

Anti-Inflammatory Effects of The Chloroform Extract of *Annona muricata* Leaves on Phospholipase A₂ and Prostaglandin Synthase Activities

Oyekachukwu AR, Elijah JP, Eshu OV* and Nwodo OFC

Faculty of Biological Sciences, Department of Biochemistry, University of Nigeria, Nsukka, Enugu State, Nigeria

Abstract

This study ascertained the mechanisms of the anti-inflammatory activity of the total lipid (chloroform) extract of *Annona muricata* leaves. The plant material was extracted with a mixture of chloroform and methanol (2:1) and partitioned with 0.2 volume water. The chloroform extract was investigated for its effect on the *in vitro* activities of phospholipase A₂, prostaglandin synthase and membrane stabilization. The extract significantly ($p < 0.05$) inhibited phospholipase A₂ activity in a concentration-related manner compared to the control, with a range of 0.2 - 0.6 mg/ml inhibiting the enzyme activity by 23.91 - 43.48%. Effect of the extract on prostaglandin synthase activity showed a significant ($p < 0.05$) inhibition of enzyme activity at the doses 0.1, 0.5 and 1.0 mg/ml compared to the control. The highest percentage inhibition (87.46%) attained at 0.5 mg/ml was comparable to that of 1.0 mg/ml indomethacin. At various concentrations (0.1-0.8 mg/ml), the chloroform extract also significantly ($p < 0.05$) inhibited heat and hypotonicity-induced haemolysis of human red blood cells (HRBCs) compared to the control. The highest percentage inhibition of heat-induced haemolysis (53.03%) was obtained at 0.4 mg/ml of the extract while the highest percentage inhibition of hypotonicity-induced haemolysis (77.91%) was obtained at 0.8 mg/ml. This study thus confirmed that the mode of action of this extract of *Annona muricata* leaves on inflammation could be through the inhibition of phospholipase A₂ and prostaglandin synthase activities and by membrane stabilization.

Keywords: *Annona muricata*; Anti-inflammatory; Phospholipase A₂; Prostaglandin synthase; Membrane stabilization; HRBCs: Human Red Blood Cell Membranes

*Corresponding author:

Okpashi Victor Eshu

✉ vic2reshu@gmail.com

Faculty of Biological Sciences, Department of Biochemistry, University of Nigeria, Nsukka, Enugu State, Nigeria.

Tel: +2348037636808

Citation: Oyekachukwu AR, Elijah JP, Eshu OV, Nwodo OFC (2017) Anti-Inflammatory Effects of The Chloroform Extract of *Annona muricata* Leaves on Phospholipase A₂ and Prostaglandin Synthase Activities. *Transl Biomed.* Vol.8 No.4:137

Received: November 14, 2017; **Accepted:** December 18, 2017; **Published:** December 26, 2017

Introduction

Inflammation is a pervasive phenomenon that operates during severe perturbations of homeostasis, such as infection, injury and exposure to contaminants [1]. The triggering of inflammation leads to the release of inflammatory mediators which can either be cell-derived or plasma-derived. Cell-derived mediators include: vasoactive amines, cytokines, nitric oxide, prostaglandins, thromboxane A₂ (T_XA₂), prostacyclin, leukotrienes and platelet-activating factor. They are released from inflammatory cells such as leukocytes, platelets and vascular endothelial cells. Mediator-producing systems in plasma include the complement, clotting, fibrinolytic and kinin systems. Acute inflammatory response is

defined as a series of tissue responses that occur within the first few hours following injury [2]. It is initiated by resident tissue macrophages, mast cells and endothelial cells (Anosike [3]). Vascular responses in the acute inflammatory response include vasodilatation and increased vascular permeability which leads to the leakage of plasma protein-rich fluid from the vascular compartment into the extravascular space. Acute inflammation also involves the emigration of leukocytes from the vascular to the extravascular compartment. There is also activation of platelets and their aggregation and the release of proteases and oxidants from phagocytes during acute inflammation [2]. The outcomes of acute inflammation are complete resolution, healing by connective tissue replacement, abscess formation

or progression to chronic inflammation. The resolution of inflammation is important to protect the host from overt tissue damage and amplification of the acute inflammatory response towards chronicity. Non-steroidal anti-inflammatory drugs (NSAIDs) and corticosteroids are classically used to alleviate inflammation. Long term use of NSAIDs causes side effects including gastric ulceration and renal toxicity [4] since they concurrently inhibit both isoforms of prostaglandin synthase or cyclooxygenase (COX). The development of NSAIDs which are selective COX inhibitors still have side-effects as they have been connected with an increased risk of heart attack and stroke [5]. There is therefore, a need for potent anti-inflammatory drugs with fewer side effects. *Annona muricata* Linn (Soursop) has potentials in curbing inflammation as it is a rich source of active phytoconstituents that provide medicinal benefits against various ailments and diseases.

Annona muricata is a lowland tropical fruit-bearing tree in the Annonaceae family, which is a family of flowering plants. Annonaceae is the largest family of the Magnoliales order and comprises of approximately 130 genera and 2,300 species [6]. *Annona muricata* is a species of the genus *Annona*, derived from the Latin word 'anon' which means 'yearly produce' or 'annual harvest'. As the name suggests, various species in this genus generally have annual fruit production [7]. Other common species include *Annona squamosa* (sweetsop), *Annona senegalensis* (wild soursop), *Annona reticulata* (custard apple), *Annona cherimoya* (cherimoya) and the hybrid, *Annona atemoya* (cross between cherimoya and sweetsop). *Annona muricata* is the most tropical of the group and bears the largest fruits. It is commonly known as Graviola, Soursop or Gunbanana. In Nigeria, it is known in Igbo, Yoruba and Hausa as Sawonsop, Ebo and Tuwon Biri respectively. It is an underutilized [8] tree grown from sea level to 1000 m and adapted to areas of high humidity and relatively warm winters. It has alternate, large, glossy, leaves oval to lanceolate in shape, which are dark green on the upper surface and lighter green beneath. They have short petioles, 14 to 16 cm in length and 5 to 7 cm in width [9]. Temperatures below 5°C will cause damage to the leaves and small branches and temperatures below 3°C can be disastrous. All parts of *Annona muricata* are extensively used as traditional medicines against an array of human ailments and diseases. Previous reports on *Annona muricata* reveal it possesses anti-oxidant effect Baskar [10] anti-proliferative effect [11,12] and anti-trypanosomal effects [13], anti-bacterial effect [14,15], anti-hepatotoxic activity [16,17], wound healing activity [18]. The antinociceptive and anti-inflammatory effect of the ethanol extract of the plant in animal models has also been reported [19-21]. Despite studies on the anti-inflammatory activity of *Annona muricata* leaves, there is little or no empirical evidence on its anti-inflammatory mechanisms of action. This study thus aimed at determining the mechanisms of the anti-inflammatory action of the chloroform extract of the leaves of *A. muricata*.

Materials and Methods

Plant materials

Fresh leaves of *Annona muricata* were collected from Onuiyi-Nsukka in Nsukka Local Government Area of Enugu State, Nigeria

at Longitude- 7.4093°E and Latitude- 6.8683°N. The leaves were authenticated by Mr. Alfred Ozioko of the Bioresources Development and Conservation Programme (BDCP) Research Centre, Nsukka, Enugu State. The plant Voucher is number- InterCEDD/16089. Bambara seed used as source of bambara oil was purchased from Orba market, Enugu State.

Blood samples

The blood samples were collected by an experienced phlebotomy, from Two (2) female volunteers, between 33 and 28 years with their consent and were free from drug treatments for at least two weeks. The blood was collected into EDTA bottles to prevent coagulation.

Bacterial organism

The bacterial organism used for the study was *Bacillus cereus*. The organism was cultured and obtained from the Department of Microbiology, University of Nigeria, Nsukka.

Bovine seminal vesicle

Bovine seminal vesicle used for this study was obtained fresh from the abattoir (Ikpa Market, Nsukka).

Chemicals

All the chemicals used were of analytical grade. They include Chloroform (Kermel), methanol (Emsure), distilled water (Lavans, Nigeria), sodium hydroxide, tween 80 (Qualikems), MacConkey agar, calcium chloride, petroleum ether, glutathione, hydroquinone (Kermel, China), citric acid, ethylacetate (JHD, China), potassium hydroxide, indomethacin (Elble Pharma Veranhandles, Germany) and prednisolone.

Extraction procedure

The fresh leaves of *A. muricata* were cleaned and shade-dried for three weeks. The dried leaves were pulverised into coarse form using a mechanical grinder after which a known weight (1,200 g) of the pulverised leaves was macerated in chloroform - methanol (2:1). The suspension was left for 48 hr with occasional stirring and subsequently filtered using a muslin cloth. Further filtration was achieved with Whatman no.4 filter paper so as to remove the fine residue. Distilled water which measured up to 20% of the volume of the filtrate was added to the filtrate and the mixture shaken vigorously so as to enable the development of two layers. The upper layer (aqueous-methanol layer) was separated from the lower layer (chloroform layer) using a separation funnel. The chloroform layer was concentrated in a rotary evaporator to obtain the crude dark-green, slurry-like extract which was weighed and stored in the refrigerator until used. The method for extraction was a modification of the method described by Odo [22].

Determination of the effect of the extract on phospholipase A₂ activity

The effect of the extract on phospholipase A₂ activity was determined using modifications of the methods of [23,24]. Fresh human blood samples were centrifuged at 3,000 rpm for 10 min and the supernatant (plasma) discarded. The red cells were washed three times with equal volume of normal saline,

measured and reconstituted as a 40% (v/v) suspension with normal saline. This served as the substrate for phospholipase A₂.

Bacterial enzyme preparation was obtained from *Bacillus cereus* strain culture. The organism was cultured in MacConkey agar for three days and the culture transferred into a test tube containing normal saline. This was centrifuged at 3,000 rpm for 10 min after which the bacterial cells settled at the bottom of the test-tube while the supernatant contained the enzyme preparation that was used for enzyme assay.

Aliquots (0.1 ml) of re-suspended erythrocytes (substrate), normal saline (1.2 ml), 2 mM calcium chloride (0.2 ml), 1 ml varying concentration of extract (0.2, 0.4 and 0.6 mg/ml) in 3% tween 80 (dissolved in normal saline) and 0.2 ml of the enzyme preparation were incubated at 37°C for 1 hr. Control tubes contained 0.1 ml of the erythrocytes, 1.2 ml normal saline, 0.2 ml calcium chloride, 1 ml 3% tween 80 and 0.2 ml of the enzyme preparation. The blanks contained everything in the test except the enzyme. After incubation, the reaction mixture was centrifuged at 3,000 g for 10 min and the absorption of the supernatant read against the blank at 418 nm. Prednisolone (0.4 mg/ml), a known inhibitor of the enzyme was used as the standard control. The percentage inhibition of enzyme activity was calculated thus:

$$\% \text{ Enzyme activity} = \frac{\text{Absorbance of test}}{\text{Absorbance of control}} \times 100$$

$$\% \text{ inhibition} = 100 - \% \text{ Enzyme activity}$$

Determination of the effect of the extract on prostaglandin synthase activity

This was assayed using the methods of [25,26]. The method of Nugteren [27] was used in the isolation of the enzyme from bovine seminal vesicle while the method of Harold [28] was used in the preparation of the substrate. The bovine seminal vesicle was frozen, partially thawed and freed of fat and connective tissues. A 50g quantity of the tissue was weighed out, sliced, allowed to chill in a freezer and homogenised in 40 ml of 0.02 M Tris-HCl buffer, pH 7.6 for 2 min at 4°C with a blender. The homogenate was centrifuged at 6,000 rpm for 10 min after which the supernatant was decanted and centrifuged again at 15,000 rpm for 10 min at 4°C. The supernatant was again decanted and centrifuged at 18,000 rpm for 10 min. The resulting supernatant was used as the crude enzyme preparation. Bambara nut oil was used as the source of the substrate (arachidonic acid). The Bambara oil was extracted using petroleum ether by soxhlet method and filtered. The filtrate was concentrated at 60°C to obtain the substrate.

The crude enzyme preparation (7 mg) was weighed into each set of test tubes followed by the addition of 1.5 ml of cofactor solution (33 mM hydroquinone, 2 mM glutathione and 40 μM haemoglobin in the ratio 1:1:8 respectively). The mixture was allowed to preincubate for 2 min at 37°C. The reaction was started by adding 0.2 ml of the substrate and allowed to proceed for 2 min at 37°C. Varying concentrations of the extract and buffer were also added to make the volume up to 2.5 ml. Only buffer was added in the control tube. The reaction mixture was

incubated for 2 min after which the reaction was terminated by the addition of 0.5 ml of 0.2 M citric acid. The reaction mixture was then extracted twice with 5 ml ethyl acetate and centrifuged at 2,500 rpm for 10 min. For each extraction, the top organic layer was pipetted out into a clean test tube. The combined ethyl acetate extract was evaporated to dryness on a sand bath. The residue was dissolved in 2 ml methanol. Then, 0.5 ml of 3 M potassium hydroxide solution was added to the solution and allowed to stand for 15 min. The absorbance of the tests against blanks at 37°C was read at 278 nm. The blanks contained exactly the same substances as in the test solution except that, the enzyme was boiled (denatured) in cofactor solution and cooled before use (Nwodo). Duplicate determinations were made with the assay mixture containing 0.1, 0.5 and 1.0 mg/ml of the extract. Indomethacin (1.0 mg/ml) was used as the standard drug. Enzyme activity and percentage inhibition of enzyme activity was calculated using the relations:

$$\% \text{ Enzyme activity} = \frac{\text{enzyme activity of test}}{\text{enzyme activity of control}} \times 100$$

$$\% \text{ Enzyme activity} = \frac{\text{enzyme activity of test}}{\text{enzyme activity of control}} \times 100$$

$$\% \text{ inhibition} = 100 - \% \text{ Enzyme activity.}$$

Determination of the effect of the extract on membrane stabilization

The effect of the chloroform extract of *A. muricata* leaves on membrane stabilization was determined using both heat and hypotonicity induced haemolysis of HRBCs.

Heat-induced haemolysis of human red blood cells

This was evaluated using modifications of the method of Shinde et al. [29]. Fresh blood samples (3 ml each) were collected from healthy adult volunteers into EDTA bottles and centrifuged at 3,000 rpm for 10 min and the supernatant discarded. A volume of normal saline equivalent to that of the supernatant was used to dissolve the red blood pellets. The volume of the dissolved red blood pellets was measured and reconstituted as a 40% (v/v) suspension with normal saline. The reconstituted red blood cells were used as such.

Samples of the extract and indomethacin used were dissolved in 3% tween 80 in normal saline. A set of five test tubes containing respectively, 5 ml graded doses of the extracts (0.1, 0.2, 0.4, 0.6 and 0.8 mg/ml) were arranged in quadruplicates (4 sets per dose). Control tubes contained 5 ml of the vehicle and 5 ml of 0.2 mg/ml indomethacin respectively. HRBC suspension (0.1 ml) was added to each of the tubes and mixed gently. A pair of the tubes was incubated in a regulated water bath at 54°C for 20 min while the other pair was maintained in the freezer at -15°C for 20 min. afterwards, the tubes were centrifuged at 1300 rpm for 3 min and the haemoglobin content of the supernatant was estimated using a spectrophotometer at 540 nm. The blanks contained varying concentrations of the extract, indomethacin and vehicle (for control) without HRBC suspension. The percentage inhibition

of haemolysis by the chloroform extract or indomethacin was calculated thus:

$$\% \text{Inhibition of Haemolysis} = 100 \left(1 - \frac{0D_2 - 0D_1}{0D_3 - 0D_1} \right) \times 100 \quad (1)$$

Where OD_1 = Absorbance of test sample (extract or indomethacin) unheated.

OD_2 = Absorbance of test sample heated.

OD_3 = Absorbance of control sample heated.

Hypotonicity-induced haemolysis of human red blood cells

This was determined using modifications of the method of [30]. Blood samples (3 ml each) obtained from healthy volunteers were placed into EDTA bottles, centrifuged at 3,000 rpm for 10 min and washed three times with equal volumes of normal saline. For each, the blood volume was measured and reconstituted as a 40% (v/v) suspension with normal saline.

Samples of the extract and indomethacin used were dissolved in 3% tween 80 in distilled water which served as the hypotonic solution. Aliquots (1 ml) of varying concentrations of the extract (0.1, 0.2, 0.4, 0.6, 0.8 mg/ml) were respectively added to each of a set of five test-tubes. Another tube contained 1 ml of 0.4 mg/ml indomethacin. The contents of the respective tubes were made up to 4.9 ml with the vehicle (3% tween 80 dissolved in distilled water). Two control tubes were used for this test. A control tube contained 4.9 ml of the vehicle while another contained 4.9 ml of 3% tween 80 dissolved in normal saline (isotonic solution). HRBCs suspension (0.1 ml) was added to each tube, and after gentle mixing, the mixtures were incubated for 1 hr at 37°C. After incubation, the reaction mixture for each tube was centrifuged at 3,000 g for 10 min and the absorbance of the supernatant measured at 418 nm using a spectrophotometer. The tests were carried out in triplicates. Reaction media containing 1 ml varying concentrations of extract or indomethacin made up to 5 ml with 3% tween 80 in normal saline, without HRBCs suspension were used as the respective blank for the tests. The blank for the control tubes contained 3% tween 80 in normal saline also without HRBCs suspension. The percentage inhibition of haemolysis was calculated using the relation:

$$\% \text{Inhibition of Haemolysis} = 100 \left(1 - \frac{0D_2 - 0D_1}{0D_3 - 0D_1} \right) \times 100 \quad (2)$$

Where OD_1 = Absorbance of control I (isotonic solution)

OD_2 = Absorbance of test sample

OD_3 = Absorbance of control II (hypotonic solution)

Statistical analysis

The data obtained were expressed as mean \pm SD and were analysed using Statistical Product and Service Solutions (SPSS), version 18. Tests of statistical significance were carried out using one-way Analysis of Variance (ANOVA). Significant levels were at $p < 0.05$.

Results

Phytochemical constituents of the chloroform extract of *Annona muricata* leaves

Phytochemical analysis showed that flavonoids and phenols were found to be present in very high concentration while terpenoids, alkaloids, tannins and soluble carbohydrates were found to be moderately present. The concentration of steroids, saponins, reducing sugars, hydrogen cyanides and glycosides were found to be minimal (**Table 1**).

Effect of the extract on phospholipase A_2 activity

The chloroform extract of *A. muricata* leaves at 0.2, 0.4 and 0.6 mg/ml significantly ($p < 0.05$) inhibited the activity of phospholipase (PLA_2) in a concentration-related manner. This is shown by the progressive increase in the percentage inhibition of enzyme activity (**Table 2**).

Effect of the extract on prostaglandin synthase activity

As shown in **Table 3**, the chloroform extract of *A. muricata* leaves at 0.1, 0.5 and 1.0 mg/ml significantly ($p < 0.05$) inhibited prostaglandin synthase activity compared to the control. The inhibition of enzyme activity at 0.5 mg/ml of the extract (87.46%) was comparable to that of 1.0 mg/ml indomethacin.

Effect of the extract on membrane stabilization

Effect of the extract on heat-induced haemolysis of human red blood cells: At various concentrations (0.1, 0.2, 0.4, 0.6 and 0.8 mg/ml), the chloroform extract of *A. muricata* leaves significantly ($p < 0.05$) inhibited heat-induced haemolysis of HRBCs compared to the control. This is shown by the reduced absorbance values of the heated solution relative to the control. The highest percentage inhibition (53.03%) of haemolysis was obtained at 0.4 mg/ml of the extract. The anti-haemolytic effect at 0.1 mg/ml of the extract was comparable to that obtained for 0.2 mg/ml indomethacin (**Table 4**).

Table 1 Phytochemical constituents of the chloroform extract of *Annona muricata* leaves.

Phytochemical constituents	Relative abundance
Flavonoids	+++
Terpenoids	++
Steroids	+
Saponins	+
Alkaloids	++
Tannins	++
Reducing sugars	+
Soluble carbohydrates	++
Hydrogen cyanides	+
Glycosides	+
Phenols	+++
*minimally present	
**moderately present	
+++highly present	

Table 2 Effect of the chloroform extract of *Annona muricata* leaves on phospholipase A₂ activity.

Treatment	Concentration (mg/ml)	OD (nm)	Percentage enzyme activity (%)	Percentage inhibition of enzyme activity (%)
Control	-	0.46 ± 0.04 ^c	100	-
Extract	0.2	0.35 ± 0.04 ^b	76.09	23.91
	0.4	0.28 ± 0.01 ^a	60.87	39.13
	0.6	0.26 ± 0.01 ^a	56.52	43.48
Prednisolone	0.4	0.29 ± 0.03 ^a	63.04	36.96

Results are expressed in means ± standard deviation; n=3. Mean values having different letters as superscripts across the column are considered significant (p<0.05).

Table 3 Effect of the chloroform extract of *Annona muricata* leaves on prostaglandin synthase activity.

Treatment	Concentration (mg/ml)	OD (nm)	Enzyme activity (IU)	Percentage enzyme activity	Percentage inhibition of enzyme activity (%)
Control	-	0.88 ± 0.03 ^c	13.64	100	-
Extract	0.1	0.20 ± 0.01 ^b	3.1	22.73	77.27
	0.5	0.11 ± 0.00 ^a	1.71	12.54	87.46
	1	0.15 ± 0.00 ^a	2.33	17.08	82.92
Indomethacin	1	0.11 ± 0.01 ^a	1.71	12.54	87.46

Results are expressed in means ± standard deviation; n=2. Mean values having different letters as superscripts across the column are considered significant (p<0.05).

Table 4 Effect of the chloroform extract of *Annona muricata* leaves on heat-induced haemolysis of human red blood cells.

Treatment	Concentration (mg/ml)	Absorbance (nm)		
		Heated solution	Unheated solution	Percentage inhibition of haemolysis (%)
Control	-	0.70 ± 0.08 ^c	0.05 ± 0.01	-
Extract	0.1	0.52 ± 0.07 ^b	0.06 ± 0.01	28.13
	0.2	0.45 ± 0.10 ^{ab}	0.04 ± 0.01	37.88
	0.4	0.35 ± 0.01 ^a	0.04 ± 0.00	53.03
	0.6	0.37 ± 0.02 ^a	0.02 ± 0.00	48.53
	0.8	0.36 ± 0.00 ^a	0.02 ± 0.01	50
Indomethacin	0.2	0.52 ± 0.00 ^b	0.07 ± 0.01	28.57

Results are expressed in means ± standard deviation; n=2. Mean values having different letters as superscripts across the column are considered significant (p<0.05).

Table 5 Effect of the chloroform extract of *Annona muricata* leaves on hypotonicity-induced haemolysis of human red blood cells.

Treatment	Concentration (mg/ml)	Absorbance (nm)	Percentage inhibition of haemolysis (%)
Control (hypotonic solution)	-	0.99 ± 0.06 ^e	-
Control (isotonic solution)	-	0.13 ± 0.04	-
Extract	0.1	0.73 ± 0.17 ^d	30.23
	0.2	0.57 ± 0.04 ^{cd}	48.84
	0.4	0.41 ± 0.06 ^{bc}	67.44
	0.6	0.45 ± 0.17 ^{bc}	62.79
	0.8	0.32 ± 0.17 ^{ab}	77.91
Indomethacin	0.4	0.75 ± 0.14 ^d	27.91

Results are expressed in means ± SD; n=2. Mean values having different letters as superscripts are considered significant (p<0.05).

Effect of the extract on hypotonicity-induced haemolysis of human red blood cells: From the data shown in **Table 5**, the chloroform extract of *A. muricata* leaves significantly (p < 0.05) protected the human erythrocyte membrane against lyses induced by hypotonic solution compared to the control. This is indicated by the reduction in the absorbance values of the extract relative to the hypotonic control solution. The highest

percentage inhibition (77.91%) of haemolysis was obtained at 0.8 mg/ml of the extract.

Discussion

This study revealed the mechanisms of the anti-inflammatory action of the chloroform extract of *A. muricata* leaves have been established using inhibition of phospholipase A₂ and

prostaglandin synthase activities, membrane stabilization and platelet aggregation. Phospholipase A₂ is an enzyme that cleaves free fatty acids from membrane phospholipids. Arachidonic acid released from these phospholipids is acted upon by COX and lipoxygenase (LOX) both of which lead to the *de novo* synthesis of lipid mediators George [31]. The action of COX on arachidonic acid produces mediators such as T_XA₂, prostaglandin E₂, D₂ and I₂, while the action of 5-LOX on arachidonic acid releases leukotrienes such as leukotriene B₄ (LTB₄). The chloroform extract of *A. muricata* leaves from 0.2 to 0.6 mg/ml exhibited significant ($p < 0.05$) and concentration-dependent inhibition of PLA₂ activity. This was shown by reduced absorbance as the inhibition prevented the action of PLA₂ on erythrocyte membrane, causing reduced leakage of haemoglobin which absorbs maximally at 418 nm. This inhibition of PLA₂ activity by the extract also implies that it was able to suppress the release of free fatty acids from red blood cell (RBC) membrane phospholipids and the consequent deprivation of COX and LOX precursors for the synthesis of inflammatory mediators, hence limiting their effects such as vasodilatation, vascular permeability, chemotaxis and pain, thus preventing inflammation. This inhibition also implies that the extract has potentials in curing atherosclerosis and cancer as PLA₂ has been implicated in their aetiology Sato [32]. The mechanism of inhibition of PLA₂ by the extract could be like that of corticosteroids which induce lipocortin [33]. This effect could be attributed to the presence in the extract of phytoconstituents such as flavonoids and tannins [34-36].

The chloroform extract also exhibited a significant ($p < 0.05$) inhibition of prostaglandin synthase activity with the inhibition at 0.5 mg/ml comparing well with that of 1.0 mg/ml indomethacin. This effect was found to be concentration-independent, contrary to that obtained by previous researchers using different plant extracts [37-39] which established a concentration-dependent inhibition of the enzyme activity. This inhibition of COX by the extract implies that, it does not only prevent the release of free fatty acids but also prevents COX from acting on the fatty acids when released, thereby preventing the synthesis of inflammatory mediators. The extract could have possibly exerted this effect by competitively inhibiting COX like indomethacin [39]. This inhibitory effect could be due to the presence of flavonoids in the extract as they can inhibit arachidonic acid metabolizing enzymes such as PLA₂, COX and 5-LOX [40,41]. The sequential inhibition of PLA₂ and COX leads to potent suppression of the synthesis of inflammatory mediators, which implies amplification of the anti-inflammatory activity of the extract. This inhibition of PLA₂ activity has previously been confirmed by Oliveira [42] using aqueous extract of *Annona muricata*. Thus, the action of PLA₂ on the erythrocyte membrane causes the release of free fatty acids from membrane phospholipids thereby causing leakage. This allows haemoglobin to flow into the medium in the process. Decrease in absorbance values relative to the control is associated to decrease in the amount of haemoglobin in the medium. From **Table 2**, the chloroform extract of *A. muricata* leaves significantly ($p < 0.05$) decreased the amount of haemoglobin in a concentration-related manner from 0.2 to 0.6 mg/ml, which corresponds to the activity of PLA₂.

The preponderance of polyunsaturated fatty acids in the RBC membrane makes the cells highly susceptible to oxidative damage [43] leading to haemolysis by which haemoglobin and other internal cell components are released into the surrounding fluids. Red blood cell membrane can be lysed due to its exposure to injurious substances such as hypotonic medium, heat and methyl salicylate. This study demonstrated the capability of the plant extract to inhibit the lyses of HRBCs membrane induced by hypotonic solution and heat. The chloroform extract of *A. muricata* leaves at concentrations of 0.1 to 0.8 mg/ml was able to stabilize significantly ($p < 0.05$) HRBC membrane, which is an indication of the ability of the plant extract to prevent haemolysis. The anti-haemolytic effect of *A. muricata* leaves against Triton-X-100 induced lyses of HRBCs has been previously confirmed by Mounnissamy et al. [44] though a concentration-related inhibition was established in contrast to this research. The inhibition of RBC membrane lysis was used as a measure of the mechanism of anti-inflammatory activity of *A. muricata* leaves extract because HRBC membranes are analogous to lysosomal membrane components [45,46]. The stabilization of erythrocyte membranes by the extract thus implies that it may also stabilize lysosomal membranes. Stabilization of lysosomal membranes is important in limiting the inflammatory response by preventing the release of lysosomal constituents of activated leukocytes such as, bactericidal enzymes and proteases, which upon extracellular release cause further tissue inflammation and damage [47,48].

Prostaglandin synthase oxidizes the free fatty acid released from membrane phospholipids leading to the production of prostaglandins. Alkaline treatment (using KOH, from the methods) of the synthesized prostaglandin E₂ results in the formation of prostaglandin B which absorbs maximally at 278 nm. Absorption at 278 nm thus corresponds to the enzyme activity. The lower the absorbance value relative to control, the lower prostaglandin B formed, the lower the enzyme activity and the higher the percentage inhibition of enzyme activity. The extract (0.5 mg/ml) produced the highest inhibition of enzyme activity while 0.1 mg/ml produced the least (**Table 3**).

Lyses of the red blood cell lead to the release of haemoglobin. The lower the quantity of haemoglobin released, the lower the absorbance value. At various concentrations (0.1, 0.2, 0.4, 0.6 and 0.8 mg/ml), the chloroform extract of *A. muricata* leaves significantly ($p < 0.05$) reduced haemolysis of HRBCs induced by heat compared to the control. This is shown by the reduced absorbance values of the heated solution relative to the control. The highest percentage inhibition (53.03%) of haemolysis was obtained at 0.4 mg/ml of the extract (**Table 4**).

From the data shown in **Table 5**, the chloroform extract of *A. muricata* leaves significantly ($p < 0.05$) protected the human erythrocyte membrane against lyses induced by hypotonic solution compared to the control. This leads to decrease in haemoglobin released, indicated by the reduction in the absorbance values of the extract relative to the control (hypotonic solution). The highest percentage inhibition (77.91%) of haemolysis was obtained at 0.8 mg/ml of the extract.

This result thus shows that membrane stabilization is part of the mechanisms of the anti-inflammatory effect of *A. muricata*. Both membrane stabilization and phospholipase A₂ activity are related to low calcium availability. The haemolytic effect of hypotonic solution could be related to the uptake of water by the cells through osmosis, resulting in swelling and the subsequent rupturing of its membrane, releasing intracellular electrolyte and fluid components. This implies that the extract could have possibly stabilized the RBC membrane by inhibiting processes which enhance the efflux of these intracellular components, such as by preventing the release of lytic enzymes and mediators of inflammation and by inhibiting the activity of phospholipase A₂. Stabilization of the membrane also prevents the leakage of serum protein and fluids into the tissues during a period of increased vascular permeability caused by inflammatory mediators [48].

References

- 1 Ashley NT, Zachary ZW, Nelson RJ (2012) Inflammation: Mechanisms, costs, and natural variation. *Annual Review of Ecology, Evolution and Systematics* 43: 385-406.
- 2 Ward PA (2010) The inflammatory Response - An overview. In: *Fundamentals of Inflammation*. Serhan CN, Ward PA and Gilroy DW (Eds) Cambridge University Press, Cambridge, New York, USA. pp. 1-10.
- 3 Anosike CA, Obidoa O Ezeanyika LUS (2012) Membrane stabilization as a mechanism of the anti-inflammatory activity of methanol extract of garden egg (*Solanum aethiopicum*). *Daru* 20: 76.
- 4 Ezekwesili CN, Obidoa O, Nwodo OFC, Ezekwesili-Ofili JO (2011) Toxicity of *Acalypha torta* (Muell) leaves ethanolic extract in mice and rat. *Animal Research International* 8: 1315-1322.
- 5 Salmon SD (2006) Cyclooxygenase-2 inhibitors and cardiovascular risk. *Curr Opin Cardiol*. 21: 613-617.
- 6 Mishra S, Ahmad S, Kumar N, Sharma BK (2013) *Annona muricata* (the cancer killer): A review. *Global Journal of Pharmaceutical Research* 2: 1613-1618.
- 7 Southampton Centre for Underutilised Crops (SCUC) (2006) *Annona: Annona cherimola, A. muricata, A. reticulata, A. senegalensis and A. squamosa*. Field Manual for Extension Workers and Farmers, University of Southampton, Southampton, UK.
- 8 Heenkenda HMS, Pushpakumara DKNG, Ranil RHG, Thantirige MK (2011) *Annona* species in: Underutilized fruit trees in Sri Lanka. Vol 2. Pushpakumara DKNG, Gunasena HPM and Singh VP (Eds.) World Agroforestry Centre, South Asia Office, New Delhi. pp. 158-182.
- 9 Pinto ACde Q, Cordeiro MCR, De Andrade SRM, Ferreira FR, Filgueiras Hade C, et al. (2005) Taxonomy and Botany. In: *Annona species*. Williams et al. (Eds.) International Centre for Underutilised Crops, University of Southampton, Southampton, UK. pp. 9-11.
- 10 Baskar R, Rajeswari V, Kumar TS (2007) *In vitro* antioxidant studies in leaves of *Annona* species. *Indian J Exp Biol*. 4: 480-485.
- 11 Moghadamtousi SZ, Kadir HA, Paydar M, Rouhollahi E, Karimian H (2014) *Annona muricata* leaves induced apoptosis in A549 cells through mitochondrial-mediated pathway and involvement of NF- κ B. *BMC Complement Altern Med*. 14: 1-13.
- 12 Pieme CA, Kumar SG, Dongmo MS, Moukette BM, Boyoum FF, et al. (2014) Antiproliferative activity and induction of apoptosis by

Conclusion

This study indicates that the mechanisms of anti-inflammatory action of the chloroform extract of *A. muricata* leaves might be via the inhibition of the synthesis of inflammatory mediators, achieved by inhibiting the activities of phospholipase A₂ and prostaglandin synthase. The extract also acts by stabilizing the RBC membrane, and by extension, the lysosomal membrane. The active agent might therefore, serve as an adjuvant to the conventional drugs. Further studies should be directed at determining if the extract inhibits the two isoforms of COX (COX-1 and COX-2) or if it is a COX selective herb. The capability of the extract to inhibit 5-LOX should also be investigated. In addition, the active agent should be isolated and studied for further drug development.

Annona muricata (Annonaceae) extract on human cancer cells. *BMC Complement Altern Med*. 14: 516-520.

- 13 Onyeyili PA, Aliyoo K (2015) *In vitro* and *in vivo* evaluation of antitrypanosomal activity of *Annona muricata* stem bark extracts. *Herba Polonica* 61: 50-62.
- 14 Vieira GHF, Mourao JA, Angelo AM, Costa RA, Vieira SDF (2010) Antibacterial effect (*in vitro*) of *Moringa oleifera* and *Annona muricata* against gram positive and gram negative bacteria. *Rev Inst Med Trop Sao Paulo* 52: 129-132.
- 15 Chukwuka KS, Ikheloa JO, Okonko IO, Moody JO, Mankinde TA (2011) The antimicrobial activities of some medicinal plants on *Escherichia coli* as an agent of diarrhoea in livestock. *Adv Appl Sci Res* 2: 37-48.
- 16 Agbai EO, Nwanegwo CO (2013) Effect of methanolic extract of *Annona muricata* seed on liver function enzymes in alloxan-induced diabetic male mice. *Journal of Science and Multidisciplinary Research* 5: 74-83.
- 17 Oyedepo TA (2014) Hepatoprotective activity of *Annona muricata* leaf extract against paracetamol induced hepatotoxicity. *Journal of Advances in Scientific Research and Applications* 1: 115-120.
- 18 Padmaa PM, Chansouria JPN, Khosa RL (2009) Wound healing activity of *Annona muricata* extract. *J Pharm Res* 2: 404-406.
- 19 De Sousa OV, Vieira GDV, De Pinho JJRG, Yamamoto CH, Alves MS (2010) Antinociceptive and anti-inflammatory activities of the ethanol extract of *Annona muricata* Linn leaves in animal models. *Int J Mol Sci* 11: 2067-2078.
- 20 Roslida AH, Tay CE, Zuraini A, Chan PF (2010) Anti-inflammatory and antinociceptive activities of the ethanolic extract of *Annona muricata* leaf. *Journal of Natural Remedies* 10: 97-104.
- 21 Chan PF, Roslida AH (2012) Evaluation of anti-inflammatory activities of ethanolic extract of *Annona muricata* leaves. *Braz. J Pharmacogn* 22: 1301-1307.
- 22 Odo CE, Nwodo OFC, Joshua PE, Ugwu OPC (2014) Acute toxicity investigation and anti-diarrhoeal effect of the chloroform-methanol extract of the leaves of *Persea americana*. *Iran J Pharm Res* 13: 651-658.
- 23 Vane JR (1971) Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. *Nat New Biol* 231: 232-235.
- 24 Morimoto S, Kazuna S, Kawai K (1979) Effects of some anti-rheumatic agents on copper-catalysed thermal aggregation of gamma albumin. *Agents Actions* 9: 375-380.

- 25 Yoshimoto A, Ito H, Tomita K (1970) Cofactor requirement of the enzyme synthesizing prostaglandin in bovine seminal vesicles. *J Biochem* 68: 487-499.
- 26 Flower RJ, Cheung HS, Cushman DW (1973) Quantitative determination of prostaglandin and malondialdehyde formed by the arachidonate oxygenase (Prostaglandin synthase) system of bovine serum vesicle. *Prostaglandins* 4: 325-341.
- 27 Nwodo OFC (1981) Elucidation of the nature of some pharmacologically active substances extractible from the seeds of *Abrus precatorius*. A Ph.D thesis submitted to the University of London, UK.
- 28 Nugteren, DH, Beerthius RK, Vandorp DA (1966) Enzymic conversion of all-8,11,14 eicosatrienoic acid into prostraglandin E. *Recueil des Travaux Chimiques Pays-Bas* 85: 1757-1765.
- 29 Harold E, Kirk RS, Sawyer R (1981) Nitrogen and crude protein. In: *Pearson's Chemical Analysis of Food*. (8th edn). Churchill, Livingstone. pp. 15-23.
- 30 Shinde UA, Phadke AS, Nari AM, Mungantiwar AA, Dikshit VJ, et al. (1989) Membrane stabilization activity - A possible mechanism of action for the anti-inflammatory activity of *Cedrus deodora* wood oil. *Fitoterapia* 70: 251-257.
- 31 Oyedepo OO, Akindede VR, Okunfolami OK (1997) Effect of extracts of *Olax subsoroides* and *Aspila africanaon* bovine red blood cells. *Phytotherapy Research* 11: 305-306.
- 32 George A, Chinnappan S, Chintamaneni M, Kotak CV, Choudhary Y, et al. (2014) Anti-inflammatory effects of Polygonum minus (Huds) extract (Lineminus™) in *in-vitro* enzyme assays and carrageenan induced paw oedema. *BMC Complement Altern Med*. 14: 1-7.
- 33 Sato H, Taketomi Y, Isogai Y, Masuda S, Kobayashi T, et al. (2009) Group III secreted phospholipase A₂ transgenic mice spontaneously develop inflammation. *Biochem J* 421: 17-27.
- 34 Schimmer BP, Parker KL (2001) Adrenocorticotrophic hormone, adrenocortical steroids and their synthetic analogs, inhibitors of the synthesis and actions of adrenocortical hormones. In: *Goodman and Gilman's the Pharmacological Basis of Therapeutics*. (10th edn). Gilman AG, Hardman JG and Limbird LE (Eds.) McGraw Hill Co., New York, USA. pp. 1649-1677.
- 35 Kim HP, Park H, Son KH, Chang HW, Kang SS (2008) Biochemical pharmacology of biflavonoids: Implications for anti-inflammatory action. *Arch Pharm Res* 31: 265-273.
- 36 Barbosa DBM, Nascimento MVM, Lino RC, Magalhães MR, Florentino IF, et al. (2012) Mechanism involved in the anti-inflammatory effect of *Spiranthera odoratissima* (Manacá). *Braz J Pharmacogn* 22: 137-143.
- 37 Iwueke AV, Nwodo OFC, Okoli CO (2006) Evaluation of the anti-inflammatory and analgesic activities of *Vitex doniana* leaves. *Afr J Biotechnol* 5: 1929-1935.
- 38 Ekwueme FN, Oje OA, Nwodo OFC, Ozoemena NF (2011) Anti-inflammatory capacity of the aqueous leaf extract of *Senna mimosoides* on inhibition of rat oedema, platelet aggregatory activity and prostaglandin synthase activity. *J Med Plants Res* 5: 3028-3036.
- 39 Enechi OC, Nwodo OFC (2015) The mechanisms of anti-inflammatory action of *Buchholzia coriacea* seed extract. *Jokull Journal* 65: 10-33.
- 40 Hemalatha K, Madhumitha G, Roopan SM (2013) Indole as a core anti-inflammatory agent - A mini review. *Chem Sci Rev Lett* 2: 287-292.
- 41 Oliveira AP, Sa I, Pereira DM, Goncalves RF, Andrade PB, et al. (2017) Exploratory studies on the *in vitro* anti-inflammatory potential of two herbal teas (*Annona muricata* L. and *Jasminum grandiflorum* L.), and relation with their phenolic composition. *Chem Biodiversity* 14: 1-11.
- 42 Nagamani JE, Divya N, Prabhu S (2012) Antioxidant and anti-hemolytic activities of *Bombax Ceiba* Pentandra spike and fruit extracts. *Int J Pharm Pharm Sci* 4: 311-315.
- 43 Muthu S, Durairaj B (2015) Inhibitory effect of hydroethanolic extracts of *Annona muricata* on human platelet aggregation and haemolysis *in vitro*. *J Pharm Pharm Sci* 2: 207-213.
- 44 Mounnissamy VM, Kavimani S, Balu V, Drlin QS (2008) Evaluation of anti-inflammatory and membrane stabilizing properties of ethanol extract of *Canjera rehedii*. *Iranian Journal Pharmacology and Therapeutics* 6: 235-237.
- 45 Mohammadi Z, Atik F (2014) Haemolytic activity of different herbal extracts used in Algeria. *International Journal of Pharmaceutical Sci Res* 5: 495-500.
- 46 Cunha TM, Verri WAJ, Poole S, Parada CA, Cunha FQ, et al. (2007) Pain facilitation by pro-inflammatory cytokine actions at peripheral nerve terminals. In: *Immune and Glial Regulation of Pain*. Sorkin L, DeLeo J, Watkins, LR and Seattle WA (Eds.) International Association for the Study of Pain, USA. pp. 67-83.
- 47 Borregaard N (2010) Neutrophils, from marrow to microbes. *Immunity* 33: 657-670.
- 48 Chaitanya R, Sandhya S, David B, Vinod KR, Murali S (2011) HRBC membrane stabilizing property of root, stem and leaf of *Glochidion velutinum*. *Int J Pharm Biomed Res* 2: 256-259.