

Full Length Research Paper

Evaluation of the anti-inflammatory properties of the hexane extract of *Hydrocotyle bonariensis* Comm. Ex Lam. leaves

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This study investigates the anti-inflammatory properties of *Hydrocotyle bonariensis* Comm. Ex Lam, a medicinal plant used by indigenous traditional healers to manage chronic inflammatory diseases especially rheumatism and arthritis. The hexane extract of *H. bonariensis* leaves was evaluated for the presence and concentration of phytochemicals. It was subjected to heat-induced albumin denaturation and human red blood cell (HRBC) membrane stabilization assays. The anti-inflammatory properties of the extract were further assessed by employing the formaldehyde induced arthritis animal model assay. The extract was finally subjected to GC/MS analysis for the tentative identification of the phytochemical constituents. Phytochemical analysis of the extract revealed the presence of saponin, phenol, flavonoid, tannin, terpenoid and sterol. This extract showed the ability to inhibit thermally-induced protein denaturation and stabilize HRBC membrane in concentration dependent manner. In the formaldehyde induced arthritis model, the paw measurements of the rats were taken, their hematological parameters were determined, and their liver function tests (aspartate aminotransferase and alanine aminotransferase activities) were also carried out. Results from the animal model indicated that oral administration of the hexane extract of *H. bonariensis* leaf at a dose of 250 mg/kg body weight had potent anti-inflammatory action. The GC/MS analysis suggested a number of anti-inflammatory compounds in the extract among which were: hexadecanoic acid methyl ester, falcarinol and phytol. Consequently, the tentative identification of phytochemicals with anti-inflammatory activity in *H. bonariensis* affirms the anti-inflammatory property of the plant and the phytochemicals could serve as lead compounds for designing anti-inflammatory drugs.

Key words: *Hydrocotyle bonariensis*, anti-inflammatory, phytochemicals, protein denaturation, membrane stabilization, arthritis.

INTRODUCTION

Inflammation is a very complex response that occurs as a result of an injury, infection or another stimulus, in which several cell types and secreted factors elicits protective immunity, tissue repair and resolution of tissue damage (Howcroft et al., 2013). It is characterized by five

pathological phenomena; *calor*-increase in tissue temperature, *rubor*-redness of vascularized tissue at inflammation site, *dolor*-intensive sensation of a noxious stimulus, *tumor*-swelling of the tissue, *functio laesa*-impaired function of the affected organ (Rather, 1971), all

of which are secondary to the direct consequence of tissue injury-enhancement of vascular permeability, protein denaturation and membrane alteration (Ryan and Majno, 1977; Majno and Plalade, 1961; Umapathy et al., 2010). It is worthy of note that though inflammatory responses are part of the body's defense mechanism, therefore important and useful, its prolonged action or escalation may cause debilitating harm to the body (Stankov, 2012). Chronic inflammation triggers casual pathways associated with aging, including physical frailty, energy imbalance, homeostatic dysregulation, changes in body composition and neuro-degeneration (Howcroft et al., 2013). Most currently used drugs for the treatment of inflammation-related diseases are the steroidal and non-steroidal drugs. These drugs have remarkable potency, however, long-term administration is required for treatments of chronic diseases. In addition, these drugs are known to have several adverse effects, and this has encouraged the use of medicinal plants with very little side effects to substitute for these chemical therapeutics.

The use of plants as medicine predates written human history itself but documentation can be found as far back as approximately 6000 years (Paulsen, 2010). Medicinal plants play an important role in the development of potent therapeutic agents and have contributed significantly towards the development of modern medicine. In recent times, use of medicinal plants worldwide was re-evaluated by extensive research on different plant species and their active therapeutic principles and it was concluded that the wealth of the plant kingdoms can represent novel sources of newer compounds with significant anti-inflammatory activities. Major advantages of the use of plants as medicine over conventional drugs seem to be their perceived efficacy, low incidence of serious adverse effects, and low cost (Sangita et al., 2012).

Hydrocotyle bonariensis, commonly known as large leaf pennywort is a member of the family *Araliaceae* and genus *Hydrocotyle*. It is a herbaceous plant with prostrate, creeping or floating stems and roots forming at nodes. There are approximately 402 species in this genus and they are mostly found in Africa and America. Its common name in West Africa, Nigeria is Karo (Ajani, 2012). It is well known for its traditional uses and medicinal properties for the treatment of various kinds of diseases such as tuberculosis, relieving the pains of inflammation, rheumatism and arthritis, to increase brain capacity and for longevity (Masoumian et al., 2011). This herb has its medicinal uses as emetics, diuretics and laxatives (Evans, 1992). Leaves of this plant have been reported to contain alkaloids, flavonoids, tannins, phenolics and saponins (Ajani, 2012). The anti-mutagenic activity of the aqueous and methanolic extracts from the leaves

and stems has been reported (Florinsiah et al., 2013). Results obtained in a study by Ajani (2012), suggested that chronic administration of *H. bonariensis* aqueous leaf extract may not contribute to liver and renal dysfunction. The study also indicated that when administered at an acute dose, the extract may not potentiate any significant toxic effect. It has also been reported that *H. bonariensis* possesses significant antioxidant property that may offer protection from galactose induced oxidative damage in both the lens and the liver (Ajani, 2012).

To the best of our knowledge, no attempts have been made to evaluate the anti-inflammatory properties of the hexane extract of *H. bonariensis* leaves. However, Ouviaña et al. (2009) explored anti-inflammatory activity of an infusion and methanolic extract of aerial parts of *H. bonariensis*. Therefore, this study aimed at identifying the phytochemicals present in the hexane extract of *H. bonariensis* leaves, identify the bioactive agents in the extract using Gas Chromatography/Mass Spectrometry analytical method and evaluate the anti-inflammatory properties of the extract using some *in vitro* and *in vivo* methods, with the objective of assessing the therapeutic use of *H. bonariensis* in the management of inflammatory diseases.

MATERIALS AND METHODS

Collection of plant material, Identification and preparation of extract

Fresh leaves of *H. bonariensis* were collected from a garden in Ilishan-Remo, Ogun State, and authenticated by a plant taxonomist, Prof E. B. Esan of the Department of Basic Sciences, Babcock University Ilishan-Remo, Ogun State of Nigeria. The leaves were washed with tap water to remove all debris, air-dried at room temperature (25°C) for four weeks and pulverized mechanically, using a warring blender. The extract was prepared according to the method of Florinsiah et al. (2013). 80 g of the dry powdered plant material was soaked in 800 ml of n-hexane with intermittent shaking for 48 hours and filtered through Whatman No. 1 filter paper. The filtrate was concentrated at 45°C using rotary evaporator (RE52-3 model, LIDA Instrument).

Preliminary screening of the extract phytochemicals

Initial screening tests of the extract were performed to ascertain the presence or absence of phenolics, flavonoids, saponins, tannins, terpenoids and sterols using standard procedures at 45°C

Determination of total phenolic content

Total phenolic content in the extract was determined according to the method of Singleton et al. (1999). An aliquot of 0.5 ml of extract (1mg/ml) was mixed with 2.5 ml Folin-Ciocalteu reagent (previously diluted with distilled water 1:10 v/v) and 2 ml (7.5% w/v) of sodium

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carbonate (Na_2CO_3). The tube was vortexed for 15 s and incubated for 40 min at 45°C for colour development. Absorbance was measured at 765 nm using double beam UV/visible spectrophotometer (T80 model, PG Instrument). A calibration curve was obtained using gallic acid as standard, the total phenolics content was expressed as mg/g gallic acid equivalent using the equation obtained from the calibration curve.

Estimation of total flavonoids

The formations of a complex aluminum chloride were estimated using the method described by Ordonez et al. (2006). 0.5 ml of the extract (1 mg/ml) was mixed with 0.5 ml of 2% aluminum chloride (AlCl_3) prepared in ethanol. The resultant mixture was incubated for 60 min at room temperature for yellow colour development to indicate the presence of flavonoid. Absorbance was measured at 420 nm using double beam UV/visible spectrophotometer (T80 model, PG Instrument). A calibration curve was obtained using quercetin as standard, and the total flavonoids content was expressed as mg/g quercetin equivalent using the equation obtained from the calibration curve.

Determination of tannin concentration

Tannin content of the sample was determined according to the modified vanillin-HCl methanol method as described by Noha et al. (2011). The vanillin-HCl reagent was prepared by mixing an equal volume of 8% HCl and 1% vanillin in methanol. The reagent was mixed just prior to use. About 0.2 g of the ground sample was placed in a small conical flask. 10 ml of 1% concentrated HCl in methanol was added. The flask was capped and continuously shaken for 20 min and the content was further centrifuged at 2500 rpm for 5 min with 1.0 ml of the supernatant was pipetted into a test tube containing 5 ml of vanillin-HCl reagent. Absorbance at 450 nm was read on the spectrophotometer (T80 model, PG Instrument) after 20 min of incubation at 30°C. A standard curve was prepared to express the result as tannic acid equivalent; tannin (%) = $C \times 10 \times 100/200$. Where C = concentration corresponding to the optical density; 10 = volume of the extract (ml); 200 = sample weight (mg).

Determination of saponin concentration

5 g of plant sample was dispersed in 50 ml 20% v/v ethanol prepared in distilled water. The suspension was heated over a hot water bath for 1 h with continuous stirring at 55°C. The mixture was filtered and the residue re-extracted with another 50 ml of 20% ethanol. The combined extracts were reduced to 20 ml over hot water bath at about 90°C. The concentrated solution obtained was shaken vigorously with 10 ml of diethyl ether in a 250 ml separating funnel; the aqueous layer was collected while the ether layer was discarded. 20 ml of but-1-ol was added to the filtrate and washed three times with 10 ml of 5% w/v aqueous sodium chloride. The whole mixture was heated to evaporation on a hot water bath and later oven-dried to a constant weight. The percentage saponins content of the sample was calculated using the formula described by Okwu and Josiah (2006).

% Saponins = Weight of final filtrate/weight of sample \times 100

Gas chromatography/mass spectrometry (GC/MS) analysis

GC/MS analysis was carried out at the Department of Chemistry, University of Lagos Akoka, by using Agilent Technology model 7890A GC/MS, MSD = 5975C (detector) Agilent Technologies,

Injector: 7683B Series, Initial temperature = 100°C held for 2 min, final temperature = 270°C at the rate 10°C/min, 1 μ l of the hexane extract of *H. bonariensis* leaves was injected. Temperature of heater was 250°C, pressure was 3.2652 psi, mode type slit less, column type (HP 5MS: 30 m \times 320 μ m \times 0.25 μ m) and carrier gas (Helium, 99.9999% purity, flow rate = 1.4963 ml/min; average velocity = 45.618 cm/s). The constituent compounds were determined by comparing their retention times and mass weights with those of authentic samples obtained by GC as well as the mass spectra National Institute of Science and Technology (NIST) version 2.0 MS database and literatures.

Human red blood cell (HRBC) membrane stabilization method

Estimation of *in vitro* anti-inflammatory activity by HRBC membrane stabilization method described by Sadique et al. (1989) was used to assess the hexane extract *in-vivo* anti-inflammatory effect. The principle involved is stabilization of the human red blood cell membrane by hypotonicity-induced membrane lysis. The assay mixture containing 1 ml phosphate buffer (pH 7.4, 0.15 M), 2 ml hypo saline (0.36%), 0.5 ml HRBC suspension (10% v/v) with 0.5 ml of the plant extract of various concentrations 50 to 250 μ g/ml; standard drug diclofenac sodium 50 to 250 μ g/ml and control (distilled water instead of hypo saline to produce 100% haemolysis) were incubated at 37°C for 30 min followed by centrifugation at 3000 rpm for 10 min. The hemoglobin content in the suspension was estimated using double beam UV- visible spectrophotometer (T80 model, PG Instrument) at 560 nm. The percentage haemolysis produced in the presence of distilled water was taken as 100%. Percentage of HRBC membrane stabilization or protection was calculated using the formula,

Percent stabilisation = $100 - (\text{optical density of drug}) \div (\text{optical density of control}) \times 100$

Preparation of red blood cells (RBCs) suspension

Fresh whole human blood was collected from a healthy human volunteer who had not taken any None Steroidal Anti-Inflammatory Drugs (NSAIDs) for two weeks prior to the experiment and centrifuged at 3000 rpm for 10 min. It was washed four four times with equal volume of normal saline. The volume of blood was measured and re constituted as 10% v/v suspension with normal saline Sadique et al. (1989).

Inhibition of protein denaturation assay

The effect of the plant extract on protein denaturation was studied using the method of Sakat et al. (2010), with minor modifications. The reaction mixtures in test tubes contained 50 μ l of various studied concentrations 10 - 80 μ g/ml of diclofenac sodium and test fractions and 50 μ l methanol as a control. 450 μ l of 5% w/v bovine serum albumin (BSA) was added to the above test tubes. The test tubes were incubated at 37°C for 20 min and then heated at 57°C for 10 min. After cooling, 2.5 ml phosphate buffered saline (pH 6.3) was added to each tube. The absorbance of these solutions was measured using double beam UV/visible spectrophotometer (T80 model, PG Instrument) at wavelength 660nm. The percent inhibition of denaturation was calculated as follows:

% inhibition = $(\text{Absorbance of control} - \text{Absorbance of treated})/\text{Absorbance of control} \times 100$

Experimental animals and care

120 to 200 g male albino rats (Wistar strain) purchased from

Babcock University animal house, Ilishan-Remo, Ogun State were used for the study. They were acclimatized for two weeks in the animal house; Babcock University Ilishan-Remo Ogun State. The animals were kept in aerated wooden cages under a natural light condition at room temperature (28 to 30°C) and fed with rat cubes (pellets) and water *ad libitum*. All the animal experiments and protocol conformed to the guidelines of National Institute of Health (NIH, 2000) for laboratory animal care and use.

Formaldehyde-induced arthritis assay

The protocol described by Eun-Michoi and Jae-Kwan (2004), was used for this experiment. Thirty (30) rats were divided based on their weights into six groups of five rats each. Arthritis was induced by injecting 0.02 ml formaldehyde (2% v/v) solution into the left hind paw of the rats; beneath the plantar aponeurosis on the first and third days 30 min after the oral administration of the standard drug and varied doses of the test extract and the administration of the drug and the extract continued for seven days.

Experimental design

Group I: Normal rats that were not induced with arthritis and given 0.5 ml of normal saline.

Group II: Control (untreated) rats that were induced with arthritis and given 0.5 ml of normal saline.

Group III: Arthritis induced rats that were treated with diclofenac sodium, a NSAID; (10 mg/kg) body weight.

Group IV: Arthritis induced rats that were treated with the hexane extract of *H. bonariensis* leaves (50 mg/kg) body weight.

Group V: Arthritis induced rats that were treated with the hexane extract of *H. bonariensis* leaves (100 mg/kg) body weight.

Group VI: Arthritis induced rats that were treated with the hexane extract of *H. bonariensis* leaves (250 mg/kg) body weight.

Measurement of paw thickness

The paw thickness of the rats was measured using a micrometer screw gauge for seven days. The change in paw size was obtained by subtracting the baseline values from values obtained during the period of the experiment. The baseline values are the values from measurements taken before the induction of arthritis.

Sacrificing of animal

Rats were made to fast overnight the seventh day, they were euthanized by cervical dislocation before being sacrificed, and blood was collected by cardiac puncture into lithium heparinised tubes for liver function tests (AST and ALT determination) and into EDTA tubes for the determination of hematological parameters.

Collection of plasma

Plasma for liver function tests was obtained by centrifuging the collected blood samples at 3000 g for 10 min.

Determination of plasma alanine amino transferase (ALT)

Assay of alanine aminotransferase (ALT) was carried out using the procedure provided by the Randox kit manufacturer and followed the principle described by Reitman and Frankel (1957).

Table 1. Phytochemicals identified in the hexane extract of *H. bonariensis* leaf.

Phytochemical	Presence	Concentration
Phenol	+	1.07 ± 0.003 mg(GE)/g
Flavonoid	+	1.36 ± 0.042 mg(QE)/g
Tannin	+	18.74 ± 0.050% *
Saponin	+	47.20 ± 1.600% *
Terpenoid	+	ND
Sterol	+	ND

Values are expressed as means ± standard deviation of the mean (n = 3). + Presence of phytochemicals; GE: Gallic acid Equivalent; QE: Quercetin Equivalent; *Phytochemicals having concentrations evaluated from the ground sample. ND: Not determined.

Determination of plasma aspartate amino transferase (AST) activity

The activity of AST was assayed using the procedure provided by the Randox kit manufacturer and followed the principle described by Reitman and Frankel (1957).

Determination of hematological parameters

The test to determine the hematological parameters of the rats was carried out at the hematological unit of Babcock University Teaching Hospital (BUTH). It was done using an auto-analyzer (Swelab Alfa 3- Part Hematology Analyzer by Boule Medicals).

Statistical evaluation

Unless otherwise stated, all values are expressed as mean ± standard deviation of triplicate readings. Where applicable, statistical evaluation was done using one way analysis of variance (ANOVA) followed by Duncan's Post Hoc Test (DPHT). The significance level was set at P<0.05.

RESULTS

Phytochemical analysis

The result of the phytochemical screening of the hexane extract of *H. bonariensis* leaves presented in Table 1 reveals the presence of terpenoid, steroids, phenol, flavonoid, tannin and saponin.

GC/MS analysis

Hexadecanoic acid methyl ester, faltarinol and phytol which are compounds that have been reported to exert anti-inflammatory effects were three of the major compounds suggested by the GC/MS analysis to be present in the hexane extract of *H. bonariensis* leaves (Table 2, Figure 1).

Table 2. Bioactivity of the compounds detected in the hexane extract of *H. bonariensis* leaf by the GC/MS.

Bioactive agent	Peak #	Retention time (min)	Area %	Reported bioactivity	References
Cyclohexasiloxane, dodecamethyl (Siloxane)	1	15.149	1.00	Antifungal properties	Mahmoud et al. (2013)
Caryophyllene (Terpenoid)	2	18.124	1.03	Anti-inflammatory, anti-carcinogenic, local anaesthetic activities, antioxidant, antibiotic	Legault and Pichette (2007)
1,3,6,10- Dodecatetraene,3,7,11-trimethyl-, (Z,E)- (Sesquiterpene)	3	18.611	1.02	Insect semiochemical; pheromones	Sobotnik et al. (2008); Hern and Dorn (1999)
3,5-Octadiene, 2,2,4,5,7,7- hexamethyl-, (E,Z)-	4	19.349	8.90	No reported bioactivity	
Spiro[5.5]undeca-1,8-diene,1,5,5,9-tetramethyl-,	5	20.138	1.27	No reported bioactivity	
Cycloheptasiloxane, tetradecamethyl-(Siloxane)	6	20.482	1.97	Antifungal properties	Mahmoud et al. (2013)
Caryophyllene oxide (Terpenoid)	7	23.068	1.47	Antifungal; Flavouring/Fragrance ingredient; Antibacterial, antitumor, anti-inflammatory	Yang et al. (1999); Antonella et al. (2013); Egharevba et al. (2012)
Silane,[[4[1,2bis [(trimethylsilyl) oxy]ethyl]-1,2-phenylene]bis(oxy)] bis[trimethyl-	8	25.334	2.37	No reported bioactivity	
5-Isopropyl-2-methylphenyl 3- methyl butanoate	9	26.570	0.70	No reported bioactivity	
Cyclononasiloxane, octadecamethyl	10, 15, 23, and 29	29.557, 33.333, 41.293, and 49.681	1.55, 1.42, 1.65, and 1.95	No reported bioactivity	
Bicyclo[3.1.1]heptane,2,6,6-trimethyl-, (1.alpha.,2.beta.,5.alpha.)	11	30.112	26.39	No reported bioactivity	
2-pentadecanone, 6, 10, 14-trimethyl	12	30.181	1.24	Antibacterial	Nurettin et al. (2006)
Bicyclo [4.1.0] heptane,3-methyl	13	30.564	0.74	No reported bioactivity	
Hexadecanoic acid,methyl ester	14	32.143	3.49	Anti-inflammatory	Cai et al. (2005)
Falcarinol	16 and 17	34.998 and 35.141	6.08 and 7.58	Anti-inflammatory; antioxidant; anticancer; antifungal; natural pesticide	Kobaek-Larsen et al. (2005); Christensen (2009)
9,12- Octadecadienoic acid,methyl ester	18	36.355	3.21	Influence synthesis of prostaglandins and other cell regulators	Magdi et al. (2009)
9,12,15- Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	19	36.555	3.18	Anti-inflammatory	Lalitharani et al. (2009)
Phytol (diterpene)	20	37.133	11.09	Transcription modulator, anti-inflammatory, fragrance	Gloerich (2005); Silva et al. (2013);IFRA (2004)
Hexasiloxane, tetradecamethyl-	21, 26, and 28	37.367, 44.382, and 46.992	1.84, 1.71, and 1.77	No reported bioactivity	
Tetracosane	25	43.988	0.72	Cytotoxicity activities	Uddin et al. (2012)
Hexadecane	27	45.893	0.57	Antibacterial, antioxidant	Yogeswari et al. (2012)
2,6,10,14,18,22- Tetracosahexane (Isomer of squalene ; a triterpene)	30	49.950	1.98	Intermediate in the biosynthesis of cholesterol, emmollient for skin, antioxidant, antitumor activities	Zih-Rou et al. (2009)

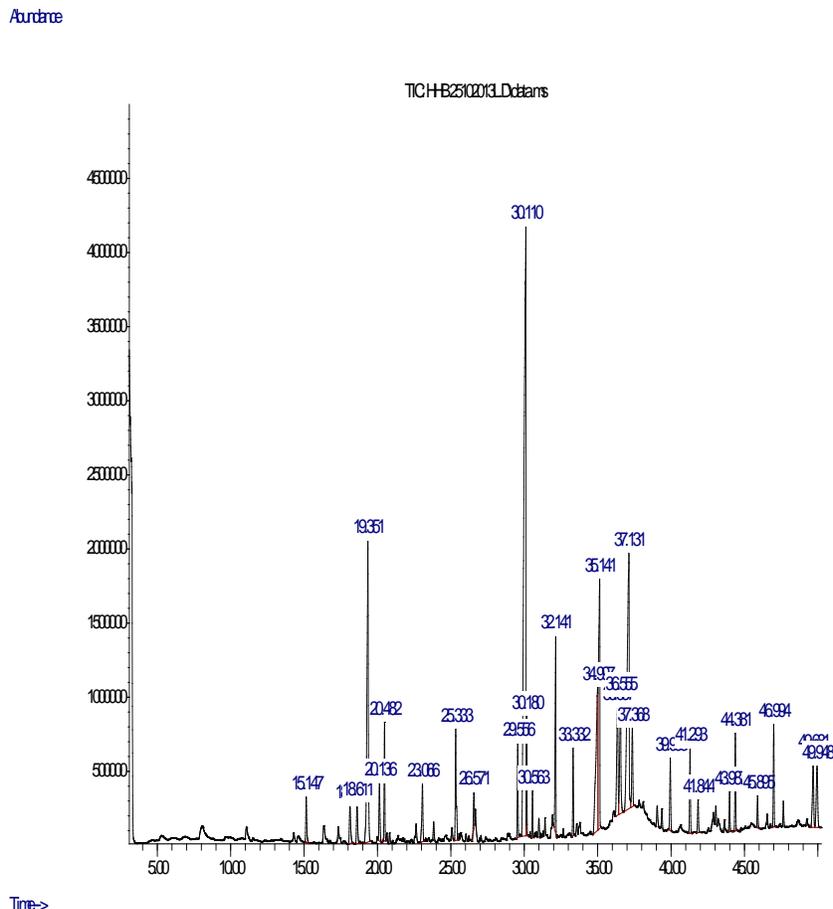


Figure 1. GCMS chromatogram of the hexane extract of *H. bonariensis* leaf.

Membrane stabilization

The hexane extract of *H. bonariensis* leaf showed stabilization with increase in concentration (30.16 ± 0.95 to $71.32 \pm 0.44\%$) and IC_{50} value of $117.37 \mu\text{g/ml}$. The standard drug, diclofenac sodium used as reference also did the same (52.65 ± 0.85 to $95.63 \pm 0.44\%$) with an IC_{50} value of $47.34 \mu\text{g/ml}$ (Figure 2).

Inhibition of protein denaturation

In this study, the hexane extract of *H. bonariensis* leaves was found to inhibit protein denaturation in a concentration-dependent manner (26.50 ± 6.45 to $69.23 \pm 5.12\%$) with an IC_{50} value of $44.84 \mu\text{g/ml}$ compared to standard drug diclofenac sodium which also did the same (46.15 ± 3.63 to $85.47 \pm 2.96\%$) with an IC_{50} value of $10.68 \mu\text{g/ml}$ (Figure 3).

Animal studies

The comparative inhibitory effect of the hexane extract of

H. bonariensis leaf and standard drug, diclofenac sodium on edema induced by the formaldehyde injection which was measured by change in paw thickness of the rats is presented in Figure 4. From the result, 250 mg/kg body weight oral administration of the hexane extract of *H. bonariensis* leaf showed significant ($P < 0.05$) reduction in the induced edema starting from the fourth day when compared to the control (untreated), thereby suggesting the anti-inflammatory action at this concentration. No significant reduction of the edema was observed from the administration of (50 and 100) mg/kg body weight of the extract.

The results from the hematological parameters shown in Figures 5 to 9 present the total White Blood Cell (WBC), the differential white cell count (lymphocytes, granulocytes, eosinophils, monocytes and basophils) and the total platelet count. From the results, consistency in reduction of these cells was indicated in the group of rats that were orally administered 250 mg/kg body weight hexane extract of *H. bonariensis* leaf, when compared to the control. Figures 10 and 11 present the results of the liver function tests (AST and ALT activity) of the rats. From the results oral administration of 250 mg/kg body weight of the hexane extract of *H. bonariensis* leaf

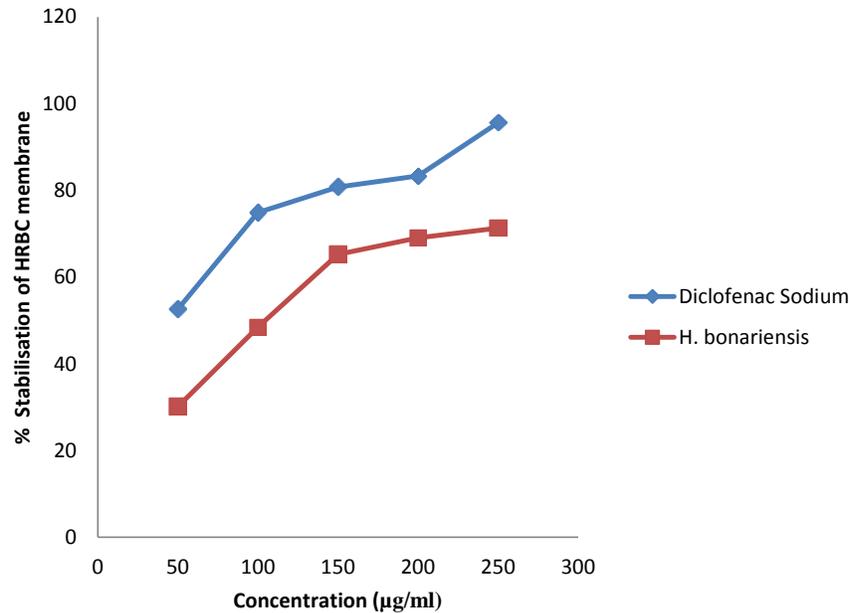


Figure 2. Percentage stabilization of human red blood cell (HRBC) membrane with diclofenac sodium and *H. bonariensis* extract. IC_{50} of diclofenac sodium = 47.34 µg/ml; IC_{50} of *H. bonariensis* leaf (hexane extract) = 117.37 µg/ml.

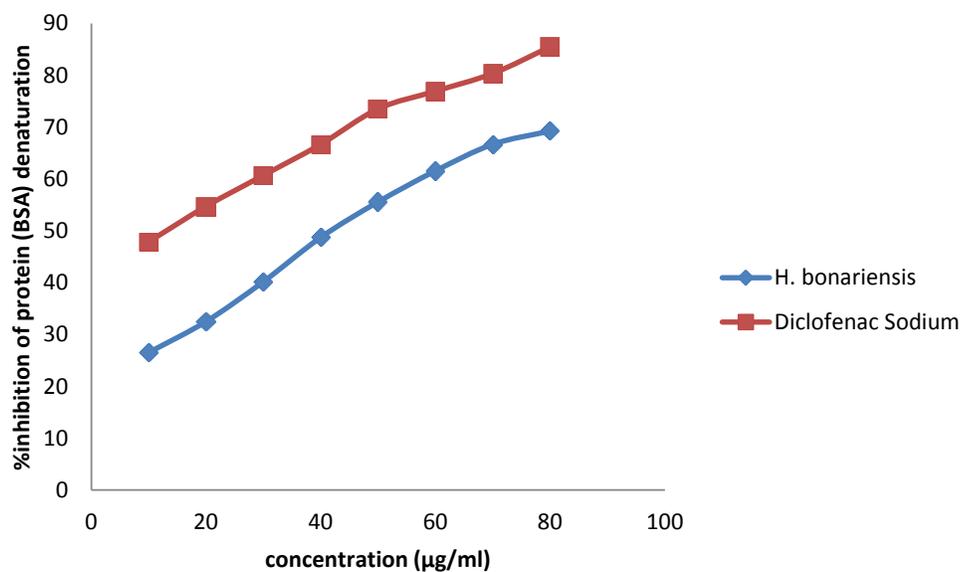


Figure 3. Percent inhibition of the denaturation of protein (BSA) by the hexane extract of *H. bonariensis* leaf and standard drug, diclofenac sodium. IC_{50} of diclofenac sodium = 10.68 µg/ml; IC_{50} of *H. bonariensis* leaf (hexane extract) = 44.84 µg/ml.

showed a significant reduction ($P < 0.05$) in AST and ALT activities in the rats' blood plasma when compared to the control. Oral administration of the extract at doses of 50 and 100 mg/kg body weight did not show a significant reduction ($P > 0.05$) in the activities of the enzymes.

DISCUSSION

The search for phytochemicals possessing anti-inflammatory properties has been on the rise due to their potential use in the therapy of various chronic and

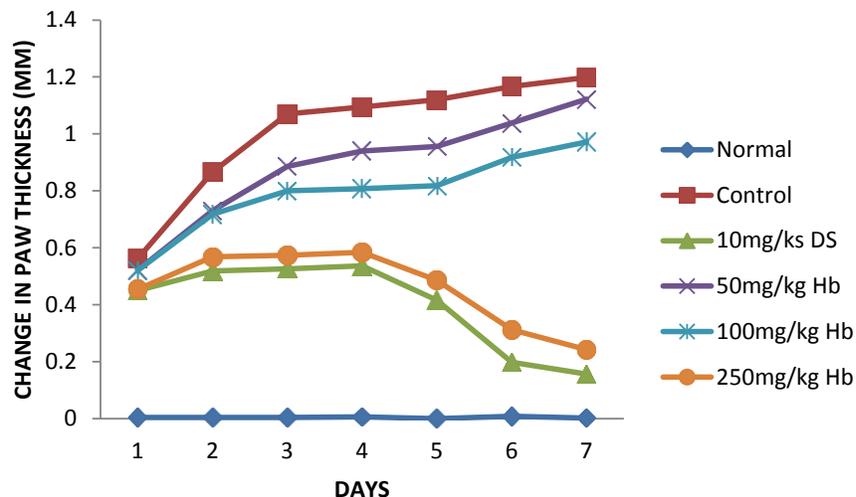


Figure 4. Comparative inhibitory effect of the hexane extract of *H. bonariensis* leaf and standard drug, diclofenac sodium on edema induced by the formaldehyde injection.

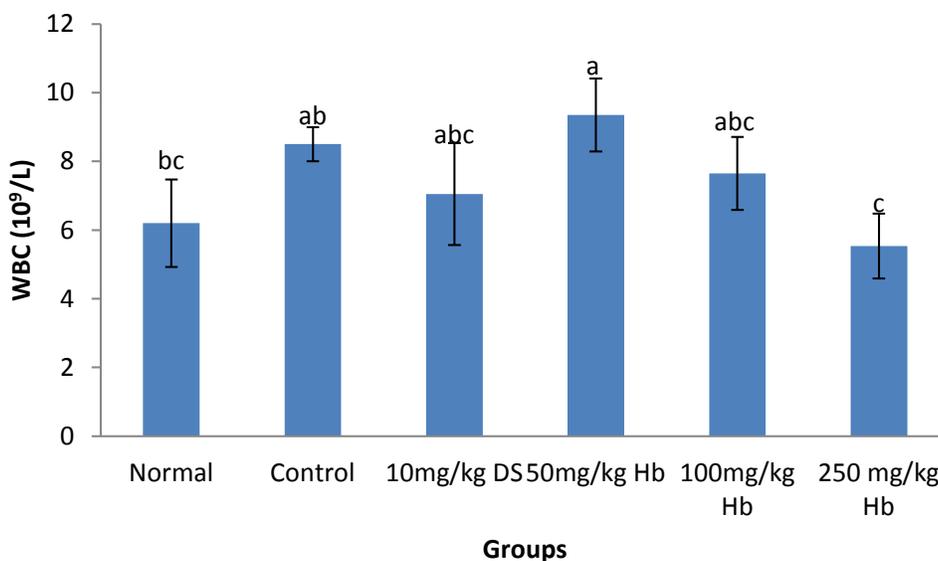


Figure 5. Total white blood cell (WBC). Values are means \pm standard deviation (n = 5); bars with different alphabets are significantly different ($P < 0.05$).

compounds have been found to possess potent anti-inflammatory activity (Sakat et al., 2010; Roy et al., 2010; Garg et al., 2010). Also, several authors have reported the anti-inflammatory properties of flavonoids in various studies (Antonella et al., 2013; Lopez-Lazaro, 2009; Yoshida et al., 2008; Amaral et al., 2009). Tannins have been reported to have potent anti-inflammatory properties (Souza et al., 2007; Fawole et al., 2010). The percentage of saponin in *H. bonariensis* leaf was found to be $47.20 \pm 1.60\%$. Saponins are well known to have anti-inflammatory activity (Uddin et al., 2012). Thus, the major

part of the anti-inflammatory properties of *H. bonariensis* could be due to the saponin content. The phytochemicals detected in this study are in agreement with those reported by Ajani (2012).

Hexadecanoic acid methyl ester, falcarinol and phytol which are compounds that have been reported to exert anti-inflammatory effects were three of the major compounds suggested in the hexane extract of *H. bonariensis* leaves. In studies with isolated kupffer cells treated with lipopolysaccharide (Cai et al., 2005), Hexadecanoic acid methyl ester showed anti-

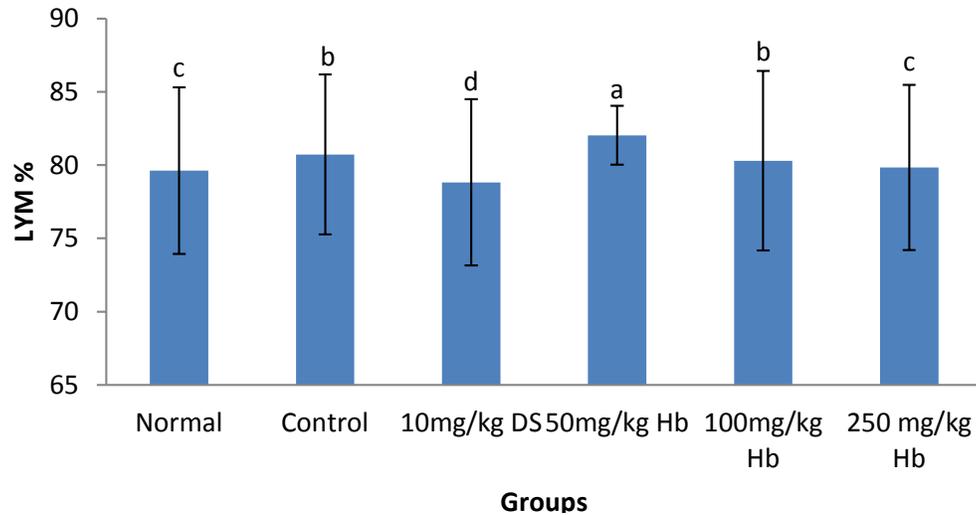


Figure 6. Percent lymphocytes (LYM). Values are expressed as means \pm standard deviation ($n = 5$); bars with different alphabets are significantly different ($P < 0.05$).

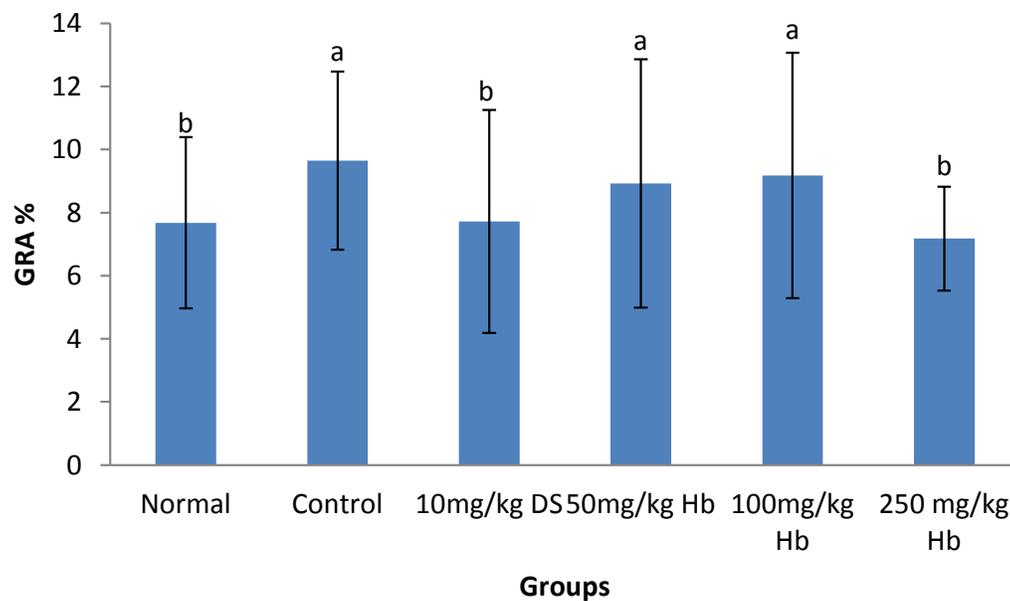


Figure 7. Percent granulocytes (GRA). Values are expressed as means \pm standard deviation ($n = 5$); bars with different alphabets are significantly different ($P < 0.05$).

1467 inflammatory activity by decreasing secretion of interleukin-10, tumor necrosis factor- α (TNF- α), nitric oxide, and prostaglandin E_2 . In a recent study by Silva et al. (2013), phytol was reported to inhibit the recruitment of total leukocytes and neutrophils; decreased MPO (myeloperoxidase) activity, tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β) levels, and MDA (malondialdehyde) concentration during carrageenan-induced acute inflammation. The study thus suggested that phytol attenuates the inflammatory response by

inhibiting neutrophil migration which is partly done by the reduction in IL-1 β and TNF- α levels. The anti-inflammatory mechanism of falcariol is still unclear, however the reactivity of falcariol towards mercapto and amino groups in proteins forming a hapten-protein complex (antigen), probably due to its hydrophobicity and its ability to form an extremely stable carbocation with the loss of water, thereby acting as a very reactive alkylating agent towards various biomolecules could explain its anti-inflammatory mechanism (Alan et al., 2006).

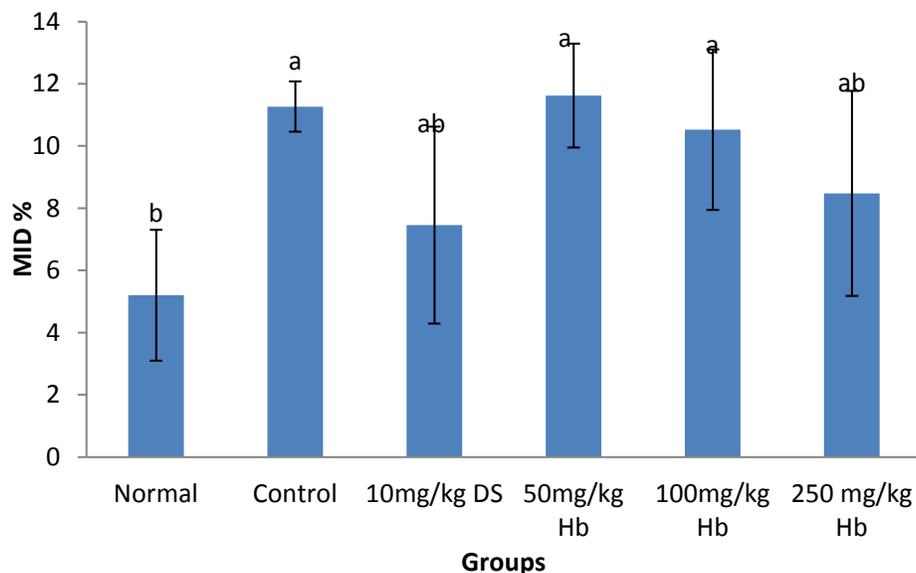


Figure 8. Percent eosinophils, monocytes and basophils (MID). Values are expressed as means \pm standard deviation ($n = 5$); bars with different alphabets are significantly different ($P < 0.05$).

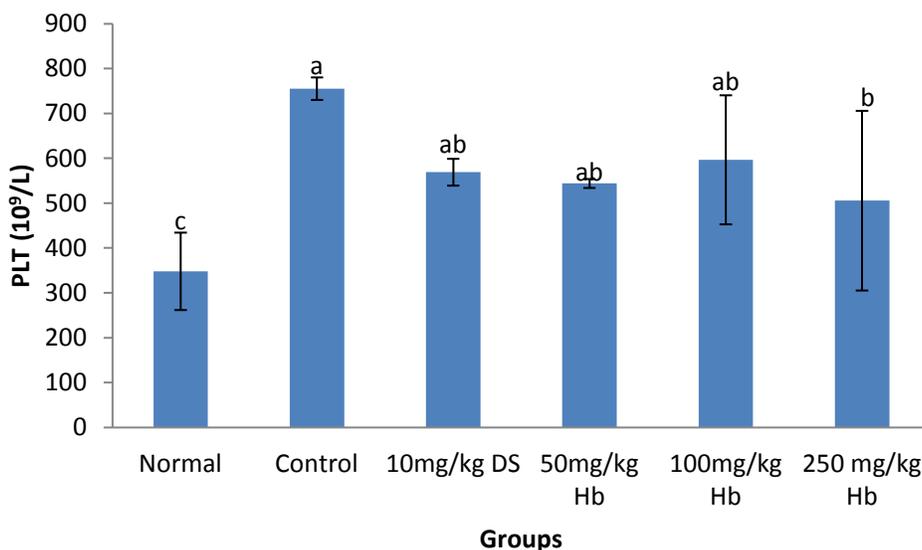


Figure 9. Total platelet count (PLT). Values are expressed as means \pm standard deviation ($n = 5$); bars with different alphabets are significantly different ($P < 0.05$).

The precise mechanism of membrane stabilization is yet to be elucidated. However, this effect could be possible when surface area/volume ratio of cells are brought about by an expansion of the membrane or the shrinkage of the cells and an interaction with membrane proteins (Shinde et al., 1999). The HRBC membrane stabilization has been used as a method to study the *in vitro* anti-inflammatory activity (Chou, 1997). Stabilization of the erythrocyte membrane is important in limiting the

anti-inflammatory response because it prevents the release of lysosomal constituents of activated neutrophils such as bactericidal enzymes and proteases, which cause further tissue inflammation and damage upon extracellular release (Chou, 1997). Some NSAIDs are known to possess membrane stabilization properties which may contribute to the potency of their anti-inflammatory effect. These nonsteroidal drugs act either by inhibiting these lysosomal enzymes or by stabilizing

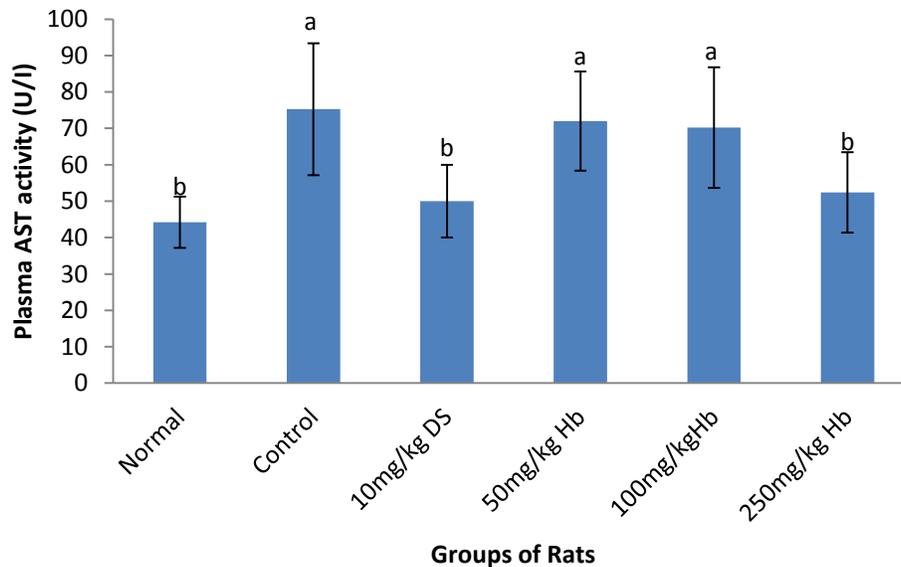


Figure 10. Plasma aspartate aminotransferase (AST) activity (UI). Values are expressed as means \pm standard deviation ($n = 5$); bars with different alphabets are significantly different ($P < 0.05$).

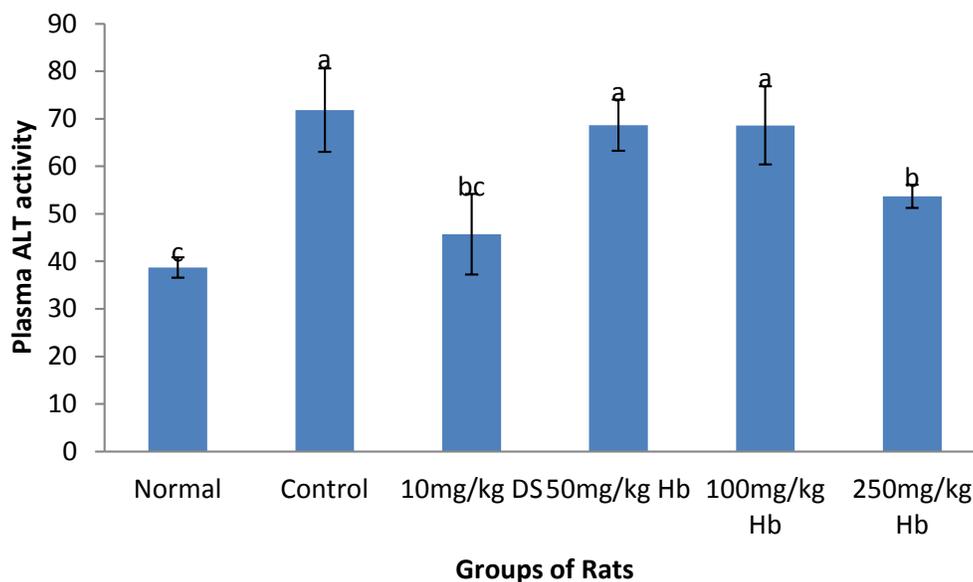


Figure 11. Plasma alanine aminotransferase (ALT) activity (UI). Values are expressed as means \pm standard deviation ($n = 5$); bars with different alphabets are significantly different ($P < 0.05$).

the lysosomal membrane (Manjunatha et al., 2013). From the results shown in this study, *H. bonariensis* appears to possess membrane stabilization properties which may contribute to the potency of the anti-inflammatory effect of the plant.

Denaturation of tissue proteins is one of the well-documented causes of inflammatory and arthritic diseases. Production of auto antigens in certain arthritic diseases may be due to denaturation of proteins *in vivo*

(Opie, 1962; Umaphy et al., 2010). Agents that can prevent protein denaturation therefore, would be worthwhile for anti-inflammatory drug development. The anti-inflammatory drugs (salicylic acid, phenylbutazone etc), have shown dose-dependent ability to inhibit protein denaturation and similar results have been observed from many reports from plant extract (Sakat et al., 2010). The result thus agrees with the earlier reports. The extract could also possibly inhibit the release of the lysosomal

content of neutrophils at the site of inflammation (Chou, 1997).

The various NSAIDs exert their effects by the pharmacological inhibition of COX (cyclooxygenase enzyme), responsible for the formation of biological mediators called prostanoids which include prostaglandins, prostacyclin and thromboxane that give rise to pain and inflammation. The consistent reduction in the studied hematological parameters (total white blood cell count, granulocytes, lymphocytes, eosinophils, monophils, basophils and total platelet count), observed with the oral administration of 250 mg/kg body weight of the hexane extract of *H. bonariensis* leaf when compared to the control could suggest that it could inhibit the cyclooxygenase (COX) enzyme, thus relieving pain and inflammation.

Enzymes are the best markers of tissue damage because of their specificity and catalytic activity to the tissue (Sivakumar et al., 2007). Increased AST levels in the plasma of the control rats and (50 and 100) mg/kg b.w hexane extract of *H. bonariensis* leaf treated rats may have been due to the leakage of this enzyme from hepatocyte cells as a result of hepatocellular damage (Xing-Jiu et al., 2006) caused as a result of the induction of arthritis. AST and ALT are membrane bound enzymes, thus the reduction in the plasma activity of these enzymes observed at the oral administration of 250 mg/kg body weight of the extract suggests its ability to stabilize the liver membrane, which could have prevented the leakage of these enzymes into the plasma. The results obtained in both of the liver function tests (AST and ALT) are in correlation with each other. This agrees with what has been reported by Xing-Jiu et al. (2006) that the amount of AST and ALT in the blood is directly related to the extent of the tissue damage.

Conclusion

Results from this study indicate that the hexane extract of *H. bonariensis* leaves could possess anti-inflammatory properties. These activities may be due to the occurrence of phytochemicals such as alkaloids, flavonoids, tannins, steroids, saponins, terpenoids and phenols present in the extract. The potent anti-inflammatory activity observed by the oral administration of the extract at a dose of 250 mg/kg body weight gives an idea that the compounds of the plant could be used as a lead compound for designing a potent anti-inflammatory drug.

Conflict of Interests

The authors have not declared any conflict of interests.

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