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ANTI-INFLAMMATORY POTENTIALS OF ETHANOL ROOT EXTRACT OF *Salacia lehmbachii* IN WISTAR RATS

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ABSTRACT

It is well known that many diseases are accompanied by inflammation and pain. Inflammation and pain results from cell injury. Inflammation is critical for the development of many complex diseases and disorders including autoimmune diseases, metabolic syndrome, neuro-degenerative diseases, cancers and cardiovascular diseases. *Salacia lehmbachii* is commonly found in the tropical forest of Cameroon and South Eastern Nigeria, it has been reported to possess Analgesic and anti-inflammatory potentials and anti-abortificient activities in rat model. In this study we compared the anti-inflammatory effects of Ethanol root extract of *Salacia lehmbachii* (ERESL), prednisolone and acetysalicyclic acid on carrageenan-induced air pouch inflammation in Wistar rats; the study focused on oxidative stress and inflammatory biomarkers in rat model since there is great similarity and homology between the genomes of humans and rodents. The anti-inflammatory activity of ERESL in Wistar rats at graded doses (200mg/kg and 400mg/kg) were compared with two standard drugs (aspirin 0.93mg/kg and prednisolone 5mg/kg) using Carrageenan-induced air pouch inflammation. The effects of ERESL and standard drugs on inflammatory biomarkers and oxidative stress markers were compared, the results were analyzed using a one-way ANOVA followed by a Bonferroni post hoc test. Pretreatment with ERESL (200 and 400 mg/kg, p.o.) and PRE (5 mg/kg, p.o.) significantly ($p < 0.05$) prevented carrageenan-induced GSH alteration when compared with carrageenan (control group). But pretreatment with ASP (100 mg/kg, p.o.) failed to prevent carrageenan-induced alteration on GSH concentration in rats. Post-hoc analysis by bonferroni test revealed that carrageenan injection into the 6th day old pouches significantly ($p < 0.05$) increase the MDA concentration when compared with vehicle group. On the other hand, pretreatment with ERSL (200 and 400 mg/kg, p.o.), PRE (5 mg/kg, p.o.) and ASP (100 mg/kg, p.o.) significantly ($p < 0.05$) inhibited carrageenan-induced increased MDA concentration when compared with carrageenan (control group), Histopathological studies revealed that administration of carrageenan to air pouches produced tissue damage characterized by prominent congested blood flow, inflammatory cellular infiltrates occupying the lower two-third of the deeper dermis tissue and cellular enlargement of pouch wall, suggesting tissue damage. However, pretreatment with ASP (100 mg/kg, p.o.), PRE (5 mg/kg, p.o.) and ERESL (200 and 400 mg/kg, p.o.) showed tissues with intact epidermis consisting of stratified squamous epithelium displaying reduced odema and inflammatory tissue infiltration. In conclusion, the results from the study provides evidence, which suggest that ethanol root extract of *Salacia lemhbachii* attenuated air pouch inflammatory reactions induced by carrageenan via mechanisms related to anti-oxidant, inhibition of cellular migration and tissue protection

INTRODUCTION

Inflammation is a protective response involving host cells, blood vessels, proteins and other mediators that is intended to eliminate the initial cause of cell injury, as well as the necrotic cells and tissues resulting from the original insult, and to initiate the process of repair (1).

It is well known that many diseases are accompanied by inflammation and pain. Inflammation results from cell injury. The components of the inflammatory reaction that destroy and eliminate microbes and dead tissues are also capable of injuring normal tissues. Inflammation is critical for the development of many complex diseases and disorders including autoimmune diseases, metabolic syndrome,

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neuro-degenerative diseases, cancers and cardiovascular diseases. If inflammation does not occur, infections would not be restrained, wounds would never heal and injured tissues would remain permanent festering (suppurative/ pus forming) sores.

The inflammatory reaction can be caused by a variety of stimuli ranging from physical injury, mechanical trauma, and infections by pathogens, and immune reactions due to hypersensitivity, chemical irritants and toxins etc.

Inflammation is characterized by five cardinal signs, namely redness (*rubor*), swelling (*tumor*), heat (*calor*; only applicable to the body' extremities), pain (*dolor*) and loss of function (*functio laesa*) (2, 3). Inflammation is divided into acute inflammation, which occurs over seconds, minutes, hours, and days, and chronic inflammation, which occurs over longer times (4, Chilton et al, 2012). Acute inflammation is a beneficial host defense mechanism protecting the body from infection and other insults, and our health entirely relies on its proper functioning. The main goals of Inflammation are to get rid of the initial cause of the cell injury (microorganism, toxin etc), and also to eliminate the consequences of the cell injury as well.

Many of the drugs used in the treatment of inflammatory conditions, are based on our understanding of the biochemical processes involved in the inflammatory reactions. Basically, the standard treatments for inflammatory conditions have been the use of a non-steroidal anti-inflammatory drug (NSAID), such as aspirin, for pain relief and also the use of glucocorticoids like cortisol and prednisolone a synthetic derivative of cortisol that reliefs other symptoms of inflammation. Glucocorticoids suppress the induced expression of COX-2, and thus COX-2–mediated PG production. They also inhibit the action of PLA_2, which releases arachidonic acid from the cell membrane. These effects contribute to the anti-Inflammatory actions of glucocorticoids. The NSAIDS exert their anti-inflammatory action by the inhibition of cyclooxygenase enzymes which are responsible for the conversion of arachidonic acid to prostaglandins (5).

Aspirin is one of the most widely used medications globally. Aspirin, also known as acetylsalicylic acid (ASA), is a medication used to treat pain, fever, and inflammation. Aspirin is used as an anti-inflammatory agent for both acute and long-term inflammation (6), as well as for treatment of inflammatory diseases, such as rheumatoid arthritis. Aspirin works similar to other NSAIDs but also irreversibly inhibits the normal functioning of platelets. Common side effects include an upset stomach. More significant side effects include stomach ulcers, stomach bleeding, and worsening asthma. Bleeding risk is greater among those who are older, drink alcohol, take other nonsteroidal anti-inflammatory drugs (NSAIDs), or are on blood thinners. Aspirin is not recommended in the last part of pregnancy. It is not generally recommended in children with infections because of the risk of Reye's syndrome. High doses may result in ringing in the ears.

Prednisolone is a synthetic glucocorticoid, a derivative of cortisol, used to treat a variety of inflammatory and autoimmune conditions and some cancers. It is the active metabolite of the drug prednisone and is used especially in patients with liver failure, as these individuals are unable to metabolize prednisone into active prednisolone; it is primarily metabolized via the liver enzyme, 11-β-hydroxydehydrogenase. Adverse effects are not generally seen with short term therapy, but weight gain, impaired immune response, and behavioral disturbances commonly occur with longer durations of treatment (7).

The use of plants to treat ailments is as old as antiquity. Records of humans using plants to treat diseases have been recorded as far back as 4000 to 6000 years ago when Ayurvedic physicians started treating tumors with extracts from *Vinca rosea* (8). *Salacia lehmbachii*, locally known by the Efik people of South South Nigeria as 'Ebananganang', is a shrub-like small tree of about three meters high belonging to the family Celastraceae. The leaves are simple, ovate oblong, opposite, acuminate and shining. The flowers are crystal yellow or orange borne on woody auxiliary tubercles. The fruits are globose, orange and contain one large seed at the centre and two-four seeds immersed almost at the periphery of the pulp (9). *Salacia lehmbachii* is commonly found in the tropical forest of Cameroon and South Eastern Nigeria, it has been reported to possess analgesic and anti-inflammatory potentials and anti-abortificient activities in rat model (10). This study focuses on the anti-inflammatory effects of Salacia lehmbachii, prednisolone and acetysalicyclic acid on oxidative stress and inflammatory biomarkers in Wistar rat model since there is great similarity and homology between the genomes of humans and rodents (11).

MATERIALS AND METHODS

Equipment

Soxhlet extractor apparatus (Friedrich Polzime, England), Whatman Filter Paper (No. 1, England), Electric Oven (Grieve Co-op, Ltd, IL USA), Neubauer chamber, Light microscope (Nikon Eclipse E200, USA), Centrifuge machine, Spectrophotometer, Weighing Balance (Electronic, Japan), Hand Grinding Mill (Corona, China), Polypropylene Cages, Beakers, Glass funnel, Animal feeders/drinkers, Hand gloves, Animal clippers, Dissecting kit, Dissection board and Forceps, Syringes and Needles

Drugs and chemicals

Carragenaan (Sigma-Aldrich, Germany), Ethanol, Ketamine, Ether, Aspirin tablets (Sigma-Aldrich, Germany), Prednisolone tablets, Normal saline, Turk’s solution, Leishman stain, Griess Reagent, Plant Extract.

Laboratory Animals

Thirty (30) albino rats of both sexes weighing between 150-250g were obtained from the animal house of the Department of Pharmacology, University of Calabar.

The animals were housed in well-ventilated cages under room temperature (28-30℃) in the animal house and feed with rat pellets and tap water *ad libitum* throughout the experimental period. The animals were left for three weeks to acclimatize prior to the experimental procedure. The 'Principles of laboratory animal care' (NIH Publication # 85-23, 1985) was followed in the study.

Collection and Identification of Plant Material

The roots of *S. lehmbachii* were purchased from Watt market, a local market in Calabar, capital of Cross River State, Nigeria. The plant was identified and authenticated by a taxonomist, Mr. Frank Apojoye of the Department of Botany of the University of Calabar where a Voucher Specimen with herbarium number 989 was deposited for reference.

Preparation of the Extract

The roots of *Salacia lehmbachii* were washed with water to remove dirt and were sliced and air dried at room temperature for 14days in a clean and dry laboratory environment. The roots were pulverized into a coarse powder using a mechanical grain mill (Corona, China) and the dry coarse root powder was weighed and stored in an airtight container. The dry coarse root powder (500 g) was defatted with 99.9% petroleum ether (Sigma Chemical Limited, USA) using a Soxhlet extractor (Friedrich Polzime, England) at 65°C for twelve hours. The petroleum ether residue was dried, weighed and re-extracted with 2 litres of 99.9 % ethanol at 60°C for 72 hours to obtain a solution of ethanol root extract of *Salacia lehmbachii,* which was then evaporated to dryness in a hot oven (Grieve Lab. Oven, Model LR 271C, Grieve Co-op. Ltd, IL, USA) at 40°C. The dried extract was weighed and was properly stored in specimen bottle and was preserved in a refrigerator at 4°C until required for administrations on the experimental animals during the research experiments.

Phytochemical screening

ARESL was qualitatively screened for the presence of phytoconstituents such as alkaloids, glycosides, flavonoids, tannins, saponins, polyphenols and glycosides following standard tests procedures (12, Trease and Evans. 2003).

Acute toxicity study

The acute toxicity study was carried out in adult female albino rats by modified 'Up and Down' procedure (13, OECD, 2006).

Carrageenan-induced air pouch inflammation

Animals were anesthetized with ketamine (100 mg/kg, i.p), and 20 mL of sterile air was injected subcutaneously on the shaved back. Four days later, the pouch was re-inflated with another 10 mL of sterile air (Martin et al., 1994). Rats were divided into 6 groups with 5 rats per group (n=5). Group 1 which served as control received normal saline (10mL/kg), group 2 which served as negative control also received 10mL/kg normal saline, group 3 received aspirin (100 mg/kg), group 4 received prednisolone (5mg/kg), group 5 received ERESL (200 mg/kg) and group 6 received ERESL (400 mg/kg). Animals were pre-treated orally from days 4-6 before the induction of inflammation. On the 6th day following the first air injection, and 30 min after the last treatment, rats in the normal control group were injected with 2 mL sterile normal saline while rats in groups 2–6 were injected with 2 mL carrageenan (1% in sterile saline) into the pouch. Twenty-four hours after saline or carrageenan injection, rats were anesthetized with deep ether anesthesia and the pouch carefully opened by a small incision. The pouch cavity was flushed with 2 mL phosphate buffered saline (0.01M, pH 7.4), and the exudates was collected and transferred into a sterile tube and the volume of the exudates was measured. Total leukocytes in exudates were determined after staining with Turk solution and number of leukocytes counted in a Neubauer chamber under a light microscope (Nikon Eclipse E200, USA). A differential cell count was performed in cytospin slide smears stained with Leishman Stain.

Exudates were centrifuged (10,000 rpm for 10 min at 48C) to obtain cell free supernatant which was used for evaluation of antioxidant and nitrosative markers. The Nitric Oxide in pouch exudates was measured using spectrophotometric methods with Griess reagent. Nonenzymic antioxidant marker, reduced gluthathione (GSH) levels in pouch exudates was determined as previously described (Sin et al., 1997). The index of lipid peroxidation was measured as the thiobarbituric reacting substance (TBARS) via the method described by (14, Nagababu et al. (2010)) and total protein content by the Biuret method.

Statistical Analysis

The result was reported as mean± standard error of mean (SEM). Data was analyzed using one-way ANOVA and a post hoc test (Bonferroni test) for evaluation of significance between mean group using SPSS Statistical package (version 22). P-values less than 0.05 (i.e $p < 0.05$) was considered statistically significant for all tests.

RESULTS

Percentage (%) Yield of Ethanol Root Extract of *Salacia lehmbachii*.

500 grams of the dried and ground root extract of *Salacia lehmbachii* was dissolved in two (2) litres of ethanol. After the extraction the dried crude extract was weighed and 125 grams of the ethanolic extract was obtained. The percentage (%) yield of the ethanolic leaf extract of *Salacia lehmbachii* was 25 %.

Quantitative Analysis of the Phytochemical Compositions of *Salacia lehmbachii*.

As reported by (10, Takem et al., 2013), Preliminary phytochemical screening of Aqueous Root Extract of *Salacia lehmbachii* revealed the presence of terpenoids, alkaloids, glycosides, flavonoids, tannins, anthraquinones, steroids and saponins in varying concentrations; with alkaloids, saponins and tannins being highly present (15, Essien et al., 2015).

Acute toxicity study

Acute toxicity study of the extract at up to a dose of 4000mg/kg revealed no observed toxic effect (16, Takem et al., 2014).

Effect of Ethanol root extract of *Salacia lemhbachi* on air-pouch carrageenan-induced alteration in glutathione concentration in rats

The effect of ERESL on air pouch carrageenan-induced GSH alteration in rats is shown in Figure. 1. One-way ANOVA revealed that there were significant differences between treatment groups [*F* (5, 24) = 5.653, p = 0.0014]. Bonferroni post-hoc analysis showed that carrageenan injection into the 6th day old pouches produced significant (p < 0.05) decrease in GSH concentration relative to vehicle group. However, pretreatment with ERESL (200 and 400 mg/kg, p.o.) and PRE (5 mg/kg, p.o.) significantly (p < 0.05) prevented carrageenan-induced GSH alteration when compared with carrageenan (control group). But pretreatment with ASP (100 mg/kg, p.o.) failed to prevent carrageenan-induced alteration on GSH concentration in rats (Figure 1).

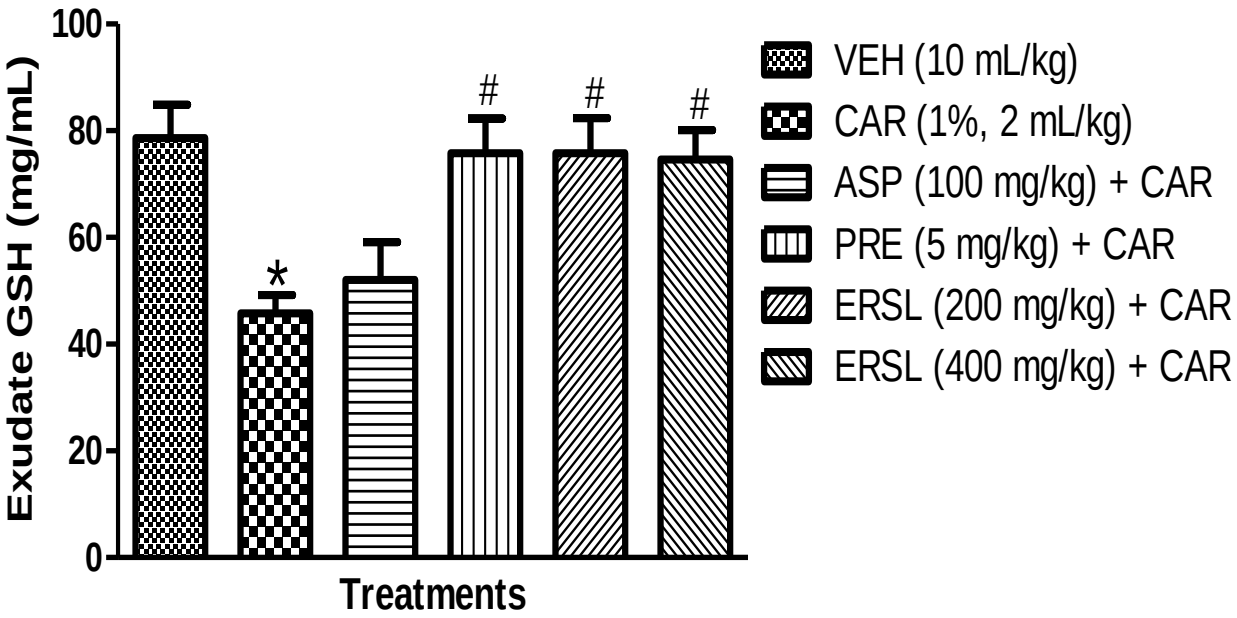

FIGURE 1: Effect of Ethanol root extract of Salacia *lehmbachii* on air-pouch carrageenan-induced glutathione alteration in rats

Bar represents the mean ± SEM of 5 animals per group. *P < 0.05 compared to vehicle group and #P < 0.05 compared to carrageenan group

VEH= Vehicle, **ERESL** = Ethanol root extract of *Salacia lemhbachi*, **ASP**= Aspirin, **PRE** = Prednisolone

Effect of Ethanol root extract of *Salacia lemhbachi* on air-pouch carrageenan-induced alteraction in malondialdehyde concentration in rats

The effect of ERESL on air pouch carrageenan-induced alteration in MDA concentration is shown in Figure. 2. One-way ANOVA revealed that there were significant differences between treatment groups [*F* (5, 24) = 10.35, p < 0.0001]. Post-hoc analysis by bonferroni test revealed that carrageenan injection into the 6th day old pouches significantly (p < 0.05) increase the MDA concentration when compared with vehicle group. On the other hand, pretreatment with ERSL (200 and 400 mg/kg, p.o.), PRE (5 mg/kg, p.o.) and ASP (100 mg/kg, p.o.) significantly (p < 0.05) inhibited carrageenan-induced increased MDA concentration when compared with carrageenan (control group) (Figure 2).

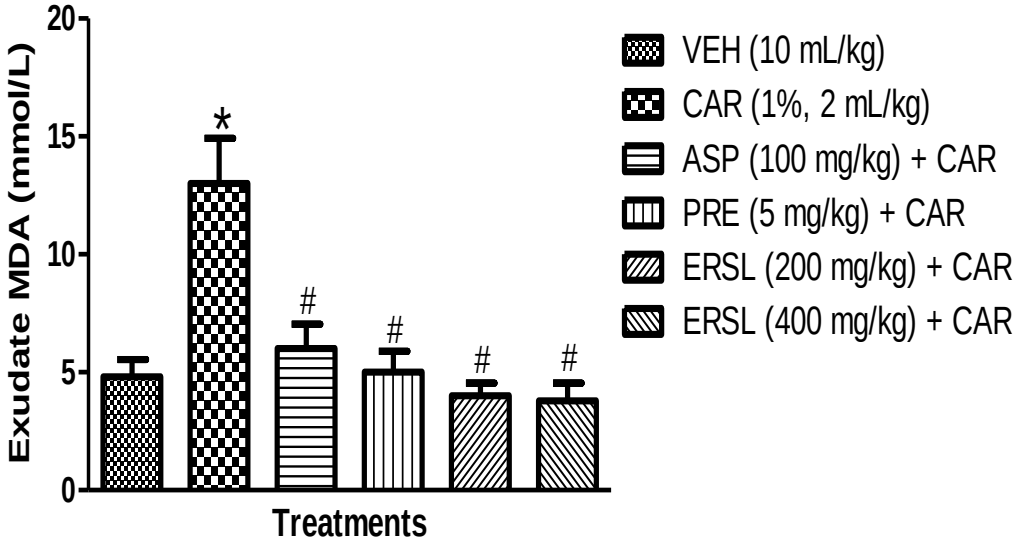

FIGURE 2: Effect of Ethanol extract of *Salacia lemhbachi* on air-pouch carrageenan-induced malondialdehyde concentration in rats.

Bar represents the mean ± SEM of 5 animals per group. *P < 0.05 compared to vehicle group and #P < 0.05 compared to carrageenan group

VEH= Vehicle, **ERSL** = Ethanol root extract of *Salacia lemhbachi*, **ASP**= Aspirin, **PRE** = Prednisolone

Effect of Ethanol extract of *Salacia lemhbachi* on air-pouch carrageenan-induced alteraction in nitrite concentration in rats.

Figure 3. Shows the effect of ERSL on air pouch carrageenan-induced alteration in nitrite concentration in rats. The result showed that carrageenan injection into the 6th day old pouches produced significant ($p < 0.05$) increase in nitrite concentration in comparison with vehicle group. However, ERSL (200 and 400 mg/kg, p.o.) significantly ($p < 0.05$) [F (5, 24) = 10.35, $p < 0.0001$] attenuated carrageenan-induced nitrite levels when compared with carrageenan. Similarly, pretreatment of rats with the standard drugs, ASP (100 mg/kg, p.o.) and PRE (100 mg/kg, p.o.) also prevented the effect of carrageenan on nitrite concentration when compared with carrageenan group alone (Figure 3.)

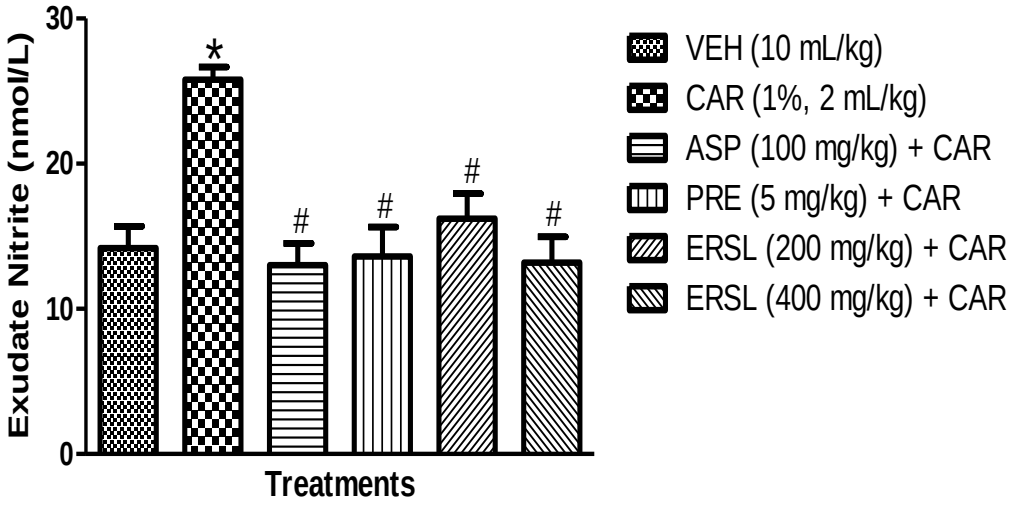

FIGURE 3: Effect of Ethanol extract of *Salacia lemhbachi* on air-pouch carrageenan-induced nitrite concentration in rats.

Bar represents the mean ± SEM of 5 animals per group. *P < 0.05 compared to vehicle group and #P < 0.05 compared to carrageenan group
VEH= Vehicle, **ERSL** = Ethanol root extract of *Salacia lemhbachi*, **ASP**= Aspirin, **PRE** = Prednisolone

Effect of Ethanol extract of *Salacia lemhbachi* on serum albumin, globulin and total protein to air-pouch carrageenan exposed rats

The effects of ERESL on air pouch carrageenan-induced serum albumin, globulin and total protein alteration in rats is shown in Figures 4, 5 and 6. Moreover, results revealed that there were significant differences between treatment groups: serum albumin [F (5, 24) = 7.142, p = 0.0003] Figures 4, globulin [F (5, 24) = 11.26, p < 0.0001] Figures 5, and total protein content [F (5, 24) = 14.31, p < 0.0001] Figures 6. Results showed that carrageenan injection into the 6th day old pouches significantly (p < 0.05) increased albumin and globulin concentrations as well as total protein contents in comparison with vehicle group. Meanwhile, pretreatment with ERESL (200 and 400 mg/kg, p.o.) and PRE (5 mg/kg, p.o.), but not ASP (100 mg/kg, p.o.) significantly (p < 0.05) reduced the increased globulin (Figure 5) and total protein content (Figure 6) in carrageenan-treated rats when compared with carrageenan group. Furthermore, pretreatment of rats with ERESL (200 and 400 mg/kg, p.o.) also prevented the effect of carrageenan on albumin concentration when compared with carrageenan group. However, PRE (5 mg/kg, p.o.) and ASP (100 mg/kg, p.o.) treatment did not reverse the effect on serum albumin caused by carrageenan treatment (Figure 4).

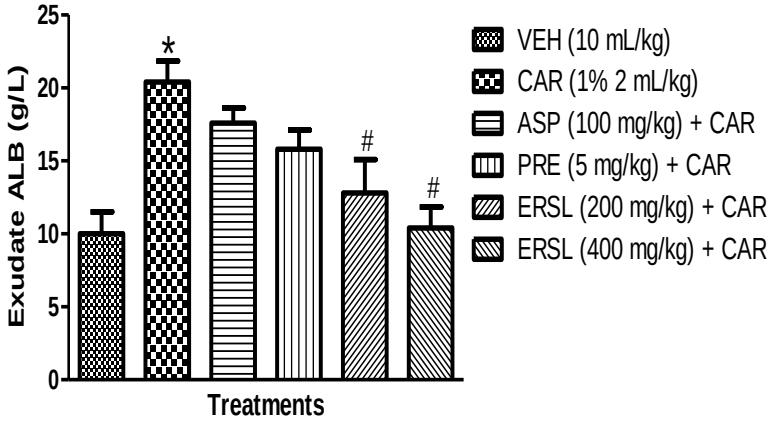

FIGURE 4: Effect of Ethanol extract of *Salacia lemhbachi* on serum albumin to air-pouch carrageenan exposed rats

Bar represents the mean ± SEM of 5 animals per group. *P < 0.05 compared to vehicle group and #P < 0.05 compared to carrageenan group
VEH= Vehicle, **ERESL** = Ethanol root extract of *Salacia lemhbachi*, **ASP**= Aspirin, **PRE** = Prednisolone

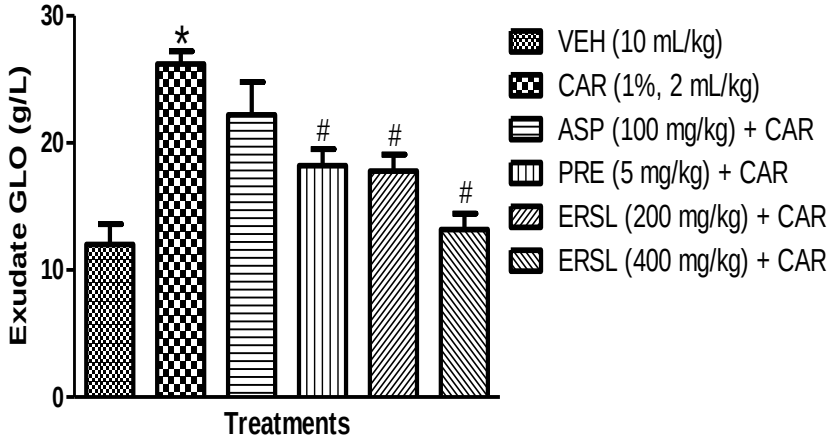

FIGURE 5: Effect of Ethanol extract of *salacia lemhbachi* on serum globulin to air-pouch carrageenan exposed rats

Bar represents the mean ± SEM of 5 animals per group. *P < 0.05 compared to vehicle group and #P < 0.05 compared to carrageenan group
VEH= Vehicle, **ERESL** = Ethanol root extract of *salacia lemhbachi*, **ASP**= Aspirin, **PRE** = Prednisolone

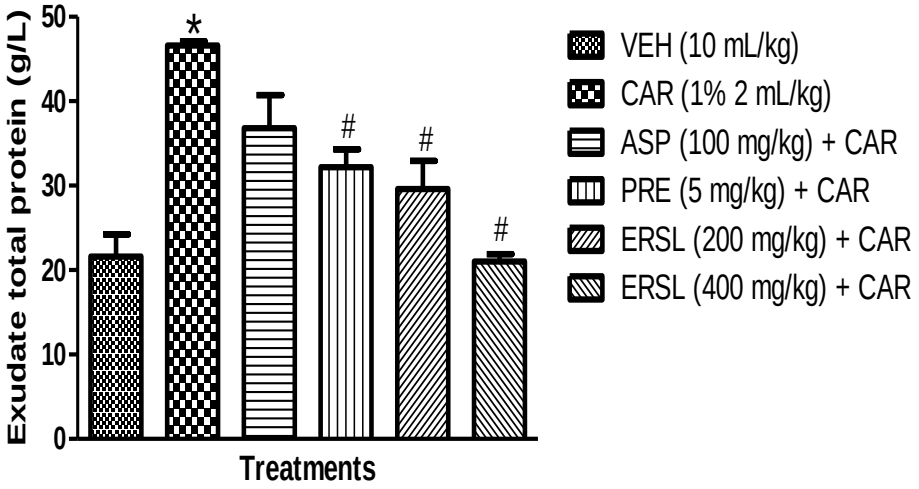

FIGURE 6: Effect of Ethanol extract of *Salacia lemhbachi* on total protein content to air-pouch carrageenan exposed rats

Bar represents the mean ± SEM of 5 animals per group. *P < 0.05 compared to vehicle group and #P < 0.05 compared to carrageenan group
VEH= Vehicle, **ERESL** = Ethanol root extract of *Salacia lemhbachi*, **ASP**= Aspirin, **PRE** = Prednisolone

Effect of Ethanol root extract of *Salacia lemhbachi* on air-pouch carrageenan-induced Alteration in exudate formation and cellular migration in rats.
Figures 7, 8, 9 and 10 show the effect of ERESL on air pouch carrageenan-induced exudate formation and cellular migration. Results showed that carrageenan injection into the 6th day old pouches produced significant ($p < 0.05$) increased exudate formation [F (5, 24) = 40.21, $p < 0.0001$] (Figure 8), and cellular migration, as it enhanced migration of neutrophil [F (5, 24) = 21.72, $p < 0.0001$] (Figure 8), lymphocyte [F (5, 24) = 13.10, $p < 0.0001$] (Figure 9) and total leukocytes [F (5, 24) = 35.34, $p < 0.0001$] (Figure 10) in comparison with vehicle groups. However, ERSL (200 and 400 mg/kg, p.o.), ASP (0.93mg/kg, p.o.) and PRE (100 mg/kg, p.o.) significantly ($p < 0.05$) [F (5, 24) = 10.35, $p < 0.0001$] decreased carrageenan-induced exudate formation as well as inhibited neutrophil, lymphocyte and total leukocytes migrations in the air-pouches when compared with carrageenan-treated groups.

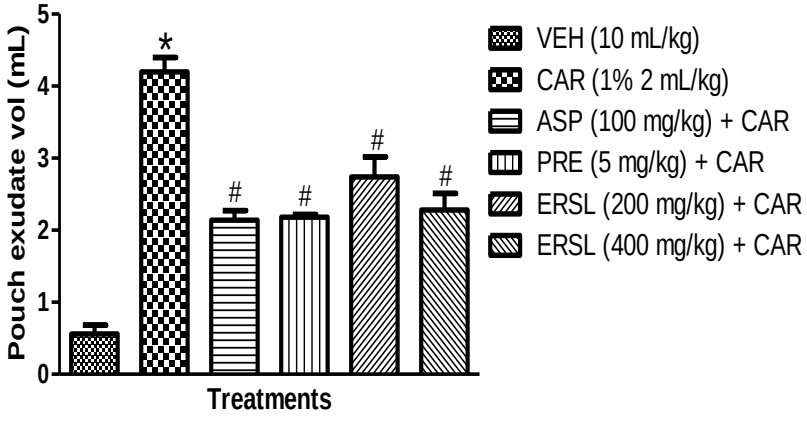

FIGURE 7: Effect of Ethanol extract of *Salacia lemhbachi* on air-pouch carrageenan-induced exudate formation in rats.

Bar represents the mean ± SEM of 5 animals per group. *P < 0.05 compared to vehicle group and #P < 0.05 compared to carrageenan group
VEH= Vehicle, **ERSL** = Ethanol root extract of *Salacia lemhbachi*, **ASP**= Aspirin, **PRE** = Prednisolone

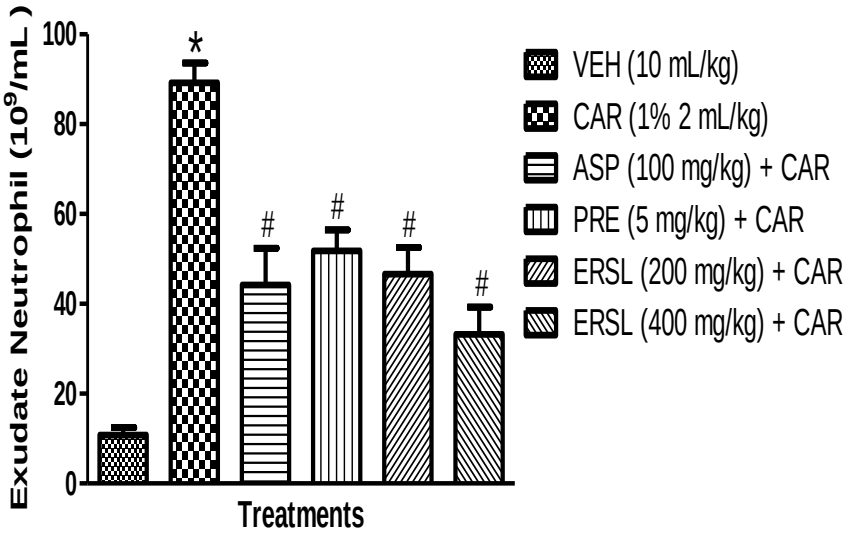

FIGURE 8: Effect of Ethanol extract of *Salacia lemhbachi* on air-pouch carrageenan-induced neutrophil migration in rats.
Bar represents the mean ± SEM of 5 animals per group. *P < 0.05 compared to vehicle group and #P < 0.05 compared to carrageenan group
VEH= Vehicle, **ERSL** = Ethanol root extract of *Salacia lemhbachi*, **ASP**= Aspirin, **PRE** = Prednisolone

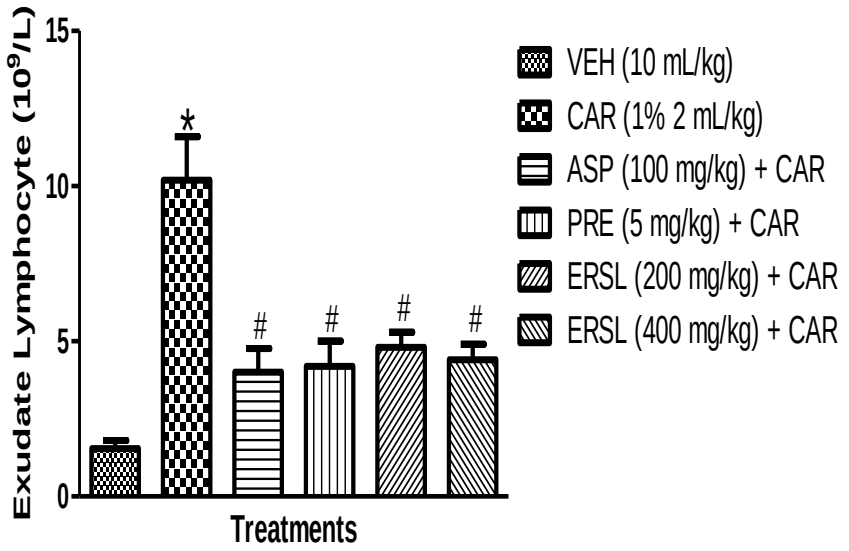

FIGURE 9: Effect of Ethanol extract of *salacia lemhbachi* on air-pouch carrageenan-induced lymphocyte migration in rats.

Bar represents the mean ± SEM of 5 animals per group. *P < 0.05 compared to vehicle group and #P < 0.05 compared to carrageenan group
VEH= Vehicle, **ERSL** = Ethanol root extract of *salacia lemhbachi*, **ASP**= Aspirin, **PRE** = Prednisolone

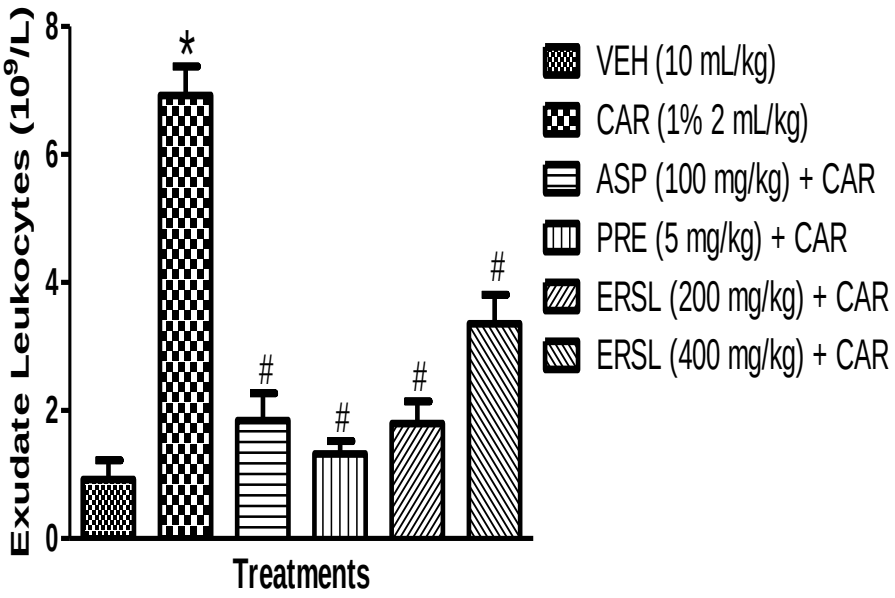

FIGURE 10: Effect of Ethanol extract of *salacia lemhbachi* on air-pouch carrageenan-induced leukocyte infiltration in rats.

Bar represents the mean ± SEM of 5 animals per group. *P < 0.05 compared to vehicle group and #P < 0.05 compared to carrageenan group
VEH= Vehicle, **ERSL** = Ethanol root extract of *salacia lemhbachi*, **ASP**= Aspirin, **PRE** = Prednisolone

Effect of Ethanol extract of *salacia lemhbachi* on carrageenan-induced histological changes in air pouch tissue lining

The photomicrographs of carrageenan-induced histological changes in air pouch tissue lining are shown in Plate 1. Histopathological studies revealed that administration of carrageenan to air pouches produced tissue damage characterized by prominent congested blood flow, inflammatory cellular infiltrates occupying the lower two-third of the deeper dermis tissue and cellular enlargement of pouch wall, suggesting tissue damage. However, pretreatment with ASP (100 mg/kg, p.o.), PRE (5 mg/kg, p.o.) and ERESL (200 and 400 mg/kg, p.o.) showed tissues with intact epidermis consisting of stratified squamous epithelium displaying reduced oedema and inflammatory tissue infiltration (Plate 1)

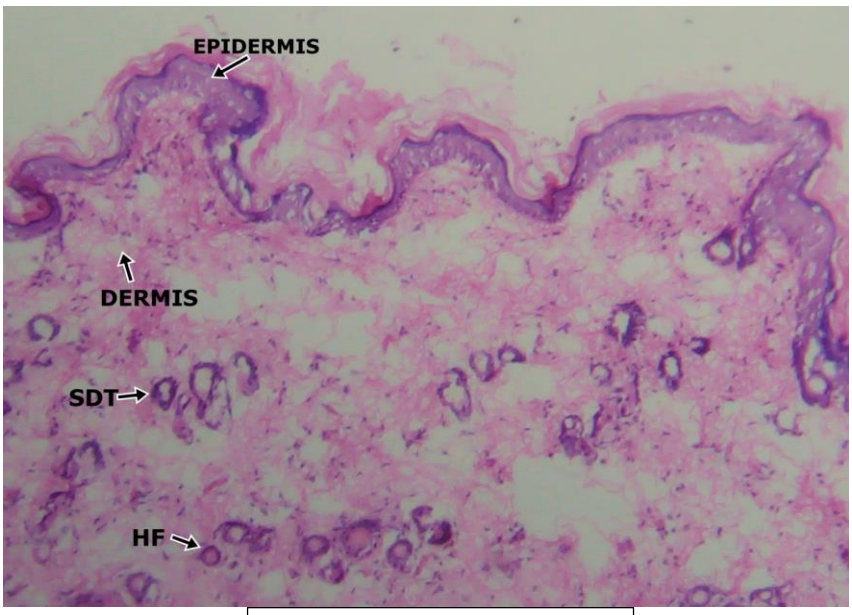

PLATE 1: VEH

Section of the skin shows the epidermis and dermis. There is mild hyperkeratosis and orderly differentiation of the epithelial cells with an intact basement. The dermis consists of prominent hair follicles, sweat ducts, sebaceous gland and ducts with a dense collagenous stroma without inflammatory cellular infiltration within the deeper dermis.

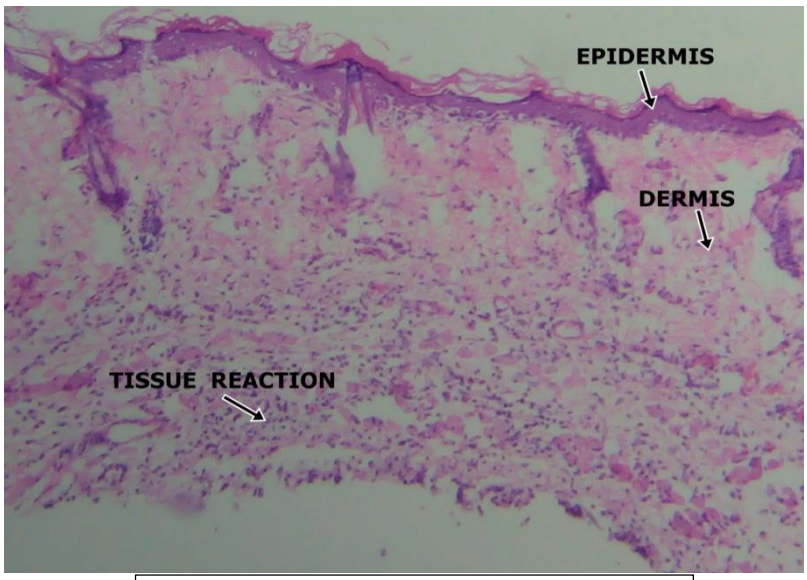

PLATE 2: CAR (1%, 2 mL/kg)

Section of the skin shows the epidermis and dermis. There is chronic hyperkeratosis. There is congested blood vessel and significant inflammatory cellular infiltrates occupying the lower two-third of the deeper dermis, suggesting tissue reactions and damages.

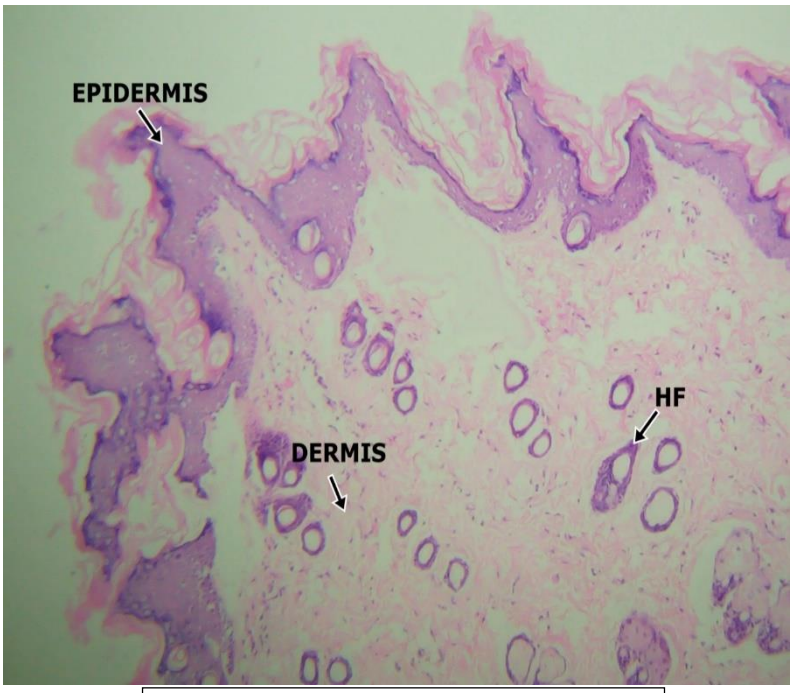

PLATE 3: ASP (100 mg/kg) + CAR

Section of the skin shows an intact epidermis consisting of stratified squamous epithelium displaying hyperkeratosis, hypergranulosis with an intact basement membrane. The underlying dermis is myxoid and oedematous containing sparse inflammatory cells, proliferating hair follicles and fibrocollagenous bundles. Finding shows mild tissue response to injury

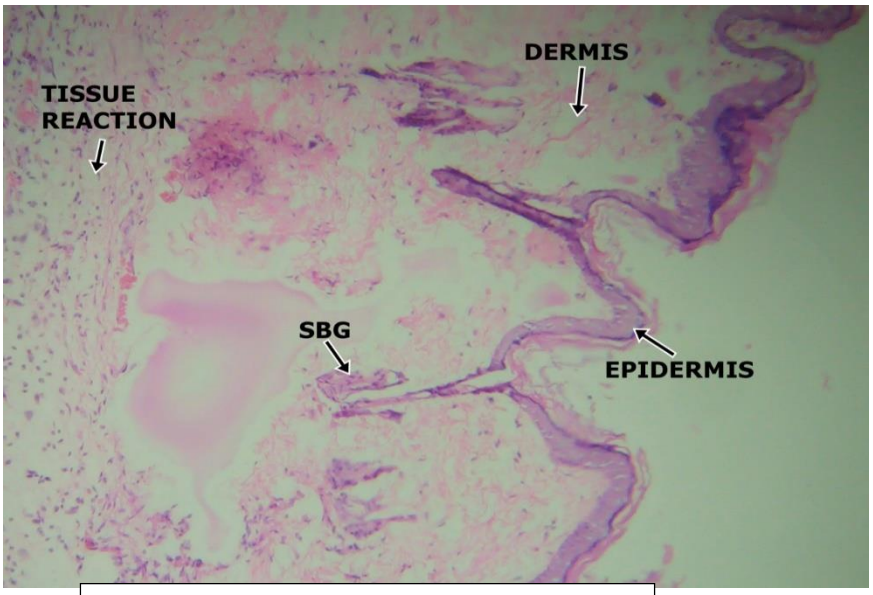

PLATE 4: PRE (5 mg/kg) + CAR

Section of the skin showing an intact epidermis consisting of straitified squamous epithelium with an intact basement layer overlying the dermis. The dermis consisting of proliferating hair follicles, sweat duct and sebaceous gland. There is moderate inflammatory tissue reaction comprising of mononuclear cells and a cellular amorphous areas surrounded by inflammatory cells. Finding suggestive of cellular tissue response and healing.

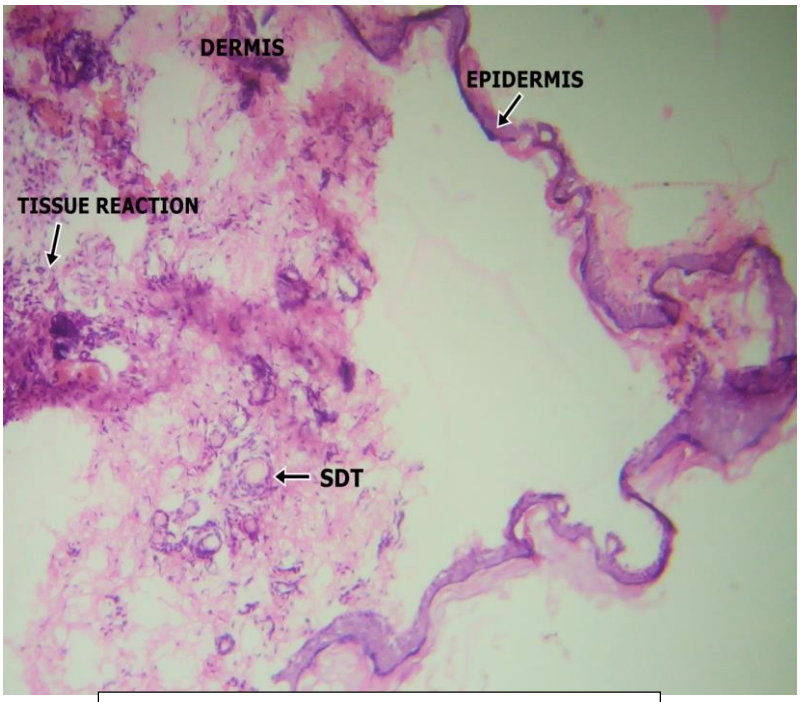

PLATE 5: ERESL (200 mg/kg) +

Section of the skin shows an intact epidermis consists of stratified squamous epithelium overlying the dermis. The dermis is loose and cellular consisting of hair follicles, sweat ducts and sebaceous glands. There are intra-dermal separation and mild tissue reaction consisting of mononuclear infiltrates.

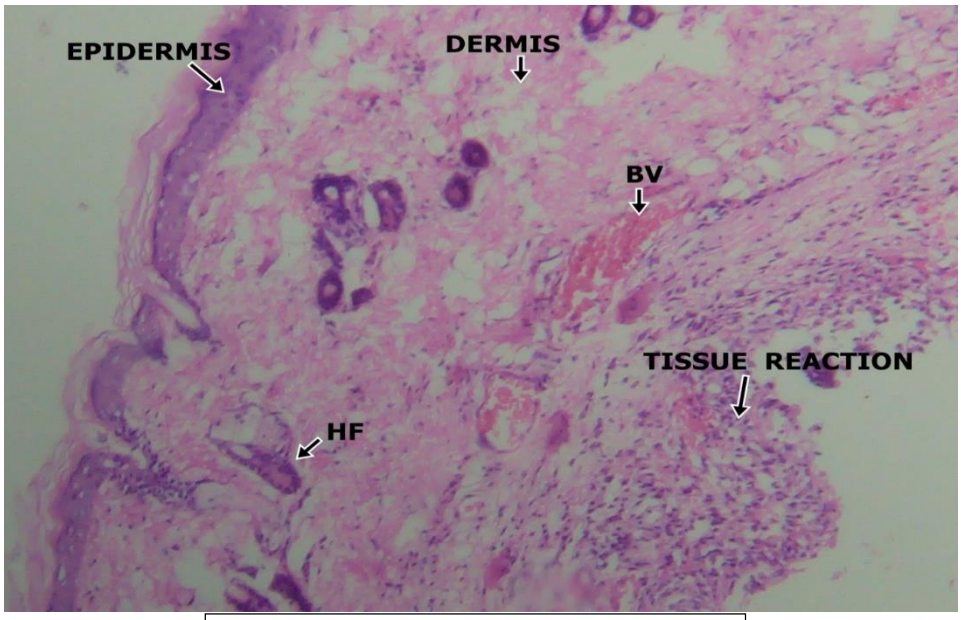

PLATE 6: ERESL (400 mg/kg) + CAR

Section of the skin shows an intact epidermis consists of stratified squamous epithelium overlying the dermis. The dermis is loose and cellular consisting of hair follicles, sweat ducts and sebaceous glands. There are intra-dermal separation and mild tissue reaction consisting of mononuclear infiltrates.

DISCUSSION

As reported by Takem *et al*., 2013, preliminary phytochemical screening of ethanol Root Extract *Salacia lehmbachii* revealed the presence of terpenoids, alkaloids, glycosides, flavonoids, tannins, anthraquinones, steroids and saponins in varying concentrations; with alkaloids, saponins and tannins being highly present. The anti-inflammatory and anti-oxidant effects of the root extract is as a result of the presence of saponins, tanins and flavonoids which are richly present in the extract (15).

Acute toxicity study of the extract at up to a dose of 4000mg/kg revealed no observed toxic effect (10).

The finding from this study revealed that ethanol root extract of *Salacia lemhbachi* (ERSL) at graded doses of 200mg/kg and 400mg/kg significantly inhibited exudate formation, as it markedly decreased the exudate volume in the air pouch. Also, ethanol root extract of *Salacia lemhbachi* attenuated carrageenan-induced air pouch oxidative stress as evidenced by increased glutathione level, and decreased malondialdehyde and nitrite concentrations in rat's pouches. The increased cellular migration as indicated by high exudate levels of neutrophil, lymphocyte and total leukocytes due to carrageenan-induced air pouch inflammation was significantly inhibited by ethanol root extract of *Salacia lemhbachi*. Furthermore, histopathological studies revealed that injection of carrageenan to air pouches produced tissue damage characterized by prominent congested blood flow, inflammatory cellular infiltrates occupying the lower two-third of the deeper dermis tissue and cellular enlargement of pouch wall, suggesting tissue damage. However, pretreatment with the ethanol root extract of *Salacia lemhbachi* similarly to aspirin and prednisolone, significantly protected the tissue lining, thereby showing tissues with intact epidermis consisting of stratified squamous epithelium displaying reduced Oedema and inflammatory tissue infiltration.

Carrageenan model of inflammation is a popular animal's model that has long been established as a phlogistic agent of inflammation, and thus it is routinely used for screening of compounds with suspected analgesic and anti-inflammatory properties (17, 18, 19). Previous studies have shown that injection of carrageenan into an air pouch produces fluid exudation and migration of inflammatory mediators to sites of inflammation (18, 20). While models such as carrageenan-induced paw edema in mice and rat are used in the screening of drugs with anti-inflammatory activity, carrageenan-induced air pouch model is popularly used as a more suitable animal model to study the mechanisms of action involved in the anti-inflammatory property of test compounds (18, 21, 20). Moreover, several studies have reported that carrageenan-induced inflammation in a 6-day old air pouch is strongly linked to increased vascular permeability, infiltration of polymorphonuclear leukocyte and bioactive mediators such as leukotrienes and prostaglandins, and expression of adhesion molecules related to edema formation at the site of inflammation (19, 22).

However, treatment with ethanol root extract of *Salacia lemhbachi* was found to significantly inhibit the edema formation induced by carrageenan in the air pouch in a similar manner to aspirin and prednisolone, confirming a significant anti-inflammatory action of the ethanol root extract of *Salacia lemhbachi* in experimental animal.

Different preclinical findings have also shown that the onset of inflammatory reaction is dependent on the levels of generation of reactive oxygen species (23, 22). Indeed, the role of oxidative stress have been consistently reported to play a prominent role in the pathogenesis of inflammatory diseases including rheumatoid arthritis, and thus, carrageenan-induced air pouch model of inflammation has been akin to an arthritic-like condition (17, 20). Air-pouches are usually composed of a lining of cells that consists primarily of macrophage-like and fibroblast-like cells, which is similar to the synovial cavity (17). Consequently, injection of a carrageenan solution into air pouch produces inflammatory reactions that are characterized by infiltration of cells, increased exudate volume and marked production of free radicals due to elevated intracellular concentrations of calcium; all of which destroy invading particles but also damage cells and tissues of the host (24, 25). Indeed, reactive oxygen and nitrogen species generation induced by intracellular calcium elevation is known to cause lipid peroxidation, which is generally regarded as the metabolic degradation of lipids (26, 25). Nitric oxide-mediated stress has been identified as a major contributor to inflammation and in the progression of multiple tissue damage, including rheumatoid arthritis and stroke (23, 27). Furthermore, high levels of generation of free radicals have also been linked to protein nitrosation (28). It is a well-known fact that proteins are important components of intracellular cell-cell communication (17). Previous studies have shown that proteins are carbonylated during inflammatory reaction and thus this result in further degradation of the microenvironment and tissue damage (25, 28). However, inhibition of free radical-mediated activity with compounds with anti-oxidant activity exerts beneficial effects in a variety of inflammation models (28). In this study, injection of carrageenan into the 6-day old air pouch was accompanied with increased oxidative and nitrergic stress as evidenced by decreased endogenous antioxidant activity (e.g., glutathione) as well as increased lipid peroxidation and nitrergic products (malondialdehyde and nitrite). This finding is in agreement with previous investigation showing that injection of carrageenan into air pouches causes depletion of antioxidant molecules and induction of lipid peroxidation (26, 25, 20). However, the finding that administration of the ethanol root extract of *Salacia lemhbachii* significantly suppressed carrageenan-induced air pouch oxidative and nitrergic stress, as indicated by increased air pouch glutathione concentration and decreased malondialdehyde and nitrite levels suggests that the extract might be useful in the management of inflammatory conditions associated with oxidative and nitrergic damages. Moreover, the finding from this study also showed that ethanol root extract of *Salacia lemhbachii* significantly decreased exudate total protein and globulin in a similar manner to aspirin and prednisolone.

Previous studies have confirmed that cellular migration during inflammation is a prominent feature of inflammatory process and infiltration of inflammatory mediators such as polymorphonuclear leukocyte and inflammatory mediators such as leukotrienes and prostaglandins are known to contribute to tissue damage (19, 23, 22). Increased activities of leukocytes accompanied by release of mediators of inflammatory reactions are known to be active in the pathway leading to chronic inflammatory disease (Yudoh et al., 2009). Of note, earlier investigation has been suggested that neutrophil activation in lung injury represents an important source of other inflammatory molecules, such as tumor necrosis factor-α and interleukins (29). Moreover, it has been reported that neutrophils and monocytes, which are involved in the overproduction of oxidant molecules are highly expressed in the bloodstream of patients with chronic inflammatory conditions (30). However, various studies have shown clearly that inhibition of cellular migration significantly prevents the development of the inflammatory process (31, 19, 32). The finding that ethanol root extract of *Salacia lemhbachii* inhibits the release of total neutrophil, lymphocytes and leukocytes in the air pouch exudate due to carrageenan injection further suggests that the extract may be eliciting its anti-inflammatory activity via mechanism related to suppression of cellular migration. This speculation is supported by the finding that the anti-oxidant activity of the extract partly due to the fact that high levels of cellular migration is usually associated with increased generation of free radical products, which was also attenuated by following administration of ethanol root extract of *Salacia lemhbachii*. In fact, one possible mechanism by which the extract attenuated carrageenan-induced air pouch inflammation including its associated increased exudate volume and cellular migration might be the reduction of lymphocyte, leukocyte and neutrophil infiltration probably via mechanism related to down-regulation of intracellular adhesion molecules. However, further studies are needed to clarify this assertion on the mechanism by which *Salacia lemhbachi* may be inhibiting cell migration in inflammatory disease conditions.

Furthermore, it has also been repeatedly shown that carrageenan-induced air pouch inflammation is highly associated with degeneration of tissue linings (27, 23, 28). Previous studies have also shown that carrageenan-induced tissue damage is linked to increased cellular migration, generation of free radicals and other inflammatory mediators (18, 23, 28).

However, there are increasing body of evidence that support the use of compounds with antioxidant potential in the prevention and treatment of inflammation-related diseases like rheumatoid arthritis (33, 28). Also, abundant evidences in literature have confirmed the ability of compounds with antioxidant property to protect tissues against the damaging effects of reactive oxygen and nitrogen species due to injection of phlogistic agents such as carrageenan, thereby preventing or delaying the onset of chronic inflammation (33, 28). In view of this, the histopathological investigation from these studies revealed that injection of carrageenan into the air pouch produced tissue damage characterized by prominent congested blood flow, inflammatory cellular infiltrates occupying the lower two-third of the deeper dermis tissue and cellular enlargement of pouch wall, suggesting tissue damage. This finding from this study further supports previous investigation, which showed that injection of carrageenan into a 6-day old air pouch, produced significant tissue damage in experimental animals (28). However, pretreatment with ethanol root extract of *Salacia lemhbachii* similarly to aspirin and prednisolone, significantly protected the tissue lining as evidenced with tissues with intact epidermis consisting of stratified squamous epithelium displaying reduced odema and inflammatory tissue infiltration. Thus, the ability of ethanol root extract of *Salacia lemhbachii* to protect against carrageenan-induced tissue damage further reinforces the fact that the plant may contain beneficial phytochemical constituents with possible anti-oxidant and anti-inflammatory activities. Moreover, it is interesting to note that *Salacia lemhbachii* has been reported to be rich in flavonoid compounds some of which have been shown to exert functional anti-inflammatory property.

CONCLUSION

In conclusion, the results from the study provides evidence, which suggest that ethanol root extract of *Salacia lemhbachii* attenuated air pouch inflammatory reactions induced by carrageenan via mechanisms related to anti-oxidant, inhibition of cellular migration and tissue protection.

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