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Research Article

ANTI- INFLAMMATORY EFFECTS OF *HIBISCUS ROSA-SINENSIS* L. AND *Hibiscus ROSA-SINENSIS* VAR. *ALBA* ETHANOL EXTRACTS

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ABSTRACT

Objective: The study was carried out to determine and compare the anti- inflammatory activities of ethanol extract of flower and leaf of *Hibiscus rosa-sinensis* var *alba* (white Hibiscus) and *Hibiscus rosa-sinensis* L. (red Hibiscus).

Methods: In the anti-inflammatory test, 0.1ml of carrageenan was injected subplantarly 30 min before administration of each extracts (5, 50 or 100 mg/kg). The animals were killed 6 hrs after carrageenan injection and polymorphonuclear infiltration (PNL) in paw tissues were counted. Phytochemical screening was also performed. Acute dose response was determined using Fixed Dose Procedure with fixed level of doses.

Results: The results revealed flavanoids, saponins and steroids presence in all extracts. Dosing of animals upto 500 mg/kg of all extracts caused no toxicity. No significant changes ($p>0.05$) in liver enzyme levels and histologically no lesions in the organs. Dosing of 50 and 100 mg/kg of flower and leaf estracts of *Hibiscus rosa-sinensis* L. caused significant inhibition ($p<0.05$) of edema. Flower and leaf of *Hibiscus rosa-sinensis* var *alba* significantly inhibited ($p<0.05$) edema in all range of testing dose. The white hibiscus revealed a more potent anti-inflammation. All extracts at various concentration caused significant reduction ($p<0.05$) on PNL infiltration with white Hibiscus also more potent than red hibiscus. All extracts showed significant reduction ($p<0.05$) on the duration of licking response. Same pattern was also observed as white Hibiscus was more potent inhibitor.

Conclusion: This study showed flower and leaf of *Hibiscus rosa-sinensis* var *alba* and *Hibiscus rosa sinensis* L. produced anti-acute inflammatory activity. It may involve the inhibition of cyclooxygenase and reduce PNL and the white Hibiscus variety was more potent than the red variety.

Keywords: *Hibiscus rosa-sinensis* L., *Hibiscus rosa-sinensis* var *alba* Anti-acute inflammatory, Cyclooxygenase, PNL

INTRODUCTION

Inflammation is a normal protective response to tissue injury caused by physical trauma, noxious chemical or microbial agents. It is the body response to inactivate or destroy the invading organisms, to remove irritant and set the stage for tissue repair. It is triggered by the release of chemical mediators from specimen injured tissue and migrating cells[1]. Although inflammation usually works to defend the body, inflammation may also be harmful. Acute inflammatory responses may be exaggerated or sustained, with or without clearance of the offending agent[2].This is explaining why control mechanisms are present to inactive chemical mediators which causing inflammation in normal body function. Acute inflammation is the immediate and early response to injury designed to deliver leucocytes to the sites of injury. Once there, leucocytes clear any invading microbes and begin the process of breaking down necrotic tissues. The vascular changes and cell recruitment account for three of five classic local signs of acute inflammation: heat (*calor*), redness (*rubor*), and swelling (*tumor*). The two additional cardinal features of acute inflammation, pain (*dolor*) and loss of function (*functiolaesa*), occur as consequences of mediator elaboration and leukocyte- mediated damaged[3]. Non-steroidal anti-inflammatory drugs (NSAIDs) are among the most important drugs used in the reduction of pain and inflammation. Their efficacy has been confirmed by a wide number of experimental and clinical studies[4-6].

Despite their efficacy, the use of NSAIDs induces serious adverse effects when administrated chronically, with the gastrointestinal tract being the most affected system[7]. For this purpose, an increasing number of studies are being carried out in search of new therapeutics from medicinal plants, especially those with proclaimed popular use as anti-inflammatory and analgesic agents[8]. *Hibiscus rosa-sinensis,* called as 'bunga raya' in Malay exhibit many variations in form and flower color[9]. The flowers are of different sizes, depending on the variety, and may be single or double. *Hibiscus rosa-sinensis* var *alba* is one type of the variant of *Hibiscus rosa-sinensis* L. (red) which is white flower in color. Ethanol extracts of flower[10] and leaf[11] of *Hibiscus rosa-sinensis* L. (red) has been reported to exhibit anti-inflammatory activity. However, there are no findings neither on the phytochemical screening nor usage of variant of *Hibiscus rosa-sinensis alba* in any biomedical research. The present study was aimed to determine and compare the anti- inflammatory activities of ethanol extract of flower and leaf of *Hibiscus rosa-sinensis* var *alba* and *Hibiscus rosa-sinensis* L. in rats at the dose predetermined in acute dose response.

METHODS

Chemicals

Ammonia, Mayer's reagent, ferric chloride, concentrated sulfuric acid (H_2SO_4), acetic anhydride, ascorbic acid, 95% ethanol, carrageenan, formalin and diclofenac sodium were purchased from Sigma-Aldrich (USA). Hydrochloric acid (HCl) and acetic acid were obtained from Merck (Darmstadt, Germany). Chloroform was obtained from Friendemann Schmidt (Germany). Liver Function Test Kit was obtained from Roche Diagnostic (USA).

Plant Material

The matured flowers and leaves of *Hibiscus rosa-sinensis* L. were collected from areas of Universiti Putra Malaysia (UPM), Serdang, Selangor, Malaysia. Whereas flower and leaves of *Hibiscus rosa-sinensis* var. *alba* were collected from the areas at Normah Medical Centre, Kuching, Sarawak, Malaysia. Voucher specimens of the flower and leaf of *Hibiscus rosa-sinensis* L. and *Hibiscus rosa-sinensis* var. *alba* have were obtained from Herbarium Institute of Bioscience, UPM, Serdang, Selangor, Malaysia (ACP0087 and ACP 0143 respectively).

Preparation of Ethanol extract of Flower and Leaf of *H.rosa-sinensis* L. and *H.rosa-sinensis* var. *alba*

The extract was prepared using the method described by Somchit et al.[12] and Zakaria et al.[13] with slight modification. In addition,

ethanol react was chosen as to extract polar compounds. The fresh samples were cleaned using running tap water and dried in a good air draft under shade overnight until constant weight or in oven at 42ºC. Then the samples grounded into fine particles using ultra centrifugal mill. The samples were soaked and stirred in 95% ethanol in a ratio of 1:10 (w/v). Then, the samples were evaporated using rotary evaporator at 40 ºC. The crude extracts were stored at 4 ºC for further use.

Phytochemical Screening

The extracts were subjected to phytochemical screening. The presence of alkaloids, flavonoids, saponins, tannins, terpenoids and steroids in the herb extracts were revealed.

Test for Alkaloids

Crude extract (0.5 g) was diluted with 10 ml of 10% acetic acid in ethanol, boiled and filtered. Hot. 2 ml of 10% dilute ammonia and 5 ml of chloroform were added to 5 ml of filtrate. The filtrate was shaken gently to extract the alkaloid base. The chloroform layer was extracted with 5% of HCl. The filtrate was treated with a few drops of Mayer's reagent. Formation of white precipitates indicated the presence of alkaloids[14,15].

Test for Flavonoids

Crude extract (1 g) was added with 5 ml ethanol, boiled and filtered. A few drops of concentrated HCl and magnesium tape ribbon (1-2 cm) were added. Colours ranging from orange to red indicated flavones, red to crimson indicated flavonols and crimson to magenta indicated flavononones[16].

Test for Saponins

Crude extract (1 g) was boiled in 10 ml of distilled water in a water bath and filtered. The filtrate was shaken vigorously (1-2 min) for a stable persistent froth (for at least 15 min) regarded as presence of saponins[14,15,17] .

Test for Tannins

Crude extract (0.5 g) was boiled in 20 ml of water and then filtered. A few drops of 0.1% ferric chloride were added. An intense blue black color was taken as an evidence for the presence of hydrolysable tannins, while brownish green indicated that of condensed tannins[14,15,17].

Test for Triterpenes

Salkowski Test: An extract (5 ml) was mixed in 2 ml of chloroform and concentrated H_2SO_4 (3 ml) was carefully added to form a layer. A reddish brown color was taken as an evidence for the presence of triterpenes[15,17].

Test for Steroids

Liebermann-Buchard Test: Acetic anhydride (2 ml) was added to 0.5 g crude extract with 2 ml H_2SO_4. The color changed from violet to blue or green was taken as an evidence for the presence of steroids[17].

Acute Dose Response Study

The Fixed Dose Procedure (FDP)[18] was performed to determine the acute dose response and the testing dose (via ratio calculation). A group of 65 Sprague Dawley male rats were divided equally at random into 13 groups composing of five rats each. The groups were supplemented with one of the following: (a) distilled water, (b) ethanol extract of flower of *Hibiscus rosa-sinensis* L. (red) (5, 50 or 500 mg/kg) or (c) leaf of *H.rosa-sinensis* L. (red) (5, 50 or 500 mg/kg) or (d) flower of *Hibiscus rosa-sinensis* var *alba* (white) (5, 50 or 500 mg/kg) and (e) leaf of *Hibiscus rosa-sinensis* var *alba* (white) (5, 50 or 500 mg/kg). The rats were kept in cage in group. Ethanol extracts of flower and leaf of *Hbiscus rosa-sinensis* L. (red) and *Hibiscus rosa-sinensis* var *alba* (white) were dissolved in distilled water and given intraperitonially to the rats. At each dose level tested, animals were categorized into one of three groups (dead; showing signs of toxicity; unaffected). Any mortality and signs of toxicity were observed within 14 days after the administration of test extracts[19].

The blood samples were collected at day 15 after the administration of test extracts and placed in plain tubes and allowed to clot for 45 minutes at room temperatures. Serum were collected by centrifugation at 3000 rpm for 10 minutes and stored at -80ºC until analyze. Serum level of Alanine transaminase (ALT), Alkaline phosphatase (ALP) and Aspartate aminotransferase (AST)[20] were analyzed. Serum level of ALT, ALP and AST obtained expressed as mean ± S.E.M. Statistical analysis were performed with three way ANOVA to determine the effect of variant, plant part and doses on liver enzyme level. Mean comparisons among the treatment effect were compare using Duncan Multiple Range Test.

Following the blood sampling, the rats were killed at day 15 after the administration of test extracts. The liver and kidney organs were isolated and firstly fixed in 10% formalin for later histopathological examination. The slides of liver specimens were prepared by routinely processed for paraffin embedding. From each sample, 5µm thick sections were obtained and stained with hematoxylin-eosin (H&E). Section were examined by light microscopy and graded for presence and intensity of lesions using severity scale from 0-5% (none, 0; minimal, involving single and a few necrotic cell, 1; mild, 10-25% necrotic cell or mild diffuse degenerative change, 2; moderate, 25-40% necrotic or degenerative cell, 3; marked, 40-50% necrotic or degenerative cell, 4; severe, more than 50% necrotic or degenerative cell, 5). Historically, section with score higher than 2 are consider as having significantly liver injury[21-23]. The grading for presence and intensity of lesions obtained expressed as mean ± S.E.M. Statistical analysis were performed with three way ANOVA to determine the effect of variant, plant part and doses on presence and intensity of lesions liver and kidney damage. Mean comparisons among the treatment effect were compare using Duncan Multiple Range Test.

Anti- Inflammatory Test

PNL Infiltration and Carrageenan-induced Paw Edema in Rats

The Carrageenan-induced Paw-edema Test was carried out as described by Zakaria *et al.*[24] with slight modification. The PNL infiltration study was done using procedure described by Ozbakis-Dengiz *et al.*[25] with slight modification. A group of 70 Sprague Dawley male rats were divided equally at random into 14 groups composing of five rats each. The groups were supplemented with one of the following: (a) distilled water, (b) ethanol extract of flower of *Hibiscus rosa-sinensis* L. (red) (5, 50 or 100 mg/kg) or (c) leaf of H. rosa-*sinensis* L. (red) (5, 50 or 100 mg/kg) or (d) flower of *Hibiscus rosa-sinensis* var *alba* (white) (5, 50 or 100 mg/kg) or (e) leaf of *Hibiscus rosa-sinensis* var *alba* (white) (5, 50 or 100 mg/kg) and (f) diclofenac sodium (10 mg/kg). The rats were kept in cage in group. Ethanol extracts of flower and leaf of *Hbiscus rosa-sinensis* L. (red) and *Hibiscus rosa-sinensis* var *alba* (white) or diclofenac were dissolved in distilled water and given intraperitonially to the rats. Thirty minutes after injection, 0.1 ml of carrageenan (1% in 0.9% NaCl) was injected subplantarly in the right hind paw of the rats. The paw volume (ΔV, ml) was measured on the principle of volume displacement using phetlysmometer immediately prior to carrageenan injection (V_0) and at hourly interval from 1 to 6 hrs afterwards (V_t). Increased in the volume of paw obtained expressed as mean ± S.E.M. Statistical analysis were performed as repeated measure with three way ANOVA to determine the effect of variant, plant part and doses on inhibition of edema. Mean comparisons among the treatment effect were compare using Duncan Multiple Range Test. The animals in each group were killed 6 hrs after carrageenan administration, and their paw tissues were excised for pathological investigation. The specimens were fixed in 10% formalin and routinely processed for paraffin embedding. From each sample, 5µm thick sections were obtained and stained with H&E to evaluate acute inflammation. PNLs were counted in 7 separate microscopic fields (× 400) from two sections of each animal. The number of PNLs was expressed as mean ± S.E.M. Statistical analysis were performed with three way ANOVA to determine the effect of variant, plant part and doses on the reduction of PNL infiltration. Mean comparisons among the treatment effect were compare using Duncan Multiple Range Test.

2.6.2 Formalin-induced Paw Licking Test in Rats

The formalin-induced paw licking was studied using the method described by Sharifzadeh *et al.*[26] and Zakaria et al.[27] with slight modification. A group of 30 Sprague Dawley male rats were divided equally at random into 6 groups composing of five rats each. The groups were supplemented with one of the following: (a) distilled water, (b) ethanol extract of 100 mg/kg flower of *Hibiscus rosa-sinensis* L. (red) or (c) 100 mg/kg leaf of *H.rosa-sinensis* L. (red) or (d) 100 mg/kg flower of *Hibiscus rosa-sinensis* var *alba* (white) or (e) 100 mg/kg leaf of *Hibiscus rosa-sinensis* var *alba* (white) and (f) diclofenac sodium (10 mg/kg). The rats were kept in cage in group. Ethanol extracts of flower and leaf of *Hbiscus rosa-sinensis* L. (red) and *Hibiscus rosa-sinensis* var *alba* (white) or diclofenac were dissolved in distilled water and given intraperitonially to the rats. Thirty minutes after injection, 50µl of formalin (5% in 0.9% NaCl) was injected subcutaneously into dorsal surface of the right hind paw of the rats. Immediately after formalin injection, animals were placed individually in glass cylinders on a flat glass floor and a mirror was placed at 45º angle under the cylinder to allow clear observation of the paw of the animals. The time the animal spent licking the injected paw was counted in two different phases: from 0-5 min post injection (early phase) and from 15-30 min post injection (late phase). Duration of licking response obtained expressed as mean ± S.E.M. Statistical analysis were performed with three way ANOVA to determine effect of variant, plant part and doses on the reduction of duration of licking response. Mean comparisons among the treatment effect were compare using Duncan Multiple Range Test.

RESULTS AND DISCUSSION

Based on phytochemical screening, flavanoids, saponins and steroids were found to be present in ethanol extracts of flower and leaf of *Hibiscus rosa-sinensis* L. (red) and *Hibiscus rosa-sinensis* var *alba* (white) (Table 1). Most of the secondary metabolites were identified in the polar extracts[28]. Flavonoids[14,29], saponins[30] and plant sterols[31] exhibited anti-inflammatory activity through different mechanism. According to Birari *et al.*[10], the phytochemical screening of ethanol extracts of flower of *Hibiscus rosa sinensis* L. demonstrated the presence of flavonoids and also in other herbal plants[32]. In addtition, Anonymous[33] also reported flowers of *Hibiscus rosa sinensis* L. have shown the presence of flavonoids and steroids. Gupta, *et al.*[8] claimed the phytochemical screening of methanol extracts of leaf of *Hibiscus rosa sinensis* L. detected flavonoids and steroids. These studies are consistent to preliminary phytochemical findings in present study. The presence of these metabolites probably explains the various uses of this plant in traditional medicine. Predominant presence of anti- inflammatory agents of flavonoids, saponins and steroids in ethanol extracts of flower and leaf of *Hibiscus rosa-sinensis* L. (red) and *Hibiscus rosa-sinensis* var *alba* (white) might be responsible for significant inhibition of inflammation of the extracts. Indeed, Pal and Verma[34] reported potent activities of flavonoids isolated from herbal plants.

Table 1: Phytochemical constituents of ethanol extracts of flower and leaf of *H. rosa-sinensis* L. (red) and *H. rosa-sinensis* var *alba* (white)

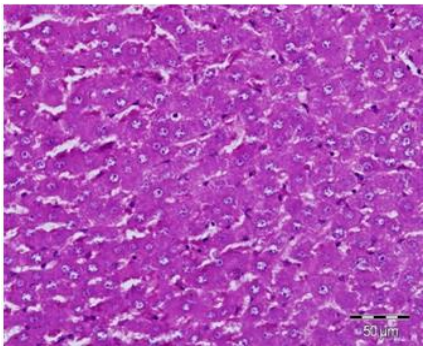
Constituents	*H. rosa-sinensis* L. (red)		*H. rosa-sinensis* var. *alba* (white)	
	Flower	Leaf	Flower	Leaf
Alkaloids	-	-	-	-
Flavonoids	++	+	++	+
Saponins	++	++	+	++
Tannins	-	-	-	-
Triterpenes	-	-	-	-
Steroids	++	+++	+	++

Note:For flavonoids, tannins, titerpenes and steroids: + weak color; ++ mild color; +++ strong color.

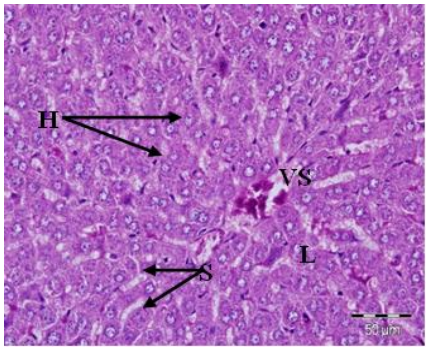
For alkaloids: + negligible amount of precipitate; ++ weak precipitate; +++ strong precipitate.

For saponins: + 1-2 cm froth; ++ 2-3 cm froth; +++ >3 cm froth.

FDP was used to determine and compare the acute dose response for ethanol extract of flower and leaf of *Hibiscus rosa-sinensis* var *alba* with *Hibiscus rosa-sinensis* L. Beneficially, it reduced the number of animals used and avoid lethality as an endpoint. This is because FDP relies on clear toxic signs observed at a number of fixed dose levels that are applicable as for the cut-off points in the European Economic Community (EEC) acute toxicity classification system[35]. At 500mg/kg dose of each extract to the rats showed some evident of altered behavior as hypoactivity, ataxia and lethargy after 1 hour of administration. The procedure was stopped and the value of 500 of dose was used for determining the testing dose which resulted (via ratio calculation) as 5, 50 and 100 mg/kg. The levels of ALT, ALP and AST for the test of liver function were not significantly ($p>0.05$) (Table 2(A)-(C)) indicated no of hepatic injury or damaged in response to the dose (up to 500 mg/kg) of all extracts. As mentioned in the study of Somchit, *et al.*[36], increasing of ALT and AST indicated hepatic injury. Supplementation of *Hibiscus rosa-sinensis* var *alba* (white) caused no significant effect ($p>0.05$) in hepatic injury as no toxicity compared with *Hibiscus rosa-sinensis* L. (red). Supplementation of flower of *Hibiscus rosa-sinensis* L. and *Hibiscus rosa-sinensis* var *alba* (white) caused no significant effect ($p>0.05$) in hepatic injury as no toxicity compared with leaf of *Hibiscus rosa-sinensis* L. and *Hibiscus rosa-sinensis* var *alba* (white). Results also indicated no significant effect ($p>0.05$) in hepatic injury as no toxicity with doses (5, 50 or 100 mg/kg) of *Hibiscus rosa-sinensis* var *alba* (white) compared with doses (5, 50 or 100 mg/kg) of *Hibiscus rosa-sinensis* L. According to the resulted of histopathological examination, no presence of lesions to the liver (Figure 1) and kidney (Figure 2) indicated these organs effect with no toxicity in response to the dose (up to 500 mg/kg) of all extracts. According to Wang, *et al.*[37], the hepatocytes necrosis in the pathological examination warranted liver injury. It can be concluded that the biochemical study (liver function test) corroborates with histopathological; no toxicity indicated in response to the dose (up to 500 mg/kg) of flower and leaf of *Hibiscus rosa-sinensis* L. (red) and *Hibiscus rosa-sinensis* var *alba* (white).



(a)



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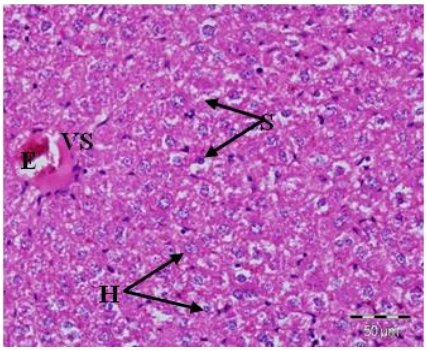

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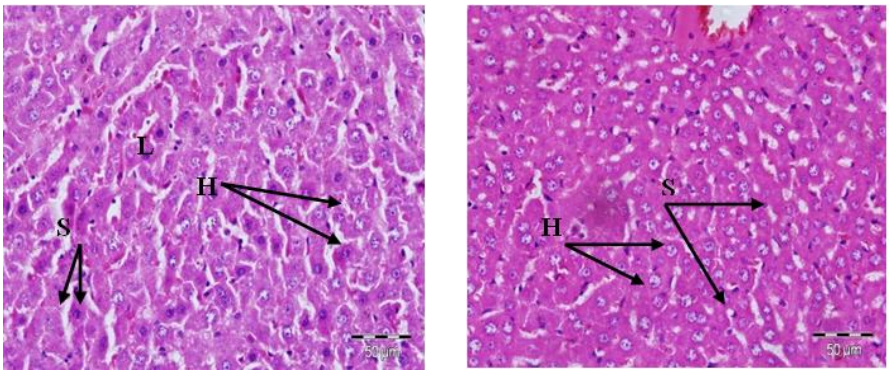


(d) (e)

Fig. 1:Histopathology of Liver of Rats Receiving 0 or 500mg/kg of *H. rosa-sinensis* L. or *H. rosa-sinensis* var *alba* extract at H&E, x400

VS: Blood vessel, E: Erythrocytes H: Hepatocytes, L: Liver lobules, S: Sinusoids.

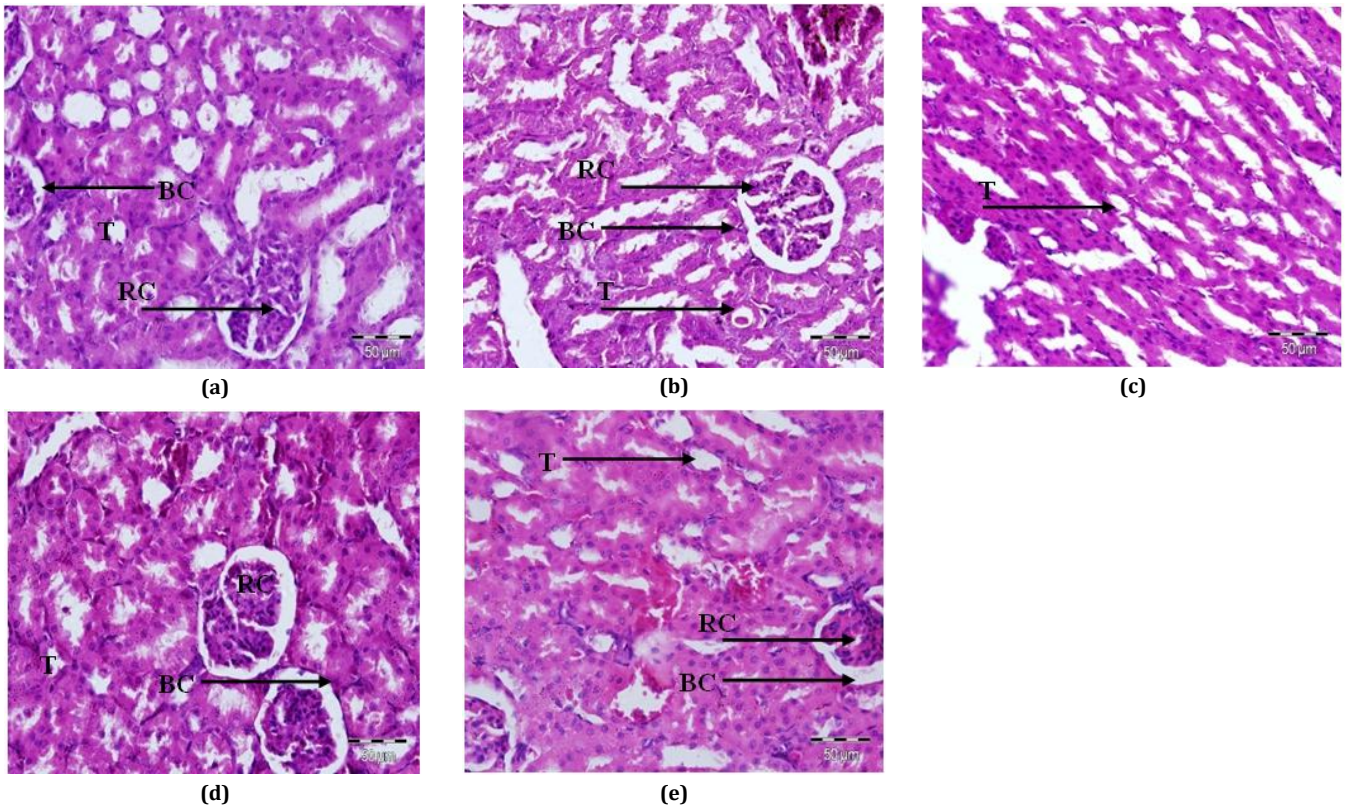
(A) Control rat liver showing normal hepatocytes.

(B) Rat treated with *H. rosa-sinensis* L. (red) flower extract. No sign of parenchymal inflammation is seen

(C) Rat treated with *H. rosa-sinensis* L. (red) leaf extract. Normal liver histology is seen

(D)Rat treated with *H. rosa-sinensis* var *alba* (white) flower extract. No sign of acute parenchymal inflammation is seen

(E)Rat treated with *H. rosa-sinensis* var *alba* (white) leaf extract. No sign of acute parenchymal inflammation is seen



(a) (b) (c)

(d) (e)

Fig. 2:Histopathology Examinations of Kidney of Rats Receiving 0 or 500mg/kg of *H. rosa-sinensis* L. or *H. rosa-sinensis* var *alba* extract at H&E, x400

BC: Bowman's space, VS: Blood vessel, RC: Renal corpuscle, T: Renal tubule

(A)Normal rat kidney.

(B)Rat treated with *H. rosa-sinensis* L. (red) flower extract. Note: No sign of acute parenchymal inflammation and acute tubular necrosis.

(C)Rat treated with *H. rosa-sinensis* L. (red) leaf extract. Note: No sign of acute parenchymal inflammation and acute tubular necrosis.

(D)Rat treated with *H. rosa-sinensis* var *alba* (white) flower extract. Note: No sign of acute parenchymal inflammation and acute tubular necrosis.

(E)Rat tested with *H. rosa-sinensis* var *alba* (white) leaf extract. Note: Normal kidney is seen

Table 2:(A)Effect of various concentration of ethanol extracts of flower and leaf of *H. rosa-sinensis* L. (red) and *H. rosa-sinensis* var. *alba* (white) on serum level of Alanine transaminase (ALT) in rats

Control	*H.rosa-sinensis* L. (red)						*H. rosa-sinensis* L. var *alba* (white)					
	Flower			Leaf			Flower			Leaf		
	5	50	500	5	50	500	5	50	500	5	50	500
0.88 ± 0.08	0.89 ± 0.06	0.90 ± 0.13	0.74 ± 0.07	0.73 ± 0.07	0.93 ± 0.03	0.95 ± 0.11	0.89 ± 0.06	0.88 ± 0.06	1.08 ± 0.13	0.83 ± 0.04	0.82 ± 0.06	0.86 ± 0.05

Table 2:(B)Effect of various concentration of ethanol extracts of flower and leaf of *H. rosa-sinensis* L. (red) and *H. rosa-sinensis* var. *alba* (white) on serum level of Alkaline phosphatase (ALP) in rats

Control	*H.rosa-sinensis* L. (red)						*H. rosa-sinensis* L. var *alba* (white)					
	Flower			Leaf			Flower			Leaf		
	5	50	500	5	50	500	5	50	500	5	50	500
294.35 ± 6.56^{a}	277.28 ± 7.93^{ab}	286.02 ± 5.69^{ab}	289.21 ± 7.61^{ab}	277.54 ± 13.16^{ab}	307.00 ± 27.36^{ab}	287.66 ± 4.46^{ab}	300.96 ± 9.96^{ab}	284.00± 20.87^{ab}	292.95 ± 6.68^{ab}	290.82 ± 8.15^{ab}	290.34 ± 11.28^{ab}	259.09 ± 24.55^{b}

Table 2:(C) Effect of various concentration of ethanol extracts of flower and leaf of *H. rosa-sinensis* L. (red) and *H. rosa-sinensis* var. *alba* (white) on serum level of Aspartate aminotransferase (AST) in rats

Control	*H.rosa-sinensis* L. (red)						*H. rosa-sinensis* var *alba* (white)					
	Flower			Leaf			Flower			Leaf		
	5	50	500	5	50	500	5	50	500	5	50	500
1.94 ± 0.11 ^{a}	1.91 ± 0.17 ^{a}	1.98 ± 0.07 ^{a}	1.75 ± 0.03 ^{b}	1.81 ± 0.04 ^{ab}	1.72 ± 0.04 ^{b}	1.73 ± 0.08 ^{b}	1.76 ± 0.03 ^{ab}	1.73 ± 0.05 ^{b}	1.92 ± 0.11 ^{ab}	2.01 ± 0.10 ^{a}	1.75 ± 0.06 ^{ab}	1.97 ± 0.04 ^{ab}

Mean + sd (n=5). Mean with different superscript differ signidicantly (P<0.05)

Table 3: Effect of various concentration of ethanol extracts of flower and leaf of *H. rosa-sinensis* L. (red) and *H. rosa-sinensis* var *alba* (white) on carrageenan-induced paw edema in rats

TIME	Control	*H. rosa-sinensis* L. (red)						*H. rosa-sinensis* var *alba* (white)						Diclofenac
		Flower			Leaf			Flower			Leaf			
	-	5	50	100	5	50	100	5	50	100	5	50	100	10
1	0.20 ± 0.05^{ax}	0.20 ± 0.03^{ax}	0.20 ± 0.03^{ax}	0.10 ± 0.01^{bx}	0.20 ± 0.03^{ax}	0.16 ± 0.02^{ax}	0.10 ± 0.01^{bx}	0.12 ± 0.02^{bx}	0.12 ± 0.02^{bx}	0.12 ± 0.02^{bx}	0.14 ± 0.02^{bcx}	0.14 ± 0.02^{bcx}	0.14 ± 0.02^{bcx}	0.10 ± 0.03^{bx}
2	0.32 ± 0.05 ^{ay}	0.28 ± 0.04^{ay}	0.22 ± 0.04^{bx}	0.10 ± 0.03^{cx}	0.24 ± 0.04^{abx}	0.20 ± 0.03^{abx}	0.10 ± 0.03^{cx}	0.22 ± 0.05^{by}	0.22 ± 0.02^{by}	0.16 ± 0.02^{dx}	0.16 ± 0.04^{dx}	0.14 ± 0.02^{cdx}	0.14 ± 0.02^{cdx}	0.08 ± 0.02^{cex}
3	0.14 ± 0.02^{az}	0.14 ± 0.02^{az}	0.12 ± 0.05^{ay}	0.10 ± 0.03^{bx}	0.14 ± 0.04^{ay}	0.14 ± 0.02^{ax}	0.08 ± 0.02^{bcx}	0.14 ± 0.04^{ax}	0.14 ± 0.02^{ax}	0.08 ± 0.04^{bcy}	0.14 ± 0.04^{ax}	0.12 ± 0.04^{abx}	0.12 ± 0.04^{ax}	0
4	0.24 ± 0.02^{axy}	0.22 ± 0.06^{ax}	0.16 ± 0.05^{bxy}	0.02 ± 0.02^{cy}	0.20 ± 0.05^{abx}	0.16 ± 0.02^{bx}	0.08 ± 0.02^{dx}	0.20 ± 0.05^{aby}	0.20 ± 0.03^{aby}	0.06 ± 0.02^{dy}	0.16 ± 0.02^{bx}	0.10 ± 0.03^{dy}	0.04 ± 0.02^{cy}	0.04 ± 0.02^{cy}
5	0.30 ± 0.03^{ay}	0.24 ± 0.05^{ax}	0.18 ± 0.04^{bxy}	0.08 ± 0.04^{cx}	0.22 ± 0.06^{abx}	0.18 ± 0.02^{bx}	0.10 ± 0.03^{cx}	0.24 ± 0.04^{aby}	0.24 ± 0.04^{aby}	0.12 ± 0.02^{cx}	0.20 ± 0.03^{abx}	0.12 ± 0.04^{cxy}	0.08 ± 0.05^{cy}	0.10 ± 0.03^{cx}
6	0.20 ± 0.03^{ax}	0.16 ± 0.04^{ax}	0.08 ± 0.04^{bcz}	0.04 ± 0.02^{cy}	0.16 ± 0.04^{axy}	0.10 ± 0.03^{by}	0.02 ± 0.02^{cy}	0.20 ± 0.03^{ay}	0.16 ± 0.04^{ax}	0.08 ± 0.02^{bcy}	0.16 ± 0.02^{ax}	0.06 ± 0.02^{bcy}	0.04 ± 0.02^{cy}	0.02 ± 0.02^{cy}

Note:Values were presented as mean ± S.D of 5 animals per group.

$^{a-e}$ Means with different superscript differs significantly in the same row at p<0.05

$^{x-z}$: Means with different superscript differs significantly in the same column at p<0.05

Table 4: Effect of various concentration of ethanol extracts of flower and leaf of *H. rosa-sinensis* L. (red) and *H. rosa-sinensis* var *alba* (white) on PNL infiltration in carrageenan-induced paw edema in rats

Control	*H.rosa sinensis* L. (red)						*H.rosa sinensis* L. var *alba (*white)						Dic
	Flower			Leaf			Flower			Leaf			
	5	50	100	5	50	100	5	50	100	5	50	100	
2379.17 ± 309.5 ^{a}	1742 ± 29.28 ^{b}	1143 ± 122.41^{c} ^{d}	409.83 ± 74.88 ^{e}	1766.67 ± 253.59^{b}	1127.33 ± 139.09^{c} ^{d}	448.17 ± 42.97 ^{e}	1765.83 ± 299.64 ^{b}	1412.17 ± 172.25^{b} ^{c}	780.5 ± 104.79^{d} ^{e}	1450 ± 202.95^{b} ^{c}	1123 ± 307.68^{c} ^{d}	761.67 ± 148.37^{d} ^{e}	296.17 ± 46.01 ^{e}

Notes:Values were presented as mean ± S.D of minimum 3 animals per group.

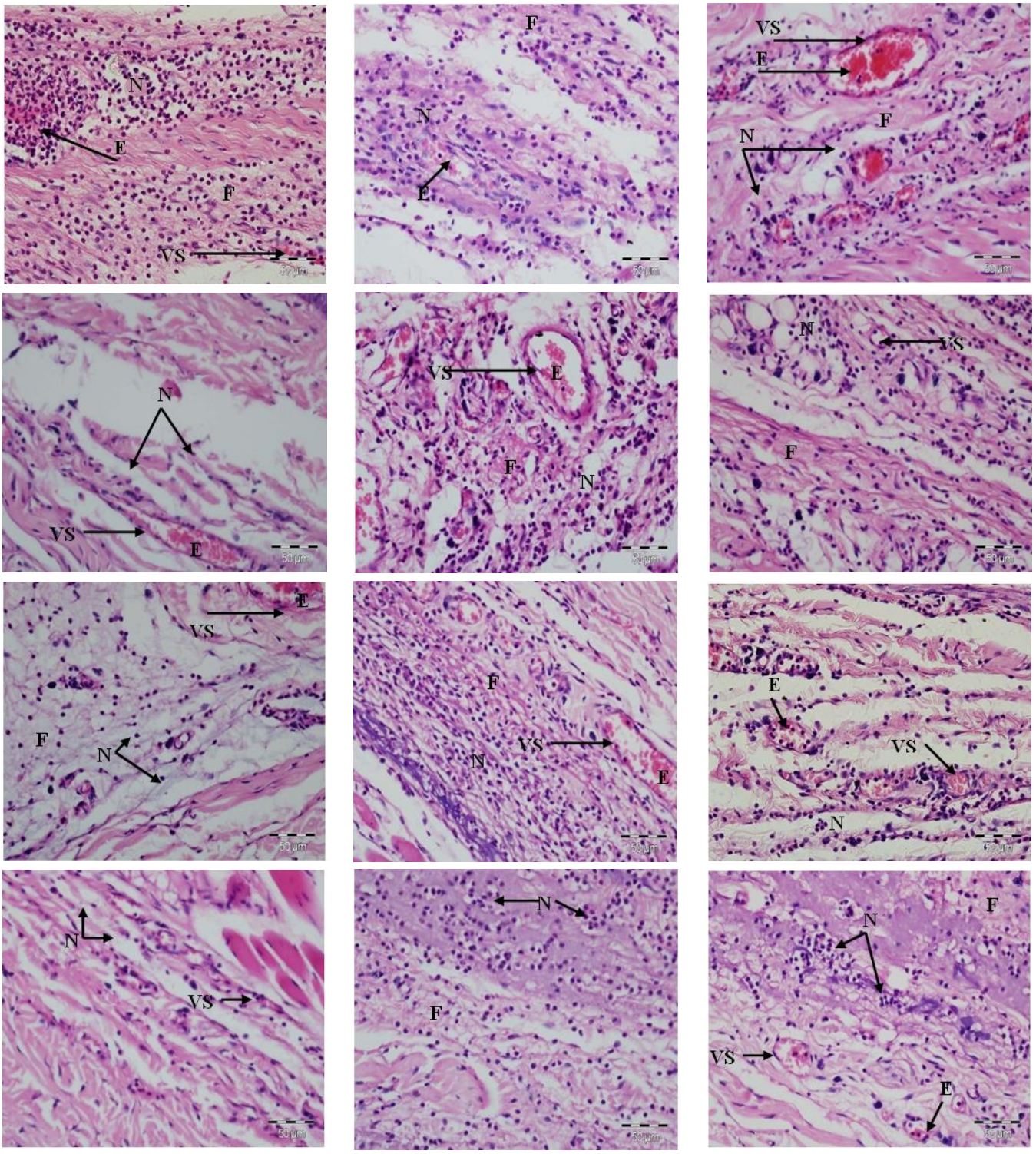
$^{a-e}$ Means with different superscript differs significantly in the same row at p<0.05

Table 5: Effects of 100mg/kg of ethanol extracts of flower and leaf of *H.rosa sinensis* L. (red) and *H.rosa-sinensis* var *alba* (white) on Formalin-induced Paw Licking in rats

Phase	Control	*H. rosa-sinensis* L. (red)		*H.rosa-sinensis* var *alba* (white)		10 mg/kg Diclofenac
		Flower	Leaf	Flower	Leaf	
		100	100	100	100	
Early	123.48 ±4.10 [a]	99.10 ±9 [bc]	94.76 ±1.88 [bc]	75.13 ±4.37 [c]	69.41 ±6.07 [c]	98.85 ±5.31 [bc]
Late	249.10 ±41.02 [a]	82.43 ±9.01 [b]	110.02 ±4.96 [b]	100.77 ±7.48 [b]	82.16 ±9.41 [b]	98.46 ±6.39 [b]

Note:Values were presented as mean ± S.D of 5 animals per group.

[a-e] Means with different superscript differs significantly in the same row at $p<0.05$



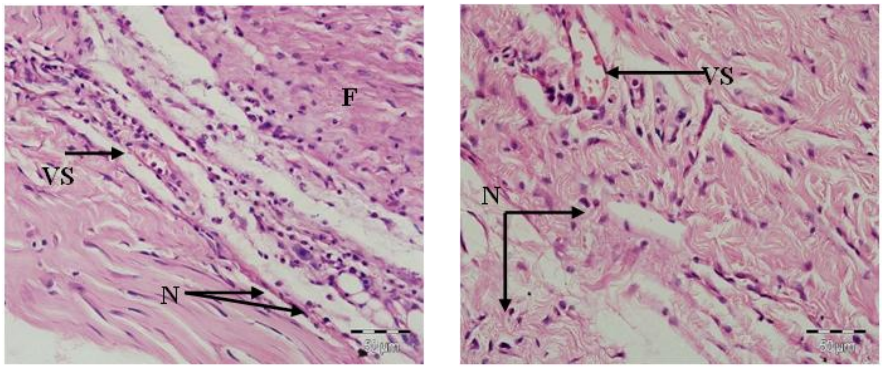


Fig. 3: Localization of PNLs in Effect of Various Concentration of Ethanol Extracts of Flower and Leaf of *H.rosa-sinensis* L. and *H.rosa-sinensis* var *alba* on Carrageenan-induced Paw Edema in Rats at H&E, x400

VS: Blood vessel, E: Erythrocytes, F: Fibrin, N: Neutrophils

(A)Control rat. Note: Acute edema, hyperemia with extensive PNLs infiltration

(B)Rat treated with 5 mg/kg *H.rosa-sinensis* L. (red) flower. Note: Reduction in inflammatory infiltrates is seen

(C)Rat treated with 50 mg/kg *H.rosa-sinensis* L. (red) flower Note: A dose dependent reduction in inflammatory infiltrates is seen

(D)Rat treated with 100 mg/kg *H.rosa-sinensis* L. (red) flower. Note: A dose dependent reduction in inflammatory infiltrates is seen

(E)Rat treated with 5 mg/kg *H.rosa-sinensis* L. (red) leaf. Note: Reduction in inflammatory infiltrates is seen

(F)Rat treated with 50 mg/kg *H.rosa-sinensis* L. (red) leaf Note: A dose dependent reduction in inflammatory infiltrates is seen

(G)Rat treated with 100 mg/kg *H.rosa-sinensis* L. (red) leaf. Note: A dose dependent reduction in inflammatory infiltrates is seen

(H)Rat treated with 5 mg/kg *H.rosa-sinensis* var *alba* (white) flower. Note: Reduction in inflammatory infiltrates is seen

(I)Rat treated with 50 mg/kg *H.rosa-sinensis* var *alba* (white) flower. Note: A dose dependent reduction in inflammatory infiltrates is seen

(J)Rat treated with 100 mg/kg *H.rosa-sinensis* var *alba* (white) flower. Note: A dose dependent reduction in inflammatory infiltrates is seen

(K)Rat treated with 5 mg/kg *H.rosa-sinensis* var *alba* (white) leaf . Note: Reduction in inflammatory infiltrates is seen

(L)Rat treated with 50 mg/kg *H.rosa-sinensis* var *alba* (white) leaf. Note: A dose dependent reduction in inflammatory infiltrates is seen

(M)Rat treated with 100 mg/kg *H.rosa-sinensis* var *alba* (white) leaf. Note: A dose dependent reduction in inflammatory infiltrates is seen

(N)Rat treated with 10 mg/kg Diclofenac. Note: Reduction in inflammatory infiltrates is seen

As shown in Table 3, supplementation of 50 and 100 mg/kg of flower and leaf of *Hibiscus rosa-sinensis* L. (red) caused significant effects on the inhibition of paw edema induced by carrageenan. Interestingly, flower and leaf *Hibiscus rosa-sinensis* var *alba* (white) showed significant inhibition of paw edema in all range of doses (5, 50 and 100 mg/kg). Based on the results, it can be concluded that all the extracts able to reverse acute inflammation through inhibition of edema induced by carrageenan. *Hibiscus rosa-sinensis* var *alba* (white) showed significant ($p<0.05$) anti-inflammatory effect compared with *Hibiscus rosa-sinensis* L. (red). Flower of *Hibiscus rosa-sinensis* L. and *Hibiscus rosa-sinensis* var *alba* (white) showed significant ($p<0.05$) anti-inflammatory effect compared with leaf of *Hibiscus rosa-sinensis* L. and *Hibiscus rosa-sinensis* var *alba* (white). Results also indicated significant ($p<0.05$) anti-inflammatory effect with doses (5, 50 or 100 mg/kg) of *Hibiscus rosa-sinensis* var *alba* (white) compared with doses (5, 50 or 100 mg/kg) of *Hibiscus rosa-sinensis* L. Interestingly, ethanol extracts of flower[10] and leaf[11] of *Hibiscus rosa-sinensis* L. (red) has also been reported to exhibit anti-inflammatory activity when assessed using the carrageenan-induced paw edema test. These studies are consistent to the present study as to determine and compare the anti-inflammatory properties of *Hibiscus rosa-sinensis* var *alba* with *Hibiscus rosa-sinensis* L. flower and leaf ethanol extract. The involvement of the cyclooxygenase-2 (COX-2) pathway in carrageenan- induced paw edema test is also worth mentioning as part of the mechanism of action of the anti-inflammatory activities of flower and leaf of *Hibiscus rosa-sinensis* L. (red) and *Hibiscus rosa-sinensis* var *alba* (white). This is based on a report indicating that carrageenan-induced edema formation involves mechanisms that include overexpression of COX-2[38]. In terms of the anti-inflammatory activity of the flower and leaf of *Hibiscus rosa-sinensis* L. (red) and *Hibiscus rosa-sinensis* var *alba* (white), the extracts ability to inhibit COX-2 is postulated. The anti-inflammatory effect at the dose of 100 mg/kg of each extract except 100 mg/kg of flower of *H. rosa-sinensis* var *alba* (white) in the carrageenan-induced paw edema test was found to be comparable to that of standard drug, Diclofenac. It is a well established fact that Non-Steroidal Antiiflammatory Drugs (NSAIDs) exert their anti-inflammatory activity by the inhibition of cyclooxygenase (COX) activity[39]. As mentioned in the study done by Masferrer *et al.*[40], NSAIDs that inhibit COX-2 may suppress the inflammation of the rat air pouch.

Various concentration (5, 50 and 100 mg/kg) of ethanol extracts of flower and leaf of *Hibiscus rosa-sinensis* L. (red) and *Hibiscus rosa-sinensis* var *alba* (white) showed significant reduction ($p<0.05$) on polymorphonuclear leucocyte (PNL) infiltration induced by carrageenan (Table 4). Based on these results, it can be concluded that all the extracts able to reverse acute inflammation through inhibition of PNL infiltration induced by carrageenan. In this study, supplementation of *Hibiscus rosa-sinensis* var *alba* (white) showed no significant ($p>0.05$) anti-inflammatory effect compared with *Hibiscus rosa-sinensis* L. (red). Supplementation of flower of *Hibiscus rosa-sinensis* L. and *Hibiscus rosa-sinensis* var *alba* (white) also showed no significant ($p>0.05$) anti-inflammatory effect compared with leaf of *Hibiscus rosa-sinensis* L. and *Hibiscus rosa-sinensis* var *alba* (white). Results indicated significant ($p<0.05$) anti-inflammatory effect with doses (5, 50 or 100 mg/kg) of *Hibiscus rosa-sinensis* var *alba* (white) compared with doses (5, 50 or 100 mg/kg) of *Hibiscus rosa-sinensis* L. PNL infiltration was observed through histopathological examination. There were evidences of

clear reduction of PNL localization in the stained neutrophil cells in slices of paw tissue of rats treated with 5, 50 and 100 mg/kg of ethanol extracts of flower and leaf of *Hibiscus rosa-sinensis* L. (red) and *Hibiscus rosa-sinensis* var *alba* (white) (Figure 3). It can be concluded that the carrageenan-induced paw edema study corroborates with PNL infiltration induced by carrageenan and histopathological; flower and leaf of *Hibiscus rosa-sinensis* L. (red) and *Hibiscus rosa-sinensis* var *alba* (white) act as anti-inflammatory agents in inhibition of paw edema concurrently inhibited PNL infiltration. Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) has been shown to trigger neutrophil migration[41-43]. Thus, inhibition of COX-2 is an important factor involved in neutrophils, indicating the importance of the COX-2 in leukocyte chemotaxis control under inflammatory conditions[44]. Flower and leaf of *Hibiscus rosa-sinensis* L. (red) and *Hibiscus rosa-sinensis* var *alba* (white) may involve in inhibiting overexpression of COX-2 in carrageenan-induced edema thus lead to the inhibition of neutrophils chemotaxis via inhibition of $PGF_{2\alpha}$. The anti-inflammatory effect 100 mg/kg of each extract in the PNL infiltration induced by carrageenan found to be comparable to that of standard drug, Diclofenac. As mentioned by Locatelli *et al.*[45], nonselective COX inhibitors by Diclofenac basically affected the reducing chemotaxis.

At 100 mg/kg of flower and leaf of *Hibiscus rosa-sinensis* L. (red) and *Hibiscus rosa-sinensis* var *alba* (white) extracts caused significant effects on the inhibition of licking response induced by formalin (Table 5). Based on the results, it can be concluded that all the extracts able to reverse acute inflammation through inhibition of licking response. *Hibiscus rosa-sinensis* var *alba* (white) showed significant ($p<0.05$) anti-inflammatory effect compared with *Hibiscus rosa-sinensis* L. (red). However, flower of *Hibiscus rosa-sinensis* L. and *Hibiscus rosa-sinensis* var *alba* (white) showed no significant ($p>0.05$) anti-inflammatory effect compared with leaf of *Hibiscus rosa-sinensis* L. and *Hibiscus rosa-sinensis* var *alba* (white). No significant ($p>0.05$) anti-inflammatory effect of flower and leaf of *Hibiscus rosa-sinensis* L. and *Hibiscus rosa-sinensis* var *alba* (white) indicated in phase 1 compared with phase 2. Interestingly, ethanol extracts of flower of *Hibiscus rosa-sinensis* L. (red) has also been reported to exhibit anti-inflammatory activity when assessed using the formalin- induced paw licking test[10]. This study is consistent to the present study as to determine and compare the anti-inflammatory properties of *Hibiscus rosa-sinensis* var *alba* with *Hibiscus rosa-sinensis* L. flower and leaf ethanol extract. In the formalin test, there is a distinctive biphasic nociceptive response termed early and late phase. Substance P and bradykinin (BK) are involved in the early phase of the formalin test (neurogenic) while the late phase (inflammatory) is due to inflammation with a release of serotonin, histamine, BK, and prostaglandins (PGs)[46]. In terms of the anti-inflammatory activity, the extracts ability to inhibit all the mediators involved in both phases is postulated. The predominant effect of the extracts in the late phase indicated its anti-inflammatory activity was an important one. It can be concluded that the formalin-induced paw licking study corroborates with carrageenan-induced paw edema study; flower and leaf of *Hibiscus rosa-sinensis* L. (red) and *Hibiscus rosa-sinensis* var *alba* (white) act as anti-inflammatory agents in inhibition of paw edema thus inhibited paw licking response.

These results suggest that the ethanol extracts of flower and leaf of *Hibiscus rosa-sinensis* L. (red) and *Hibiscus rosa-sinensis* var *alba* (white) possesses anti-inflammatory activities which may involve inhibition of prostaglandin. However, *Hibiscus rosa-sinensis* var *alba* (white) revealed a more potent inhibitor than *Hibiscus rosa-sinensis* L. (red). Future study of combination therapy maybe of benefit[47]. Further studies extending these findings could lead to improvement in the currently available approaches for the treatment of inflammation.

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