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Evaluation of Phytochemicals And Anti-Inflammatory Effects of Hexane *Extracts* from *Borreria stachydea* [(DC) Hutch. and Dalziel] (Rubiaceae)

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Abstract

This studies evaluated the preliminary phytoconstituents of hexane extract of Borreeia stachydea (Rubiaceae), toxicity studies using OECD guideline, acute and chronic inflammation. This revealed the presence of alkaloids, carbohydrate, steroid, triterpenes, and tannins. Acute toxicity study showed no symptoms of toxicity even at 5000 mg/kg for the period of fourteen days. Its efficacy was significant at doses of 125 at p< 0.01 and 500 mg/kg at p< 0.05 when compared to distilled water treated groups. It is also significant at 125 mg/kg at p< 0.05 in 4 hr. Also 250 and 500 mg/kg at p< 0.001 in 5th hr compared to 1 hr treated group. The standard drug Diclopenac (10 mg /kg) provided a significance difference at (p< 0.001). Also at (4th hour), the extract at 500, 250 and 125 mg/kg inhibited inflammation induced by carrageenan by 92.16, 88.24 and 88. 24 % respectively compared to standard drug that has the inhibition by 90.20 %. Chronic inflammation that served as a long term inflammation of this studies indicated a significance difference at p< 0.05 with 500 mg/kg on day 4, 5 and 6 at p<0.01 with 250 mg/kg on day 5 and 6. Consequently, a significance was also observed at p<0.01 and p<0.001 on day 5 and 6 All compared to day 1 treated group. Acute toxicity study were observed that the extracts did not show any behavioral changes or mortality even at a dose of 5000 mg/kg this indicated its safety and efficacy. However, further studies can be carried out to reveal the exact mechanisms of action responsible to manage inflammation. This study has highlighted that the anti-inflammatory activity of this plant could be a potential new source as well as scientific proof of its ethno-pharmacological use in inflammatory disorders.

Keywords: *Anti-inflammatory, Borreria stachydea, Phytochemicals, Toxicity*

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INTRODUCTION

Inflammation is a response to tissue injury or infection and it is characterized in its acute phase by an increase in vascular permeability and plasma extravasation, resulting in accumulation of fluid, leukocytes and mediators to the inflamed site (Guo *et al*., 2012). A variety of soluble mediators is involved in the recruitment of circulating leukocytes and in the regulation of the activation process of resident cells in the early stages of inflammation (Morais-Lima *et al*., 2011). These soluble mediators involve lipid metabolites such as: platelet-activating factor (PAF) and arachidonic acid derivatives (eicosanoids), proteases/substrates related to coagulation and complement system cascade, kinins, nitric oxide and a group of polypeptide derived cells called cytokines (Kim *et al*., 2010). In addition an inflammatory response is related to reactive oxygen species (ROS) released by neutrophils and acti- vated macrophages (Conforti *et al*., 2008). In order to alleviate this situation, anti-inflammatory drugs are used, represented by steroidal agent (SAs) and non-steroidal drugs (NSAIDs), on symptomatic effects (GautamandJachak, 2009). A prolonged use of these agents is followed by severe side effects such as gastro-duodenal and kidney damage, bone marrow depression, retention of salts and water, among others (Qandil, 2012). There is a clinical need to identify new compounds that are safe, for the prevention and treatment of inflammatory diseases (Hur *et al*., 2012). Medicinal plants are viable alternative to the discovery of new safer bioactive compounds (GautamandJachak, 2009). In fact, there are evidences that drugs derived from natural products modulate various inflammatory mediators, including their effects on the expression of pro-inflammatory molecules that are key to inflammation, such as inducible nitric oxide synthase(iNOS), cyclooxygenase(COX-2),IL-1β, TNF-α andIL-10cytokines (Bellik *et al*., 2013).

Medicinal plant species has pharmacologically important phytochemical. The primitive man used herbs as therapeutic agents and medicament, which they were able to procure easily. The nature has provided abundant plant wealth for all living creatures which possess medicinal virtues. Phytochemicals are bioactive constituents of plants. Some plants possess some useful phytochemicals which are of high medicinal values to human and veterinary animals, some which are alkaloids, tannins, flavonoids, saponins and phenolic compounds (Gislence *et al*., 2000). Plant materials contain thousands of chemicals which act against diseases and infections of humans and animals when properly used. Plant contains different types of compounds such as resins, rubbers, gums, waxes, dyes, flavours, fragrances, proteins, amino acid; bioactive peptides, sugars, flavonoids and biopesticides (Gulfraz *et al*., 2006).

Borreria is relatively large genus of herbs or half-shrubby plants. This genus consists of about 100 species distributed throughout the tropics (Neoh *et al*., 2010). In northern Nigeria *Borreria stachydea* is popularly known as "alkamar tururuwa" while fulanis called it "fairare". Little work was done on this plant which was basically on its preliminary phytochemical analysis and Anti-microbial efficacy by Paul *et al*., 2016. Anti-inflammatory aspect was first exploited scientifically on Methanol extract by Anas *et al*., 2019. Subsequently, this studies intended to explore the non-polar region of the plant constituents with regard to the traditional uses in most cases when it comes to inflammation management the usual route of application is poultices (topical) a times using dried pounded drug mixed with some natural additive to rub on the affected areas to attain healing. We want establish scientific basis in this regard to safeguard the humanity, hence it was mentioned by Qandil, (2012) that prolong intake of

steroidal and non-steroid drugs (orthodox drugs) had great risk to our health, this could serve as an alternative.

MATERIALS AND METHODS

Plant collection, Identification and Preparation

Fresh plant material were collected from Ringim Local Government Area of Jigawa State, Nigeria in May, 2016. It was identified by taxonomist at the herbarium section of the Department of Botany, Faculty of Life Science, Ahmadu Bello University Zaria, Nigeria. The plant material of 1 kg were collected and dried at room temperature, powdered and stored at a room temperature, in a closed container for further use.

Extraction of the Leaves of *Borreria stachydea*

The plant material (1000 g) were extracted with 2.5 litres of n-Hexane using cold maceration successively for 72 hours each and the extracts were filtered and dried over water bath and the percentage yield were calculated using the formula below:
Percentage Yield of extracts = (Weight of total extract) / (weight of powdered material) ×100 (%w/w) and labelled in which the marc was pressed to dryness and then kept in a safe place.

Preliminary Phytochemical Evaluation

This procedure were carried out on the Methanol extract according to Abimbola *et al*., (2013) and Evans (2009) as outlined below.

Alkaloids

Dragendorff's test; to 2 mg of the hexane extract 5 mL of distilled water will be added, 2 ml of Hydrochloric acid will be added until an acid reaction occurs. To this 1 mL of Dragendorff's reagent will be added. Formation of orange or orange red precipitate indicates the presence of alkaloids.

Wagner's test; 2 mg of hexane extract will be acidified with 1.5 % v/v of hydrochloric acid and a few drops of Wagner's reagent will be added. A yellow or brown ppt. indicates the presence of alkaloids.

Mayer's test; to a few drops of the Mayer's reagent, 2 mg of hexane extract will be added. Formation of white or pale yellow precipitate indicates the presence of alkaloids.

Flavonoids

Shinoda's test; 2 mg of hexane extract will be dissolved in 5 mL of ethanol and to this 10 drops of dilute hydrochloric acid followed by a small piece of magnesium will be added. Formation of pink, reddish or brown colour indicates the presence of flavonoids.

Triterpenoids

Liebermann - Burchard's test; 2 mg of dry extract will be dissolved in acetic anhydride, heated to boiling, cooled and then 1 mL of concentrated sulphuric acid will be added along the sides of the test tube. Formation of a pink colour indicates the presence of triterpenoids.

Saponins

In a test tube containing about 5 mL of hexane extract, a drop of sodium bicarbonate solution will be added. The test tube will be shaken vigorously and left for 3 minutes. Formation of honey comb like froth indicates the presence of saponins.

Steroids
Liebermann-Burchard's test; 2 mg of dry extract will be dissolved in acetic anhydride, heated to boiling, cooled and then 1 mL of concentrated sulphuric acid will be added along the sides of the test tube. Formation of green colour indicates the presence of steroids.
Salkowski reaction; 2 mg of dry extract will be shaken with chloroform, to the chloroform layer sulphuric acid will be added slowly by the sides of test tube. Formation of red colour indicated the presence of steroids.

Tannins
To 1-2 mg of the hexane extract, few drops of 5% w/v $FeCl_3$ solution will be added. A green Colour indicated the presence of gallotannins, while brown colour indicates the presence of pseudotannins.

Toxicity Studies of the Plant Extracts
The growing number of herbal drug users around the globe and lack of scientific data on the safety profile of herbal products make it necessary to conduct toxicity study of herbal products (Saad *et al*., 2006).

Acclimatisation of the Animals
Animals was allowed for a one week of acclimatization period prior to the study. They were having free access to food and water and maintained under standard laboratory conditions which included 12-hour light-dark cycle and temperature of 28-30 degrees centigrade. The animals were maintained under standard environmental conditions and fed with standard rodent pellet diet (Vital feed, Jos-Nigeria) and water *ad libitum*. Swiss albino mice (16-32g) of either sex were obtained from the Animal House Facility of the Department of Pharmacology and Therapeutics. The experiments were carried out in Bayero University, Kano, Nigeria in accordance with the criteria outlined in the Guide *for the Care and Use of Laboratory Animals* by the National Institutes of Health (Publication No. 80-23, revised 1996).

Acute Toxicity Studies
Acute toxicity studies of *B. stachydea* extracts was carried out in female mice by using Organization for Economic Co-operation and Development (OECD) guideline 425 (OECD, 2008). Before oral administration of a single dose of the test samples, the mice were deprived of food for 3 h. Doses of 5000 mg/kg (known as limit test) of the test samples was given using oral gavage to mice. All the mice was observed for general behavioral changes; symptoms of toxicity and mortality after treatment for the first four (critical) hours, then over a period of 24 hours, thereafter daily for 14 days.

Determination of Acute Inflammation using Carrageenan induced Paw Edema in Mice
Freshly prepared 1% carrageenan was injected on subplantar region of the right paw of mice to induce acute inflammation (Winter *et al*., 1962 and Maity *et al*., 1998). The animals were divided in to 5 groups, 3 animals each. Group I remained as control for carrageenan. Group II, III and IV received 125, 250 and 500 mg/kg body weight extract (0.1 ml in distilled water) orally by intubation guage 1 hr before carrageenan administration and group V positive control received 10 mg/kg body weight diclofenac. The thickness of the paw was measured using a digital Vernier caliper before and after carrageenan administration and thereafter at every hour up to 6 hrs. The percentage of inhibition of paw thickness was calculated using the formula below:

%inhibition of paw thickness = {[(Ct – C0) control – (Ct – C0) treated] ÷ [(Ct – C0) control]} × 100

Where Ct = mean edema index for each group at time t, and C0 = mean edema index for each group before carrageenan injection.

Determination of Chronic Inflammatory using Formalin induced Paw Edema in Mice

Single dose of freshly 50 µl of freshly prepared 2 % formalin was used to induce chronic inflammation in mice. The animals were divided in to 5 groups of 3 each. Group I remained as control. Group II, III and IV will receive 125, 250 and 500 mg/kg of body weight extract (0.01 ml) orally and V received 10 mg/kg of body weight of diclofenac (oral). Drug treatment was started 1 hr prior to formalin administration and continued for 6 consecutive hours and the thickness of paw was measured using Vernier caliper every day for 6 days as described by (Chau, 1989).

Statistical Analysis

All data was expressed as mean ± SEM with SPPS using one way and repeated measure ANOVA followed by Dunnett and Bonferroni post hoc tests. Values of $P<0.05$ were considered significance.

RESULTS AND DISCUSSION

Table 1: Mass and Percentage Yield of *Borreria stacchydea*

S/N	Solvent used	Weight of extracts (g)	Yield (%w/w)
	Hexane	7.6	0.76

Table 2: Showing Preliminary Phytochemical Evaluation from the Hexane extract of *Borreria stachydea*

Phytoconstituents/Test	Remark
Alkaloid	
Dragendoff's	-
Mayer's	-
Wagner's	-
Anthraquinones	
Bontrager's	-
Modified Bontrager's	-
Carbohydrate	
Molish	+
Fehling	-
Cardiac Glycosides	
Keller-Killian	-
Keddes	-
Flavonoids	
Sodium hydroxide	-
Ferric chloride	-
Saponin Glycosides	
Frothing	-
Haemolysis	-
Unsaturated Steroid & Triterpenes	
Lieberman Bucchard	+
Sarkwoski	+
Tannins	
Lead sub-acetate	-
Ferric chloride (hydrolysable)	+

+ = present, - = absent

Table 3: Showing Acute Toxicity Study of Hexane Extract of *B. stachydea*

Fourteen Days Observation (2 Weeks)	
Dose (mg/kg)	**Mortality**
5000	0/9

Table 4: Effect of Hexane Extract of *Borreria stachydea* on Carrageenan-induced Paw Oedema in Mice

Treatment (mg/kg)	Oedema Index (mm)					% inhibition
	1 hr	2 hr	3 hr	4 hr	5 hr	
D/W1ml/kg	0.51±0.15	0.76±0.19	0.50±0.29	0.70±0.18	0.29±0.02^{c}	
HFBS 500	0.67±0.10	0.83±0.03	0.67±0.14	0.23±0.13**	0.04±0.01^{c}	92.16
HFBS 250	0.79±0.03	0.90±0.08	0.47±0.11	0.31±0.06$^{*\,c}$	0.06±0.03^{c}	88.24
HFBS 125	0.64±0.04	0.84±0.06	0.53±0.17	0.21±0.02$^{**\,a}$	0.06±0.03^{b}	88.24
DICLO 10	0.12±0.01	0.39±0.11	0.21±0.06	0.08±0.03$^{***\,a}$	0.05±0.02^{a}	90.20

Values are Mean ± S.E.M., * = p<0.05, ** = p<0.01, *** = p<0.001 compared to D/W, ^{a} = p<0.05, ^{c} = p<0.001 compared to 1 hr - Repeated measure ANOVA followed by Bonferroni post hoc test, n=5, D/W = Distilled water, HFBS = Hexane Fraction of *BS*, DICLO = Diclofenac

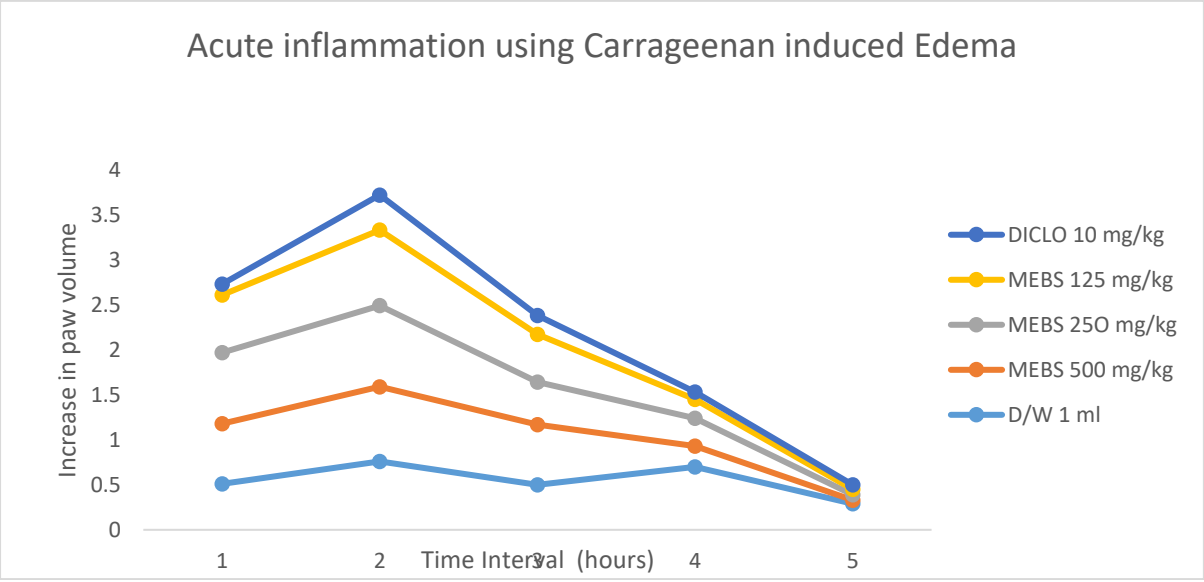

Figure 1: Chart showing Acute Inflammation using Carrageenan induced Edema by Hexane Extracts of BS

Table 5: Effect of Hexane Extract of *Borreria stachydea* on Formalin-induced Chronic Pain in Mice

Treatment (mg/kg)	Oedema Index (mm)					
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
D/W1ml/kg	0.83±0.03	0.95±0.04	0.98±0.05	1.00±0.05	1.26±0.10[c]	1.43±0.12[c]
HFBS 500	0.85±0.03	0.77±0.01	0.86±0.05	1.32±0.26[a]	1.21±0.12[b]	1.30±0.08[b]
HFBS 250	0.96±0.01	0.85±0.02	0.91±0.05	1.27±0.32	1.29±0.03[b]	1.42±0.02[b]
HFBS 125	0.87±0.03	0.87±0.03	1.01±0.05	1.14±0.05	1.39±0.04[c]	1.30±0.03[b]
DICLO 10	0.80±.018	0.86±0.06	0.87±0.06	0.75±0.02	0.99±0.01	1.05±0.10

Values are Mean ± S.E.M., [a] = $p<0.05$, [b] = $p<0.01$, [c] = $p<0.001$ compared to Day 1 - Repeated measure ANOVA followed by Bonferroni post hoc test, n=5, D/W = Distilled water, HFBS = Hexane fraction of *Borreria stachydea*, DICLO = Diclofenac

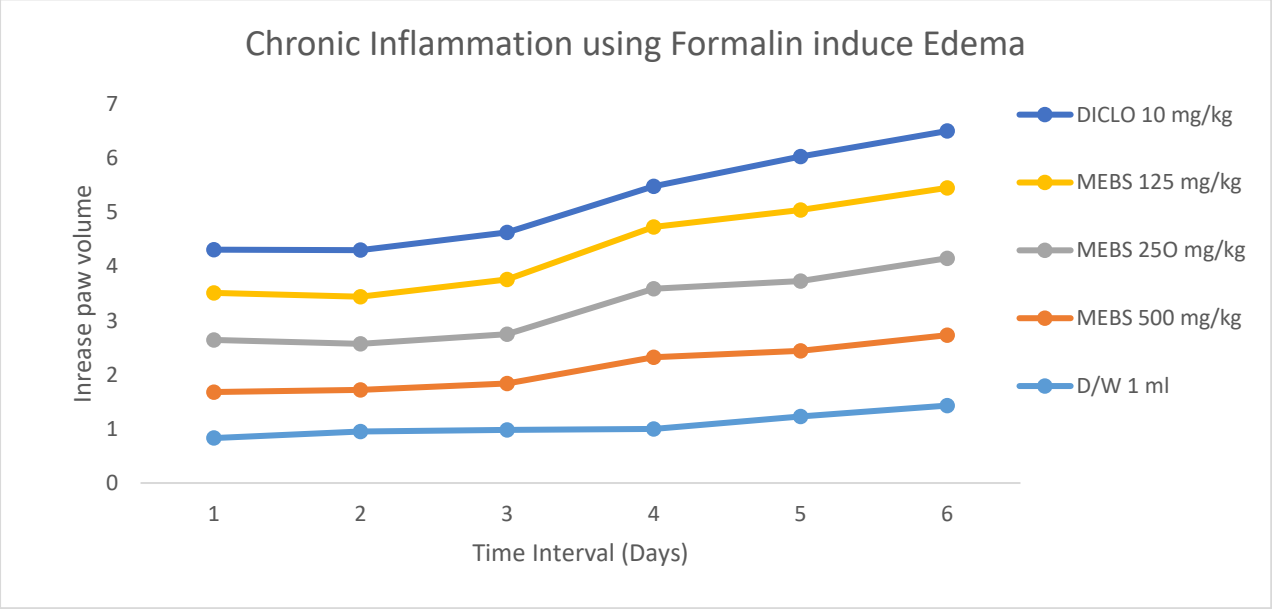

Figure 2: Chart showing Chronic Inflammation using formalin induced Edema by Hexane extract of BS

This study revealed the presence of usaturated steroids, triterpenes, tannins and alkaloid. These may probably have aid in the pharmacological activity of the plant. Presence of those phytochemicals justifies the use of the plant in curing certain diseases.

Acute toxicity studies of this plant was estimated to be > 5000 mg/kg in the mice and no adverse symptoms or death was observed with 5000 mg/kg using nine mice for the period of 14 days observation in line with OECD, (2008) guide lines rather it stimulates the experimental animals to be more energetic during the study period as shown in Table 3. This showed that the methanol tuber extract of *B. stachydea* is relatively safe (Lorke, 1983) when administered orally, as no mortality was recorded at the dose of 5,000 mg/kg.

This study revealed that the acute anti-inflammation effects was significant at doses of 125 at p< 0.01 and 500 mg/kg at p< 0.05 when compared to distilled water treated groups (Table:4). It is also significant at 125 mg/kg at p< 0.05 in 4 hr. Also 250 and 500 mg/kg at p< 0.001 in 5th hr compared to 1 hr treated group. The standard drug Diclopenac (10 mg /kg) provided a significance difference at (p< 0.001). Also at (4th hour), the extract at 500, 250 and 125 mg/kg inhibited inflammation induced by carrageenan by 92.16, 88.24 and 88. 24 % respectively compared to Standard drug that has the inhibition by 90.20 % (Table 4). This is in conformity with finding found in Anas *et al*., 2019.

B. stachydea extract significantly decreased paw edema in mice at different dosages and at different time intervals. At the 2nd hour, which marks the peak of inflammation due to carrageenan, the extract at all the doses tested reduced the edema index (Fig.1).

Formalin induced paw oedema is one of the most suitable test procedures to test chronic inflammation agent as it resembled human arthritis (Greenwald, 1991). It served as a long term inflammation of this studies indicated a significance difference at p< 0.05 with 500 mg/kg on day 4, 5 and 6. Also is significance at p<0.01 with 250 mg/kg on day 5 and 6. Consequently, a significance was also observed at p<0.01 and p<0.001 on day 5 and 6 All compared to day 1 treated group. This finding is in accordance with the Vadivelan *et al*., (2007) results in which a different plant from the same family exhibited anti-inflammatory properties.

CONCLUSION

This is the first time to our knowledge that *B. stachydea* anti-inflammatory activities have been evaluated. Acute toxicity study was observed that the extracts did not show any behavioural changes or mortality even at a dose of 5000 mg/kg indicated its safety and efficacy. However, further studies can be carried out to reveal the exact mechanisms of action responsible to treat inflammation. Though the study has highlighted that the anti-inflammatory activity of *this plant* could be a potential new source as well as scientific proof of its ethno-pharmacological use in inflammatory disorders.

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