of treatments vs Celecoxib; *b denotes P<0.5 comparison of treatments vs Aspirin. NSS was used as negative control while Aspirin and Celecoxib were used as positive controls.*

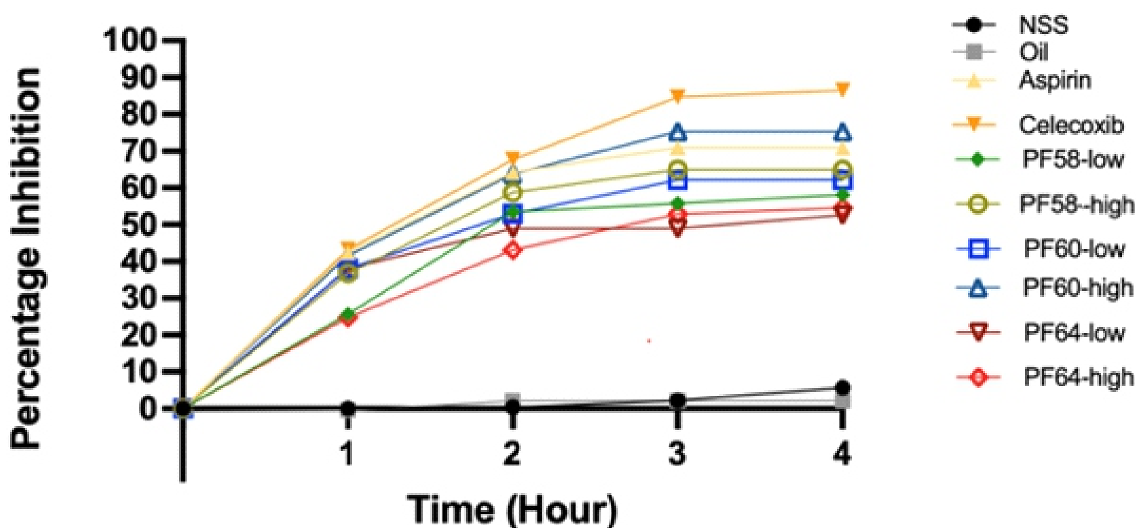

Figure 3. *Inhibitory effect of selected fractions of P. umbellatum ethyl acetate extracts in carrageenan induced paw edema in mice; NSS and oil were used as a negative control while Aspirin and Celecoxib were used as positive controls.*

dose) ($p<0.05$). The PF58 produced an inhibition of 50.97% (low dose) and 66.21 % (high dose) while the PF64 had a 51.96 % (low dose) and 54.76 % (high dose) inhibition. The inhibition of the PF60 low dose (250 mg/kg BW) was significantly lower compared with celecoxib ($p<0.05$). Similarly, the inhibition of PF60 high dose was comparable with aspirin and celecoxib ($p>0.05$).

Discussion

The study examined the anti-inflammatory activity of *P. umbellatum* extracts of the leaves and selected fractions of the ethyl acetate extract through COX inhibition assay and carrageenan-induced paw edema in mice. Results of the extraction process showed that different solvents produced different amounts of percentage yield. The aqueous extract of *P. umbellatum* leaves exhibited the highest amount of percentage yield indicating that the extraction process favors the highly polar solvents. Possibly, the plant contains high levels of polar compounds that are soluble in polar solvents such as aqueous [16].

Steroids, triterpenes, carbohydrates, cardiac glycosides, phenols and flavonoids were present in all extract types of *P. umbellatum*. Among these phytochemicals, phenols and flavonoid are commonly found in the Piper species and are also known to exert strong antioxidant activities that are correlated to numerous anti-inflammatory effects [17-18] . Thus, the phenolic and flavonoid content were determined. The result revealed that *P. umbellatum* contained remarkable levels of phenols and flavonoids in ethyl acetate and hexane extracts. This may be attributed to the phenolic compounds which have higher molecular weights or possess more phenol groups in ethyl acetate and hexane extracts (Do *et al.*, 2014). Other species of Piper with reported anti-inflammatory activities also have high phenolic and flavonoid content such as *Piper nigrum*, *Piper sarmentossum*, *Piper guineense* and *Piper betle* [19-22] .

This study tested the anti-inflammatory activity of the five extracts of *P. umbellatum* through carrageenan-induced paw edema in mice. As a background, the administration of carrageenan into the mouse hind paw induces a biphasic inflammatory edema. The initial phase of inflammation (1-2 hrs) releases a cascade of mediators like histamine, bradykinin, and serotonin by the cells near the carrageenan injection. These mediators promote an increase in vascular permeability and signal for arachidonate metabolites (prostaglandins and leukotrienes) and nitric oxide release, until the 6th hour, and for the 2nd phase of inflammation [23].

The ethyl acetate crude extract of *P. umbellatum* significantly inhibited inflammation, dose-dependently, up to 5-6 hours by which prostaglandins are supposed to be released. During this time, the release of prostaglandins causes inflammation and pain to mice and the activity of the ethyl acetate extract must be seen. The inhibition of low and medium doses (500 mg/kg bw and 1,000mg/kg bw) of ethyl acetate extract plateaued from the 4th to 5th hour but there was a decrease in activity at the 6th hour. However, the high

dose (1,500 mg/kg bw) of ethyl acetate extract maintained its activity even beyond the 6th hour. The same trend has been shown by other species of Piper such as *Piper chaba* which reduces paw edema at 1,200 mg/kg bw until the 5th hour [24]. Piper sarmentossum inhibits paw edema at 100 mg/kg [25]. In addition, the ethyl acetate extract of *P. umbellatum* was found to have no adverse toxic effects on mice and fit for consumption.

Since the ethyl acetate extract inhibited paw edema in the 1st and 2nd phase of inflammation, these findings suggest that this effect is similar to the action of the non-steroidal anti-inflammatory drugs. The ethyl acetate crude extract and pooled fractions significantly inhibited both COX-1 and COX-2 enzymes, which have similar actions with aspirin. Among the pooled fractions of the ethyl acetate extracts, the PF58, PF60, and PF64 showed the highest inhibition against COX-2 enzymes. These pooled fractions have the same trend with celecoxib 20 mg/kg in inhibiting COX-2 enzymes. Thus, the pooled fractions may have selective COX enzyme activity in inhibiting the anti-inflammatory response. When the COX-2 inhibition of the active pooled fractions was compared to celecoxib, a lower difference was seen than the crude ethyl acetate extract. The same trend was seen with other species of Piper such as the crude extract of *Piper nigrum* which inhibited the COX-2 enzymes at 200 ug/mL by 31 % and its active compound at 25 ug/mL by 80% [26].

Among the active pooled fractions, the PF60 had the highest inhibition of both COX-2 enzymes and on carrageenan-induced paw edema test in mice. A variety of phenolics, flavonoids and catechol present in *P. umbellatum* could be the reason for these activities. The least amount of 16.02 mg GAE/g of phenols, 3.61 mg QE/g of flavonoids and 3.16 mg CE/g of catechol is shown to provide an anti-inflammatory effect [27] The phytochemical screening confirms the presence of phenolic compounds such as flavonoids. They are present in all varieties of the extracts of *P. umbellatum* from nonpolar to polar extracts. The anti-inflammatory mechanism of action of phenols is known to reduce NF-kB transcription by the inhibition of I-kB kinase activity [28-30]. Furthermore, the anti-inflammatory activity has been reported by Manthey, Grohamanu & Guthric (2001) that phenolics and flavonoids can interrupt the oxidative generation of the arachidonic acids (AA) from phospholipids and reduce the production of inflammatory metabolites from AA metabolism due to their potent antioxidant capacity [31].

In addition, Yu (2020) states that the methanolic extract of *P. umbellatum* leaves has more than 50% free radical scavenging even at the lowest concentration of 33.33 µg/mL in DPPH and FRAP assays [32]. Studies also show that there is a strong correlation with the amount of phenolic/flavonoid with the antioxidant activities of *P. umbellatum* as supported by Geetha, Irulandi & Mehalingam (2017)[33]. Thus, the effectiveness of the *P. umbellatum* as used in folk medicine to suppress inflammatory responses may be due to the phytochemicals present in the plant and the capacity to reduce oxidative stress.

Studies also state that the *P. umbellatum* extract contains β- sitosterol and catechin that could be responsible for the inhibition of arachidonate metabolites generation [34]. Earlier studies showed that catechins inhibit the adhesion and migration of neutrophils that includes the suppression of the production of chemokines at the site of inflammation and reduction of the level of PGE2 in rats [35-37]. Based on the study of Nuñez *et al.* (2005), an isolated 4-nerolidylcatechol from *P. umbellatum* significantly inhibited the edema-forming activity of *Bothrops asper* and *Bothrops atrox* mycotoxins injected in mice, which could be one of the reasons for the inhibitory effect on inflammation in mice models [38].

These evidences clearly reflect the ability of *P.umbellatum* to be a potential anti-inflammatory agent through inhibiting paw edema in mice and both COX-1 and COX-2 enzymes. The findings of this study indicate that *P. umbellatum* can be a potential alternative source of anti-inflammatory agent and may contribute for future drug discovery.

Conclusion

In conclusion, this study was able to determine that the ethyl acetate extract of *P. umbellatum* leaves and its active pooled fractions showed anti-inflammatory activity in the carrageenan-induced paw edema test in mice and inhibitory activity of the COX-1 and COX-2 enzymes. The anti-inflammatory effects observed using the ethyl acetate extract showed a dose-dependent inhibition of up to 5-6 hours. The ethyl acetate extracts also revealed a comparable inhibition with aspirin against the two isoforms of cyclooxygenase enzymes, while its active fractions showed a similar trend with celecoxib. The inhibitory activities of *P. umbellatum* seen in *in vitro* and *in vivo* assays may be caused by the presence of phenols, catechins or flavonoids in the plant.

This study highlights the importance of *P. umbellatum* as a potential anti-inflammatory agent and its use as a traditional medicine for inflammatory disorders. The

isolation and characterization of the active pure compounds are recommended. In addition, analysis on the mechanism of actions of these compounds responsible for the anti-inflammatory activity of *P. umbellatum* is suggested.

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References

1. Riciotti E, Fitzgerald G. (2011) Prostaglandins and Inflammation. Arteriosclerosis, Thrombosis and Vascular Biology. 31(5):986–1000. doi: 10.1161/ATVBAHA.110.207449
2. Morteu O. (2000) Prostaglandins and inflammation: the cyclooxygenase controversy. Archivum Immunologiae et Therapiae Experimentalis. 48(6):473-80.
3. Smith JB. (1990) Prostaglandins and related eicosanoids. Basic Pharmacology in Medicine, 3rd Ed. McGraw-Hill Publication, New York, USA.181–192.
4. Ilic N, Dey M, Poulev A, Logendra S, Kuhn P, Raskin I. (2014) Anti-inflammatory Activity of Grains of Paradise (Aframomum melegueta Schum) Extract. Journal Agricultural and Food Chemistry. 62(43): 10452–10457. doi: 10.1021/jf5026086
5. Yap PR, Goh KL. (2015) Non-steroidal Anti-inflammatory Drugs (NSAIDs) Induced Dyspepsia. Current Pharmaceutical Design. 21(35):5073-81. doi: 10.2174/1381612821666150915105738
6. Goldstein Jl, Cryer B. (2015) Gastrointestinal injury associated with NSAID use: a case study and review of risk factors and preventative strategies. Drug Healthcare and Patient Safety. 7:31–41. doi: 10.2147/DHPS.S71976
7. Solomon D, Husni ME, Libby P, *et al.* (2017). The Risk of Major NSAID Toxicity with Celecoxib, Ibuprofen, or Naproxen: A Secondary Analysis of the Precision Trial. American Journal of Medicine. 130(12):1415-1422.e4. doi: 10.1016/j.amjmed.2017.06.028
8. Oguntibeju O. (2018) Medicinal Plants with Anti-inflammatory Activities from Selected Countries and Regions of Africa. Journal of Inflammatory Research. doi: 10.2147/JIR.S167789
9. Stuart GU. (2017) Kubamba. Philippine Medicinal Plants.
10. Salehi S, Zakaria ZA, Gyawali R, *et al.* (2019) Piper Species: A Comprehensive Review on Their Phytochemistry, Biological Activities and Applications. Molecules. doi : 10.3390/molecules24071364
11. Belewu MA, Olatunde, OA, Giwa TA. (2009) Underutilized medicinal plants and spices: Chemical composition and phytochemical properties. Journal of Medicinal Plants Research. 3(12):1099-1103.
12. Bhandary S., Kumari S, Bhat V, Sherly S. (2012) Preliminary phytochemical screening of various extracts of Punica granatum peel, whole fruit and seeds. Journal of Health and Allied Sciences. NU 2(4). doi: 10.1055/s-0040-1703609
13. Iwamoto L, Vendramini-Costa DB, Monteiro PA, et al, (2015). Anti-cancer and Anti-inflammatory Activities of a Standardized Dichloromethane Extract from *Piper umbellatum* Linn. Leaves. Hindawi Publishing Corporation. Evidence Based Complementary and Alternative Medicine. doi: 10.1155/2015/948737
14. Osbou K, Khodaverdi M, Akbari AF. (2010) Antibacterial Effect of Effective Compounds of Satureja Hortensis and Thymus Vulgaris Essential Oils Against Erwinia Amylovora. Journal of Agricultural Science and Technology. 12(1).
15. COX (ovine) Colorimetric Inhibitor Screening Assay Kit. (2017) Cayman Chemical Company.
16. Truong DH, Nguyen DH, Anh Ta NT, Vo Bui Anh, Ha Do Tuong, Ngyuyen HC. (2018) Evaluation of the Use of Different Solvents for Phytochemical Constituents, Antioxidants, and *In Vitro* Anti-Inflammatory Activities of Severinia buxifolia. Hindawi. doi: 10.1155/2019/8178294
17. Salehi S, Zakaria ZA, Gyawali R, Ibrahim S, Rajkovic J, Shinwari ZK, Khan T, Sharifi-Rad J, Ozleyen A, Turkdonmez E, Valussi M, Tumer TB, Fidalgo LM, Martorell M, Setzer W. (2019) Piper Species: A Comprehensive Review on Their Phytochemistry, Biological Activities and Applications. Molecules. doi : 10.3390/molecules24071364
18. Sharma AK, Gangwar M, Chaturvedi AP, Sinha AS. (2012) Tripathi YB. Comparative analysis of phenolic and flavonoid content of Jatropha curcas Linn. Avicenna Journal of Phytomedicine.12:823–26. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4967832/
19. Ganesh P, Kumar R, Saranraj P. (2014) Phytochemical analysis and antibacterial activity of Pepper (*Piper nigrum* L.) against some human pathogens. Central European Journal of Experimental Biology, 3 (2):36-41
20. Yosuf H, Radzi NSM, Richard RL. (2019) Qualitative Phytochemical Analysis and Antimicrobial Activity of Piper sarmentosum Leaves Extract Against Selected Pathogens. Jurnal Sains Kesihatan

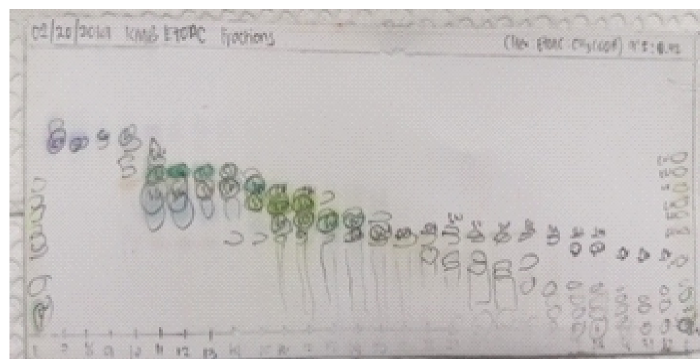
Malaysia 17(1) 2019: 67-72. DOI : http://dx.doi.org./10.17576/JSKM-2019-1701-09.

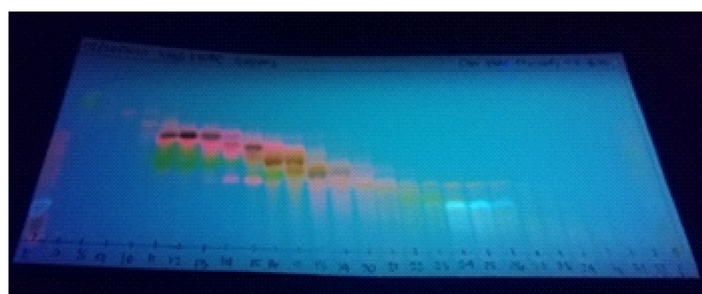
21. Ogunmefun O, Akharaiyi F, & Adegunle S. (2017) Phytochemical and Antimicrobial Properties of *Piper guineense* (Shumach and Thonn) on Selected Human Pathogens. Journal of Chemical and Pharmaceutical Research 9(11):180-186.
22. Sundang M, Nasir S, Sipaut C, Othman H. (2014) Antioxidant Activity, Phenolic, Flavonoid and Tannin Content of Piper Betle and Leucosyke Capitella. Malaysian Journal of Fundamental and Applied Sciences 8(1). DOI:10.11113/mjfas.v8n1.115
23. Moreira L, Vilela F, Orlandi L, *et al.* (2011). Anti-inflammatory Effect of Extract and Fractions from the Leaves of Byrsonima intermedia A. Juss in Rats. Journal of Ethnopharmacology. 138: 610–615. doi:10.1016/j.jep.2011.10.006
24. Itharat A, Lerdvuthisopon N, Piyabhan P, Khonsung P, Boonraeng S, Jaijoy K. (2012) Anti-inflammatory, Analgesic, and Antipyretic Activities of the Ethanol Extract of Piper interruptum Opiz. and *Piper chaba* Linn. Hindawi Publishing Corporation. doi: 10.5402/2012/480265
25. Ridtitid W, Peerati R, Wantana R, Wongnawa M. (2007) Studies of the anti-inflammatory and antipyretic activities of the methanolic extract of Piper sarmentosumRoxb. Leaves in rats. Songklanakarin Journal of Science and Technology. 29(6).
26. Liu Y, Yadev V, Aggarwal BB, Nair MG. (2010) Inhibitory Effects of Black Pepper (*Piper Nigrum*) Extracts and Compounds on Human Tumor Cell Proliferation, Cyclooxygenase Enzymes, Lipid Peroxidation and Nuclear Transcription Factor-kappa-B. SAGE Life Sciences Journals. doi:10.1177/1934578X1000500822.
27. Ruiz-Ruiz JC, Matus-Basto AJ, Acereto-Escoffie P, Segura-Campos MR. (2017) Antioxidant and anti-inflammatory activities of phenolic compounds isolated from Melipona beecheii honey. Food and Agricultural Immunology. doi: 10.1080/09540105.2017.1347148
28. Yang F, de Villiers W, McClain C, Varilek G. (1998) Green tea polyphenols block endotoxin-induced tumor necrosis factor-production and lethality in a murine model. The Journal of Nutrition. 12 8(12):2334-2340. doi: 10.1093/jn/128.12.2334
29. Pan M, Lin-Shiau S, Ho C, Lin JH, Lin J. (2000) Suppression of lipopolysaccharide-induced nuclear factor-kappa B activity by theaflavin - 3,3'-digallate from black tea and other polyphenols through down-regulation of I-kappa B kinase activity in macrophages. Biochemical Pharmacology. 59 (2000): 357-367. doi: 10.1016/s0006-2952(99)00335-4
30. Ichikawa D, Matsui A, Imai M, Sonoda Y, Kasahara T. (2004) Effect of various catechins on the IL-12p40 production by murine peritoneal macrophages and a macrophage cell line. Biological Pharmaceutical Bulletin. 27: 1353-1358. doi: 10.1248/bpb.27.1353
31. Manthey JA, Grohamanu K, Guthric, N. (2001) Biological Properties of Citrus Flavonoids Pertaining to Cancer and Inflammation. Current Medical Chemistry. 8:135-153. doi: 10.2174/0929867013373723.
32. Yu GFB. (2020) The In Vitro Antioxidant Activity and Phytochemicals of Locally Consumed Plant Foods from Quezon Province, Philippines. Acta Medica Philippina. doi: 10.47895/amp.v54i2.1531
33. Geetha S, Irulandi K, Mehalingam P. (2017) Evaluation of Antioxidant and Free Radical Scavenging Activities of Different Solvent Extracts of Leaves of Piper umbellatum. Asian Journal of Pharmaceutical and Clinical Research. 10(2). doi: 10.22159/ajpcr.2017.v10i2.15570
34. Perazzo FF, Souza GHB, Cardoso LGV, Carvalho JCT, Nanayakkara NDD, Bastos JK. (2005): Anti-inflammatory and analgesic properties of water – ethanolic extract from Pothomorphe umbellatum (Piperaceae) aerial parts. Journal of ethnopharmacology 99(2):215-200. doi: 10.1016/j.jep.2005.02.023
35. Hofbauer R, Frass M, Gmeiner B, Handler S, Speiser W, Kapiotis S. (1999) The green tea extract epigallocatechin gallate is able to reduce neutrophil transmigration through monolayers of endothelial cells. Wiener Klinische Wochenschrift. 111: 278-282.
36. Takano K, Nakaima K, Nitta M, Shibata F, Nakagawa H. (2004) Inhibitory effect of (−)- epigallocatechin 3-gallate, a polyphenol of green tea, on neutrophil chemotaxis *in vitro* and *in vivo*. Journal of Agricultural and Food Chemistry. 52: 4571-4576. doi: 10.1021/jf0355194.
37. Tang, L, Wei W, Wang X. (2007) Effects and mechanisms of catechin for adjuvant arthritis in rats. Advances In Natural Therapy. 24:679-690. doi: 10.1007/BF02848793
38. Nunez V, Castro V, Nurillo R, Ponce-soto LA, Merfoot I, Lomonte B. (2005) Inhibitory effects of Piper umbellatum and Piper peltatum extracts towards myotoxic phospholipase A2 from Bothrops snake venoms: isolation of 4-nerolidylcatchol as active Principle Phytochemistry. 66:1017-1025. doi: 10.1016/j.phytochem.2005.03.026

APPENDIX I (PROOF OF COLUMN CHROMATOGRAPHY)

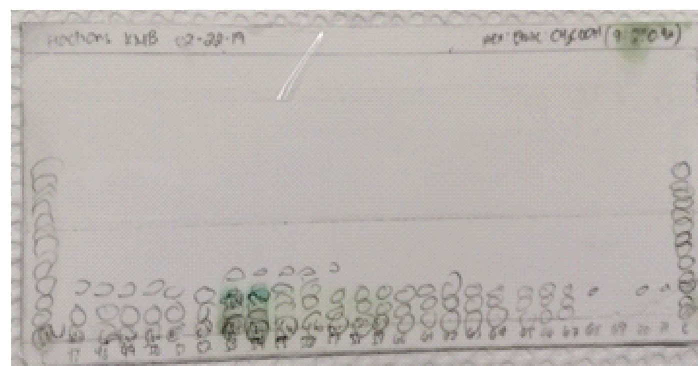
THIN LAYER CHROMATOGRAPHY PLATES

TLC- extracts (under UV 365nm)
Hexane: Ethyl acetate: Acetic acid
(9:3.5:0.1)





TLC-fractions (under UV 365nm
Hexane: Ethyl acetate: Acetic acid
(9:5:0.1)



TLC-fractions
Hexane: Ethyl acetate: Acetic acid (9:5:0.1)

Table 5. *TLC profile of the ethyl acetate of Piper umbellatum leaf extracts.*

Solvent System	Visualizing Reagent	No. of Spots under UV lights	Rf values
Hexane: Ethyl acetate: Acetic acid (9:3.5:0.1)	5% sulfuric acid acid-methanol	5	0.09, 0.22, 0.33, 0.49, 0.62
Hexane: Ethyl acetate: Acetic acid (9:5:0.1)	5% sulfuric acid acid-methanol	11	0.04, 0.08, 0.10 0.20, 0.26, 0.30, 0.32, 0.40, 0.46, 0.50, 0.64

APPENDIX

Table 6. *Rf values of partially purified fractions of ethyl acetate extract*

Pooled Fraction	Fraction #	No. of Spots	Rf values
PF7	7 8 9	2 2 2	0.64, 0.66 0.64, 0.66 0.64, 0.66
PF10	10	4	0.54, 0.58, 0.64, 0.66
PF11	11 12 13 14	5 5 5 5	0.36, 0.42, 0.52, 0.58, 0.64 0.36, 0.42, 0.52, 0.58, 0.64 0.36, 0.42, 0.52, 0.58, 0.64 0.36, 0.42, 0.52, 0.58, 0.64
PF15	15	3	0.30, 0.36, 0.42
PF16	16 17	4 4	0.30, 0.34, 0.40, 0.46 0.30, 0.34, 0.40, 0.46
PF18	18 19 20	3 3 3	0.30, 0.34, 0.36 0.30, 0.34, 0.36 0.30, 0.34, 0.36
PF21	21	3	0.30, 0.34, 0.38
PF22	22 23	3 3	0.22, 0.28, 0.30 0.22, 0.28, 0.30
PF24	24 25 26	4 4 4	0.20, 0.22, 0.28, 0.30 0.20, 0.22, 0.28, 0.30 0.20, 0.22, 0.28, 0.30
PF27	27 28 29	5 5 5	0.02, 0.06, 0.14, 0.22, 0.24 0.02, 0.06, 0.14, 0.22, 0.24 0.02, 0.06, 0.14, 0.22, 0.24
PF30	30 31 32 33 34	4 4 4 4 4	0.02, 0.06, 0.08, 0.24 0.02, 0.06, 0.08, 0.24 0.02, 0.06, 0.08, 0.24 0.02, 0.06, 0.08, 0.24 0.02, 0.06, 0.08, 0.24
PF35	35 36 37 38	3 3 3 3	0.02, 0.04, 0.08 0.02, 0.04, 0.08 0.02, 0.04, 0.08 0.02, 0.04, 0.08
PF39	39 40 41 42 43 44	4 4 4 4 4 4	0.02. 0.04, 0.08, 0.34 0.02. 0.04, 0.08, 0.34 0.02. 0.04, 0.08, 0.34 0.02. 0.04, 0.08, 0.34 0.02. 0.04, 0.08, 0.34 0.02. 0.04, 0.08, 0.34
PF45	45 46 47 48 49 50 51 52	2 2 2 2 2 2 2 2	0.02, 0.06 0.02, 0.06 0.02, 0.06 0.02, 0.06 0.02, 0.06 0.02, 0.06 0.02, 0.06 0.02, 0.06
PF53	53 54	5 5	0.02, 0.08, 0.10, 0.14, 0.20 0.02, 0.08, 0.10, 0.14, 0.20
PF55	55 56 57	6 6 6	0.02, 0.06, 0.08, 0.10, 0.14, 0.20 0.02, 0.06, 0.08, 0.10, 0.14, 0.20 0.02, 0.06, 0.08, 0.10, 0.14, 0.20
PF58	58 59	3 3	0.02, 0.06, 0.10 0.02, 0.06, 0.10
PF60	60 61 62 63	3 3 3 3	0.02, 0.04, 0.08 0.02, 0.04, 0.08 0.02, 0.04, 0.08 0.02, 0.04, 0.08
PF64	64 65 66 67	3 3 3 3	0.02, 0.06, 0.08 0.02, 0.06, 0.08 0.02, 0.06, 0.08 0.02, 0.06, 0.08
PF68	68 69 70 71	1 1 1 1	0.10 0.10 0.10 0.10