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ANTI-INFLAMMATORY ACTIVITY OF ETHANOL LEAF EXTRACT OF *HYMENODICTYON FLORIBUNDUM* (Hochst. & Steud) B. L. Rob IN RATS IS NOT DUE TO THE INHIBITION OF PROINFLAMMATORY BIOMARKERS PRODUCTION

DANRAKA, RABIU NUHU*, SALAUDEEN, MARYAM ADENIKE, MAJE, IDRIS MUHAMMED AND EJIOFOR JANET I

Department of Pharmacology and Therapeutics, Ahmadu Bello University, Zaria.

ABSTRACT

Hymenodictyon floribundum is a common shrub in Africa that has been used traditionally in the treatment of pain and inflammatory diseases. In this study, the anti-inflammatory activity of the ethanol leaf extract of *Hymenodictyon floribundum* (EEHF) in rats was studied following phytochemical screening and LD_{50} determination. The inflammatory activity of EEHF (375, 750 and 1500 mg/kg *p.o*) was evaluated using carrageenan-induced inflammation model in rats. The preliminary phytochemical screening of the extract revealed the presence of flavonoids, tannins, saponins, steroids glycosides, anthraquinones and phenols. The oral median lethal dose (LD_{50}) of the extract in rats was found to be greater than 5,000 mg/kg body weight. The result of this research revealed the ability of the extract to significantly ($p<0.05$) reduce paw oedema at the highest used doses, thus supporting the ethnomedicinal claims of the plant for the management of inflammatory conditions.

KEYWORDS: ***Hymenodictyon floribundum*; Inflammation; Cytokines; Carrageenan.**

INTRODUCTION

Inflammation is one of the most central processes required in defense of animal cells against certain injuries or microbial infections [1,2]. Nevertheless, inflammation regularly progresses to acute [3] or chronic processes [1]. The main anti-inflammatory drugs are either steroidal [4] (e.g., betamethasone, prednisolone, and dexamethasone) or nonsteroidal [5] (e.g., aspirin, diclofenac, ibuprofen, indomethacin. naproxen, and celecoxib) and are used to treat both acute and chronic inflammatory diseases such as osteoarthritis and rheumatoid arthritis [6]. However, their prolonged use is associated with various side effects; for example, steroidal drug causes adrenal atrophy [7], osteoporosis, suppression of response to infection or injury, euphoria, cataracts and glaucoma, while the non-steroidal drugs cause peptic ulcers and bronchospasm due to blockade of both the physiological and inflammatory prostaglandins and concurrent production of leukotrienes [8]. Thus, taking into account the adverse effects [9] and high cost of available steroidal or non-steroidal drugs [10], the search for new anti-inflammatory agents from herbal sources becomes imperative, with the objective of obtaining better efficacy, greater safety, and a more economical way to treat inflammation and related conditions.

Majority of the available orthodox medicines are derived from natural products prototype including atropine from *Atropa belladonna* (Solanaceae), reserpine from *Rauwolfia serpentina* (Apocynaceae), vincristine and vinblastine from *Vinca rosea* (Apocynaceae) [11] and many more. Phytomedicine has gained recognition as the epitome of alternative medicine and have been utilized for the search of many bioactive substances used as medicines [12]. There are over 30 species

***Corresponding author:** danrakarabiu@gmail.com*

of Hymenodictyon and several ethnobotanical claims have pointed to the use of *H. floribundum* as an effective anti-inflammatory and analgesic plants. *H. excelsum* for example has been scientifically proven to possess significant anti-inflammatory and analgesic properties [13]. *H. floribundum* has been reported to have significant analgesic potential [14] and as such, its anti-inflammatory properties are worth investigating.

MATERIALS AND METHODS

Drugs and Chemicals

Drugs and chemicals used for the studies include ethanol, carrageenan, chloroform (Sigma Aldrich, St. Louis Mo, USA), piroxicam (Rotex Medica, Germany), hydrochloric acid, sulphuric acid (May and Baker, UK), ferric chloride anhydrous (Avishkar, India), ammonia (Lobachemie, India), all the reagents were of analytical grade.

Animals

Adult male Wistar rats weighing between 150 and 200 g were obtained from the Animal House Facility of the Department of Pharmacology and Therapeutics, Ahmadu Bello University, Zaria, Nigeria. The rats were housed in a well-ventilated room in their cages and provided with a normal rodent feed and water *ad libitum* until the end of the study. All experiments were carried out in the main laboratory of the Department of Pharmacology and Therapeutics, Ahmadu Bello University, Zaria and were conducted in accordance with the guideline of National Institute of Health (NIH, 1998). Ethical approval for the use of animals was given with an approval number of ABUCAUC/2020/011.

Collection and identification of plant material

The plant material was collected from Kargi Hill along Birnin Gwari road, Zaria Local Government Area, Kaduna state in October 2019. The plant was identified and authenticated by a taxonomist, Malam Sanusi Namadi of the Department of Botany, Faculty of Life Sciences, Ahmadu Bello University, Zaria, and a voucher number of ABU900124 was assigned to the plant.

Plant extraction

Plant extraction was carried out according to the method described by Kupchan [15]. The leaves of *H floribundun* were shade-dried to constant weight, and then size-reduced into fine powder. 700 g of the powdered leaf material was extracted exhaustively with ethanol (70%$^{w}/_{v}$) using continuous Soxhlet apparatus for 72 hours. The solvent was removed by placing the extract on a water-bath set at 50°C, the dry extract was packed and stored in a desiccator until needed. The extract was henceforth referred to as ethanol leaf extract of *Hymenodictyon floribundun* (EEHF). Fresh solutions of the extract were prepared with distilled water for each study. The percentage yield of the extract was calculated using the formula:

$$\%\ Yield = \frac{weight\ of\ the\ extract}{weight\ of\ the\ powdered\ material}\ x\ 100$$

Qualitative phytochemical analysis

The extract EEHF was screened for the presence of different phytochemicals using simple chemical tests according to standard protocol described by Evans [16]. The secondary metabolites that were screened for include alkaloids, flavonoids, saponins, cardiac glycosides, tannins, anthraquinones, carbohydrates, steroids and triterpenes.

Acute toxicity study

Median lethal dose (LD_{50}) determination was conducted using Organization for Economic Co-operation and Development (OECD 423) guidelines in rats. In this method, two groups of three (3) animals each were fasted prior to dosing (food but not water was withheld for 3 hours). The fasted body weight was determined for each animal and the dose was then calculated according to the body weight. In the first phase of LD_{50} determination, 2000 mg/kg of EEHF was administered to each rat using oral canula and the rats were observed for 48 hours for clinical sign and symptoms of toxicity including death. The second phase was conducted in a similar manner to the first but with dose of 5000 mg/kg.

At the end of this test, rats that survived were euthanized with chloroform and subsequently incinerated.

Evaluation of anti-inflammatory activities

Carrageenan-induced paw oedema in rats

The anti-inflammatory study was carried out using the carrageenan-induced paw oedema in rats according to the method described by Winter *et al*. [17]. Thirty male rats were divided into 5 groups of six (6) rats each. Group I which served as a negative control was administered normal saline (10 ml/kg) orally, while group V received piroxicam (10 mg/kg; *p.o*) and served as the positive control. Group II, III and IV received graded doses of EEHF (375, 750 and 1,500 mg/kg respectively; *p.o*). Sixty (60) minutes post treatment, each rat was injected with 0.1 ml of 1% carrageenan into sub-plantar surface of the right hind paw. The diameter of the hind paw

oedema was measured and recorded at times 0, 1, 2, 3, 4 and 5 hours after carrageenan administration using vernier caliper. The increase in paw diameter (oedema index) for each rat was calculated as the difference in paw diameter before carrageenan injection and after carrageenan injection at each time interval, while the percentage inhibition of oedema was calculated for each group using the following relationship;

$$\% \text{ Inhibition } = \frac{m - n}{z} \times 100$$

Where;
m = Mean control increase in paw volume of control
n = Mean increase in paw volume of treated
z = Mean control increase in paw volume of control

Effect of ethanol leaf extract of *Hymenodictyon floribundum* on some inflammatory biomarkers

The described by Santos *et al*., [18] was used to investigate effect EEHF on some inflammatory biomarkers. Thirty (30) adult male rats were divided into five (5) groups of 6 rats each. Rats in group I received normal saline (10 ml/kg) administered orally, rats in groups II, III and IV received 375, 750 and 1,500 mg/kg of EEHF respectively while group V rats received piroxicam (10 mg/kg). All drug administrations were via oral route. An hour after drug administration, each rat was injected with 0.1 ml of 1% carrageenan into the sub-plantar surface of the rat's right hind paw [17]. The diameter of the hind paw oedema of each was measured and recorded at times 0-, 1-, 2-, and 3-hours using Vernier caliper. Three (3) hours after carrageenan injection, the rats were anaesthetized using chloroform and blood was collected from orbital sinus into the EDTA bottle. The blood was centrifuged at 3000 rpm for 20 minutes and the plasma was stored at -20 °C for the estimation of plasma levels of prostaglandin E_2 (PGE_2), interleukin 1β (IL-1β), interleukin 6 (IL-6), and tumour necrotic factor alpha (TNF-α) using ELISA kits by following the instructions described by the manufacturers of the kits.

Statistical analysis

Data obtained were analyzed using one-way analysis of variance (ANOVA) and Mixed design ANOVA in SPSS version 25. Results are presented as tables and graph. P values ≤ 0.05 were considered statistically significant.

RESULTS

Phytochemical constituents and acute toxic effect of EEHF

The preliminary phytochemical screening of the ethanol leaf extract of *H. floribundum* revealed the presence of alkaloids, cardiac glycosides, saponin, tannins, flavonoids, steroids/triterpenes, terpenoids carbohydrates and anthraquinones. The LD_{50} of the methanol leaf extract of *H. floribundum* was also estimated to be greater than 5000 mg/kg body weight.

Effect of *H. floribundum* on carrageenan-induced paw oedema in rats

Data analysis revealed that rats in all groups had comparable paw-size at base line, T0 ($p>0.05$). However, as time progressed, there was gradual increase in paw oedema in all groups which started declining after the third hour (see T4 and T5). After first hour (T1), only morphine-treated rats had significantly ($p<0.05$) smaller paw oedema than N/saline-treated group. But at 2nd, 3rd, 4th and 5th hours (T2, T3, T4, T5), Morphine-treated and extract-treated groups had significantly lower paw oedema sizes than N/saline ($p<0.05$) - At the 3rd hour (T3), only the high doses of extract-treated groups (EEHF750 and EEHF1500) had significantly ($p<0.05$) less paw oedema sizes than the N/saline group whereas at T4 only EEHF1500 had significantly lower paw oedema size than N/Saline group. At all the time points, none of the treatment was able to significantly reduce the paw oedema size to the baseline size except morphine at the 5th hour. Figure 1.

Effect of ethanol leaf extract of *H. floribundum* on pro-inflammatory biomarkers

All the assayed inflammatory biomarkers (IL-6, IL-β, TNF-α, PgE) had comparable levels in both the extract-treated group (EEHF750 and EEHF1500) and normal saline group. However, morphine significantly reduced the levels of PgE while ethanol extract of *H. floribundum* caused a significant rise in the level of IL-6. Figure 2.

DISCUSSION

The anti-inflammatory activity of *Hymenodictyon floribundum* was successfully investigated. Oral LD_{50} of the extract was estimated to be greater than 5000 mg/kg. The Organization for Economic Cooperation and Development (OECD, Paris, France) recommended chemical labeling and classification of acute systemic toxicity based on oral LD_{50} values as: very toxic, < 5 mg/kg; toxic, > 5 < 50 mg/kg; harmful, > 50 < 500 mg/kg; and not toxic or harmful, > 500 < 2,000 mg/kg. In line with this, the oral LD_{50} up to 5,000 mg/kg established for the treated rats, together with an absence of overt toxicity signs is indicative of relative oral safety [19].

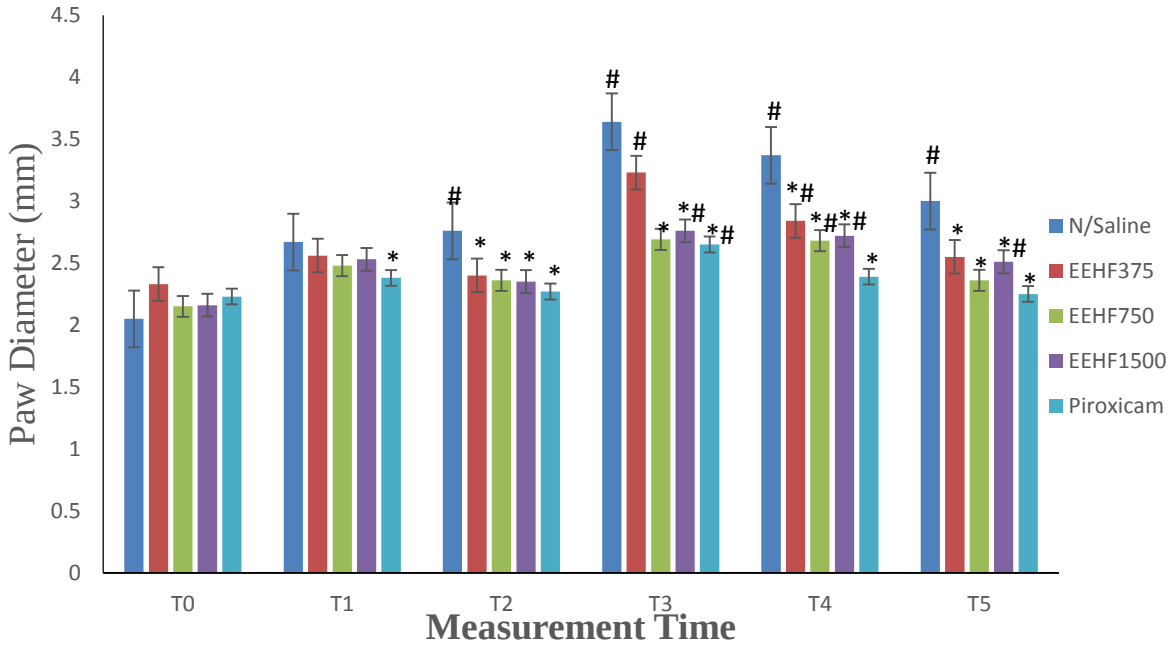

Figure 1: Effect of *Hymenodictyon floribundum* on paw size in carrageenan-induced paw oedema in rats.

Data was analyzed using Mixed-Design ANOVA followed by Bonferroni Posthoc test. * P< 0.05 compared to N/Saline, # p<0.05 compared to TI. EEHF = Ethanol Extract of *Hymenodictyon floribundum,* ANOVA = Analysis of Variance, N/Saline = Normal Saline. T0, T1, T2, T3, T4, T5 represent baseline, 1 hour, 2 hours, 3 hours, 4 hours and 5 hours respectively after carrageenan administration.

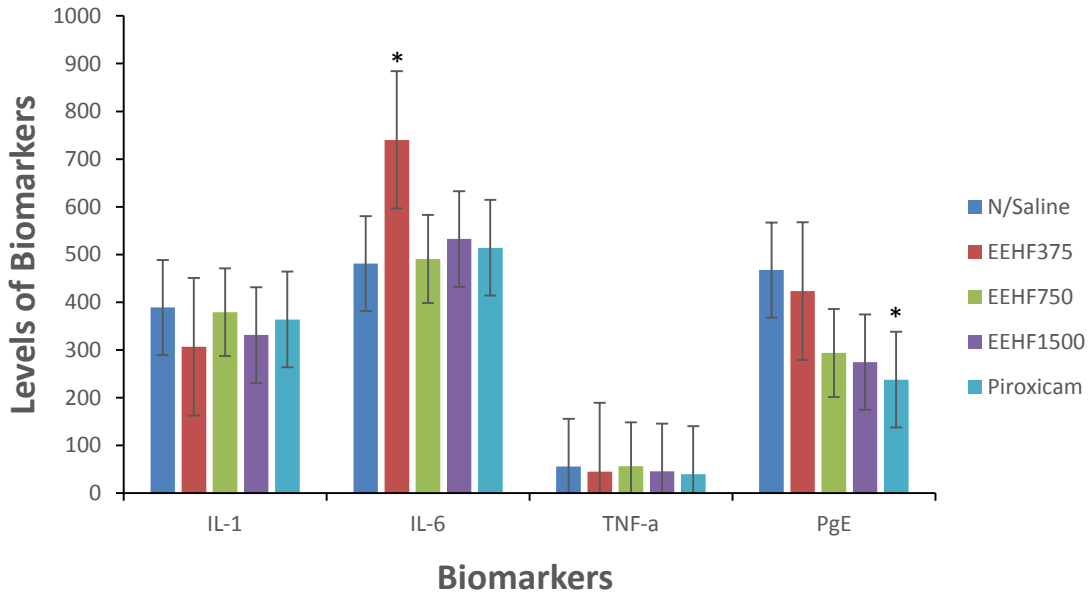

Figure 2: Effect of ethanol leaf extract of *H. floribundum* on pro-inflammatory biomarkers.

Data were analyzed using one-way Analysis of Variance (ANOVA) followed by Bonferroni Post-hoc test. * is p< 0.05 compared to N/Saline. N/Saline = Normal Saline; EEHF = ethanol Extract of *Hymenodictyon floribundum*; IL= Interleukin, PG= Prostaglandin.

Carrageenan-induced paw oedema is the most common test for the screening anti-inflammatory agents that exhibits a high degree of reproducibility [17]. Carrageenan is a phlogistic agent that is known to be non-antigenic and does not have any apparent systemic effect [20]. Swelling or edema, which is a prime indication of acute inflammation is a major parameter to consider when testing compounds with a potential anti-inflammatory activity [21]. The oedema caused by carrageenan is simple, rapid and gives reasonable results that can be relied upon [22]. After the carrageenan injection, oedema develops mainly in two phases: the first 30 minutes after the injection, the second beginning at the end of the first hour and lasting until the third hour after injection [23]. The first phase has been attributed to the release of histamine, serotonin and bradykinin on vascular permeability and the later phase has been due to over production of prostaglandin in tissues [24]. The results of this study indicate that the ethanolic extract of *H. floribundum* significantly reduced carrageenan-induced paw oedema in rats. In general, non-steroidal anti-inflammatory drugs produce their antipyretic action through the inhibition of prostaglandin synthetase within the hypothalamus [25]. More so, inflammatory cytokines are known to be upregulated during pain and inflammation [26], and the inhibition of this process can contribute to the anti-inflammatory activity of several agents. In this study, there was no significant reduction in the level of these cytokines in the extract-treated groups, an indication that the anti-inflammatory activity of the extract is not likely mediated via interference with the cytokines production, but most probably via the inhibition of inflammatory mediators such as histamine, serotonin and prostaglandins.

Preliminary phytochemical screening gives an idea of qualitative nature of active phytochemical constituents present in the extract that are responsible for several pharmacological activities that will stimulate further investigation and/or isolation of the relevant active principles. Several phytochemicals such as saponins, tannins, and cardiac glycosides which are present in the extract are known to have analgesic and inflammatory activities [27, 28]. Saponins have been reported to inhibit inflammatory mediators [29]. Flavonoids are known to be effective against acute inflammation [30]. The anti-inflammatory effect of the extract may therefore, be due to the presence of flavonoids, tannins, alkaloid or saponins acting in synergy or otherwise.

CONCLUSION

Ethanol leaf extract of *H. floribundum* has significant anti-inflammatory activities which may be attributed to the presence of flavonoids, tannins, alkaloid and saponins acting together or otherwise in the extract. The extract is unlike to produce its anti-inflammatory activity by preventing the production and generation of proinflammatory cytokines.

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