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Aqueous Ethanol Fruit Extract of *Xylopia aethiopica* and Xylopic Acid Exhibit Anti-inflammatory Activity Through Inhibition of the Arachidonic Acid Pathway

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Abstract

In the form of a decoction, *X. aethiopica* is traditionally employed in the treatment of bronchitis, asthma, arthritis and rheumatism in Ghana, Nigeria and Cameroon. This study establishes the inhibitory effect of *X. aethiopica* and its principal constituent, xylopic acid, on the arachidonic acid pathway of inflammation. Hydrogen sulphide is endogenously produced in mammalian tissues from the enzymatic activity of *cystathionine β-synthase* and *cystathionine γ-lyase* on L-cysteine. Its involvement in the local and systemic inflammatory process has been documented in animal models. Recent studies have identified its activation of phospholipase A_2 as the mechanism involved in its anti-inflammatory role. We henceforth conducted this study to investigate the inhibitory role of *X. aethiopica* and its principal constituent xylopic acid on hydrogen sulphide-induced inflammation and eventual effect on the activation of phospholipase A_2. Sodium hydrogen sulphide (NaHS) was injected into the mouse hind paw and oedema was monitored for 60 min. Paws were examined by the histological method. *X. aethiopica* extract (30, 100, 300 mg kg^{-1}) suppressed the mean maximal swelling attained at 15 min to 42.58±1.66%, 42.58±1.66% and 34.91±2.39%, respectively compared to the mean inflamed control response of 61.51±3.90%. The total paw swellings induced over the 1 h were also significantly suppressed by 31.82±5.28%, 36.19±6.01%, and 31.65±5.16%, respectively. The xylopic acid (10, 30, 100 mg kg^{-1}) suppressed the mean maximal swelling attained at 15 min to 41.75±1.24%, 40.26±1.68% and 38.26±2.52%, respectively relative to the control response while the total paw swelling was significantly suppressed by 34.08±4.49%, 38.19±4.50% and 43.40±5.09% respectively relative to the inflamed control response. Histologically, there was a significant ($P < 0.0001$) reduction in cytoplasmic vacuolation, inflammation, cellular degeneration, loss of tissue organisation and necrotic tissues with administration of *X. aethiopica* extract and xylopic acid in the inflamed paws of the mice. This study establishes that *X. aethiopica* extract, and xylopic acid mediate their anti-inflammatory actions in part through inhibition of hydrogen sulphide-induced inflammation and subsequently has an inhibitory role on phospholipase A_2 activation.

1 Introduction

Xylopia aethiopica is a tropical evergreen plant with fragrant seeds; generally utilized as a part of the type of the dried organic product decoction to treat bronchitis, asthma, joint inflammation and ailment in Ghana, Nigeria and Cameroon[1].

We have previously documented its anti-inflammatory activity in both acute[2] and chronic[3] inflammation in murine models of

inflammation. Present in *Xylopia aethiopica* are kaurane diterpenes, mainly kaurenoic acid and xylopic acid, which showed a noteworthy part in the established biologic impact of the plant[4]. This study, therefore tend to illustrate some instrument of mitigating activity of the aqueous ethanol fruit extract of *Xylopia aethiopica* and its vital constituent obtained from its bio-fractionation, xylopic corrosive.

The pro-inflammatory role of hydrogen sulphide (H_2S) has been well documented in different animal models[5-7]. Hydrogen sulphide is endogenously produced in mammalian tissues from the enzymatic activity of *cystathionine β-synthase* and *cystathionine γ-lyase* on L-cysteine. A marked increase in H_2S production in murine hind paw injected with carrageenan has been investigated by Bhatia and co-authors[6]. Recent studies have identified its activation of phospholipase A_2 as the mechanism involved in its anti-inflammatory role. Indeed, Western blot analysis has shown the expression of *cystathionine γ-lyase* (CSE) and *cystathionine β-synthase* (CBS) in acute inflammatory response induced with carrageenan, signifying the involvement of the L-cysteins/H_2S pathway[8]. Intraplantar injection of H_2S or L-cysteine induces oedema which is of rapid onset (0-60 min) which implies the involvement of the H_2S/L-cysteine pathway. The H_2S-oedema has been shown to have a similar profile to that of PLA_2-induced oedema.

The actuation of secretory PLA_2 ($sPLA_2$) continues through the introduction of a water particle by hydrogen bond association to the active site histidine of the enzyme. Adjoining this histidine, there is a conserved aspartate residue, which, together with a Ca^{2+}-binding loop, acts as a ligand cage for Ca^{2+} [9]. Thus, Ca^{2+} and water are two key elements implicated in PLA_2 activation. Interestingly, H_2S could provide both these elements necessary for PLA_2 activation. H_2S has a three-dimensional structure close to that of H_2O, but weaker intermolecular forces, and H_2S also induced entry of extracellular Ca^{2+} [10]. Thus, it is feasible that H_2S could activate PLA_2, either through Ca^{2+} entry and/or substituting for a molecule of H_2O. Additionally, oxidative adjustment of phospholipids can change the physiological condition of the plasma membrane, which influences the vulnerability of oxygenated and non-oxygenated fatty acid residues towards sPLA2 assault multifacetedly[9]. Therefore, an alternative or additional explanation for PLA_2 activation by H_2S could be that H_2S, being a reducing agent, may alter the cellular redox status, triggering prostaglandin production.

The isoform $sPLA_2$ assumes a noteworthy part in the incendiary procedure through an activity on the arachidonate pathway[11,12]. Hence if a drug significantly attenuates H_2S-induced inflammation, then it possibly has an inhibitory effect on $sPLA_2$ activation. That was the basis of our investigation of the possible inhibitory role on H_2S-induced inflammation by the fruit extract of *Xylopia aethiopica*, and its principal constituent, xylopic acid.

2 Materials and Method

2.1. Materials

2.1.1. Preparation of plant extract

The fruits of *Xylopia aethiopica* were collected from the Kwame Nkrumah University of Science and Technology (KNUST) Botanical Gardens (6°41'7" N 1°33'48" W), Kumasi between September and November, 2011 and identified by anatomical observation and direct comparison with the authentic specimens, stored in the Herbarium in the Department of Herbal Medicine, KNUST, Kumasi. A voucher specimen (No. FP/09/77) was deposited in the same department. The fruit was sun dried and 3 kg of the dried fruit was ground using heavy duty blender (37BL85 (240CB6), WARING Commercial, USA) and extracted with 70% $^{v}/_{v}$ ethanol (5 L) by maceration for 24 h. The ethanol filtrate was concentrated under reduced pressure at 60 °C by a vacuum rotary evaporator (R-210, BUCHI, Switzerland) and further dried in an oven (Gallenkamp OMT, SANYO, Japan) to yield a solid mass of weight 167 g. The dried extract, XAE was freshly emulsified with Tween-80 and prepared with normal saline before use.

2.1.2 Extraction and purification of xylopic acid

The extraction of xylopic acid was carried out based on the method described by Ekong and Ogan[13]. About 1.37 kg of the pulverized dried fruit of *Xylopia aethiopica* was placed in cylindrical jars, soaked with 5 L of petroleum ether (40-60 °C) and allowed to stand for 3 days. The petroleum ether extract was collected and concentrated using a vacuum rotary evaporator (Rotavapor R-210, BUCHI, Switzerland) at a temperature of 50°C. Five (5) ml ethyl acetate was added to the concentrate and allowed to stand for 2 days after which the xylopic acid crystals formed were washed with petroleum ether (40-60 °C).

The xylopic acid obtained was purified by recrystallization. The recrystallization process involved the dissolution of 32 g the impure xylopic acid in 96% ($^{v}/_{v}$) ethanol. The resulting concentrated solution was filtered while hot, and crystals of xylopic acid were deposited after the solution cooled and stood for two days. The yield of the isolated/purified xylopic acid was 1.47% ($^{w}/_{w}$). The purity of the isolated xylopic acid was assessed using high performance liquid chromatography (HPLC) as described by Woode *et al.* (2012)[14].

The chromatograph consisted of LC-10AT Shimadzu pump with a programmable absorbance detector (783A Applied Biosystems) and Shimadzu CR501 Chromatopac. Phenomenex Hypersil 20 micron C18 200 × 3.20 mm column was used. The mobile phase comprised of methanol, and water (9:1) eluted

isocratically at 0.5 ml min^{-1}. 20 µl portions of a suitable concentration of pure xylopic acid were loaded and injected unto the column after dissolving in the mobile phase at 60 °C. The eluent was monitored at 206 nm. Portions of the *Xylopia aethiopica* extract and xylopic acid isolated were loaded and injected. The peak(s) were noted as component(s) of the *Xylopia aethiopica* and xylopic acid. The purity of the isolated xylopic acid was 95% ($^{w}/_{w}$). The pure compound when required was constituted as an emulsion and henceforth referred to as xylopic acid (XA).

2.2. Experimental animals

Institute of Cancer Research (ICR) mice (25-30 g) were purchased from Noguchi Memorial Institute for Medical Research, University of Ghana, Legon, Accra, Ghana and kept in the Animal facility of the Department of Pharmacology, College of Health Sciences, KNUST, Kumasi, Ghana. All animals were humanely handled throughout the experimental period in accordance with Animal Welfare Regulations (USDA 1985; US Code, 42 USC § 289d) and the Public Health Service Policy on Humane Care and Use of Laboratory Animals (PHS 2002). Additionally, all animal experiments were approved by the Department of Pharmacology, KNUST Ethics Committee. Animals were acclimated upon arrival for a week before assigned randomly to their respective groups. Mice (5/cage) were housed in a polypropylene cage in a temperature-controlled room (22±3 °C) on a 12-h light-dark cycle with free access to commercial pellet diet purchased from Agricare, Tafo, Ghana and water *ad libitum*. All animals were euthanized at the end of each experiment. Each animal was therefore used only once.

2.3. Chemicals and reagents

Aspirin, Sodium hydrogen sulphide and Dexamethasone were purchased from Sigma-Aldrich (St Louis, USA). Potassium phosphate buffer was a kind donation from Department of Pharmaceutical Chemistry, KNUST, Kumasi.

2.4 Methods

2.4.1 Hydrogen sulphide-induced acute inflammation

This study employed the method described by Bianca *et al.* (2010)[8]. ICR mice (20-30 g) received intraplantar injection of NaHS (500 µg per paw) as an exogenous source of H_2S or vehicle [100 µl potassium phosphate buffer (PPS), pH 7.4]. Aspirin (100 mg kg^{-1} *p. o.*), dexamethasone (1 mg kg^{-1} *p.o.*), XAE (30, 100 and 300 mg kg^{-1} *p.o.*) or XA (10, 30 and 100 mg kg^{-1} *p.o.*) were given 90 min before the NaHS injection. Paw oedema was measured by means of water displacement plethysmometry (Ugo Basile, Comerio, Italy) at 15, 30, 45 and 60 min after the intraplantar injection of NaHS. Raw scores for right foot volume were individually normalised as percentage of change from their values at time 0 and then averaged. Percentage changes in paw volume was calculated, and the maximal oedema and total oedema responses calculated as described below:

The maximal oedema response was calculated from the formula:

$$\% \text{ change in paw thickness} = 100 \times \left[\frac{(V_t - V_i)}{V_i}\right]$$

Where V_i is paw volume before hydrogen sulphide injection

V_t is paw volume at time T.

Total pedal oedema was calculated in arbitrary units as the area under the curve (AUC) and to determine the percentage inhibition of oedema, the following equation was used:

$$\% \text{ inhibition of oedema} = \left[\frac{AUC_{(control)} - AUC_{(treatment)}}{AUC_{(control)}}\right] \times 100$$

2.4.2 Histological analysis

In this study, mice treated with 300 $mgkg^{-1}$ XAE and 100 $mgkg^{-1}$ XA were used. Acute inflammation was induced with hydrogen sulphide as described above. The mice were sacrificed by cervical dislocation 30 min after the NaHS injection. Mice paws were fixed in neutral buffered formalin before being embedded in paraffin. Sections (4 mm) were stained with haematoxylin-eosin and analysed, by an observer unaware of the treatment protocol. Histological slides were scored according to the following parameters: loss of tissue organization, inflammation, necrosis, cellular degradation and cytoplasmic vacuolation. The degree of the disorganization was quantified on a scale of 0 – 4 (i.e. 0– not present, 1– very mild, 2– mild, 3– moderate, 4– extensive).

2.5 Statistical analysis

All data are presented as the mean ± SEM (n = 5). The time-course curves for paw volume were subjected to two-way (treatment x time) repeated measures analysis of variance with Bonferroni's *post hoc* test. Differences in AUCs and histological scores were analysed by one-way ANOVA followed by Dunnett's *post hoc* test. All graphs were plotted using GraphPad Prism for Windows Version 5.01 (GraphPad, San Diego, CA).

3 Results

3.1 Measurement of paw oedema

With the intra-plantar injection of NaHS, the peak of the oedematogenic response was evident as early as 15 min after injection and had declined by 60 min (Fig 1A). XAE at doses 30 - 300 mg kg^{-1} there was a significant suppression of the mean maximal swellings attained at 15 min to 42.58±1.66%, 42.58±1.66% and 34.91±2.39%, respectively compared to the mean inflamed control response of 61.51±3.90% (Fig 1A). The total paw swellings induced over the 1 h were also significantly

suppressed by 31.82±5.28%, 36.19±6.01%, and 31.65±5.16%, respectively (Fig 1B). Similarly, XA administered at 10-100 mg kg^{-1} also suppressed the mean maximal swellings attained at 15 min to 41.75±1.24%, 40.26±1.68% and 38.26±2.52%, respectively relative to the control response (Fig 1C) while the total paw swelling was significantly suppressed by 34.08±4.49%, 38.19±4.50% and 43.40±5.09%, respectively relative to the inflamed control response (Fig 1D).

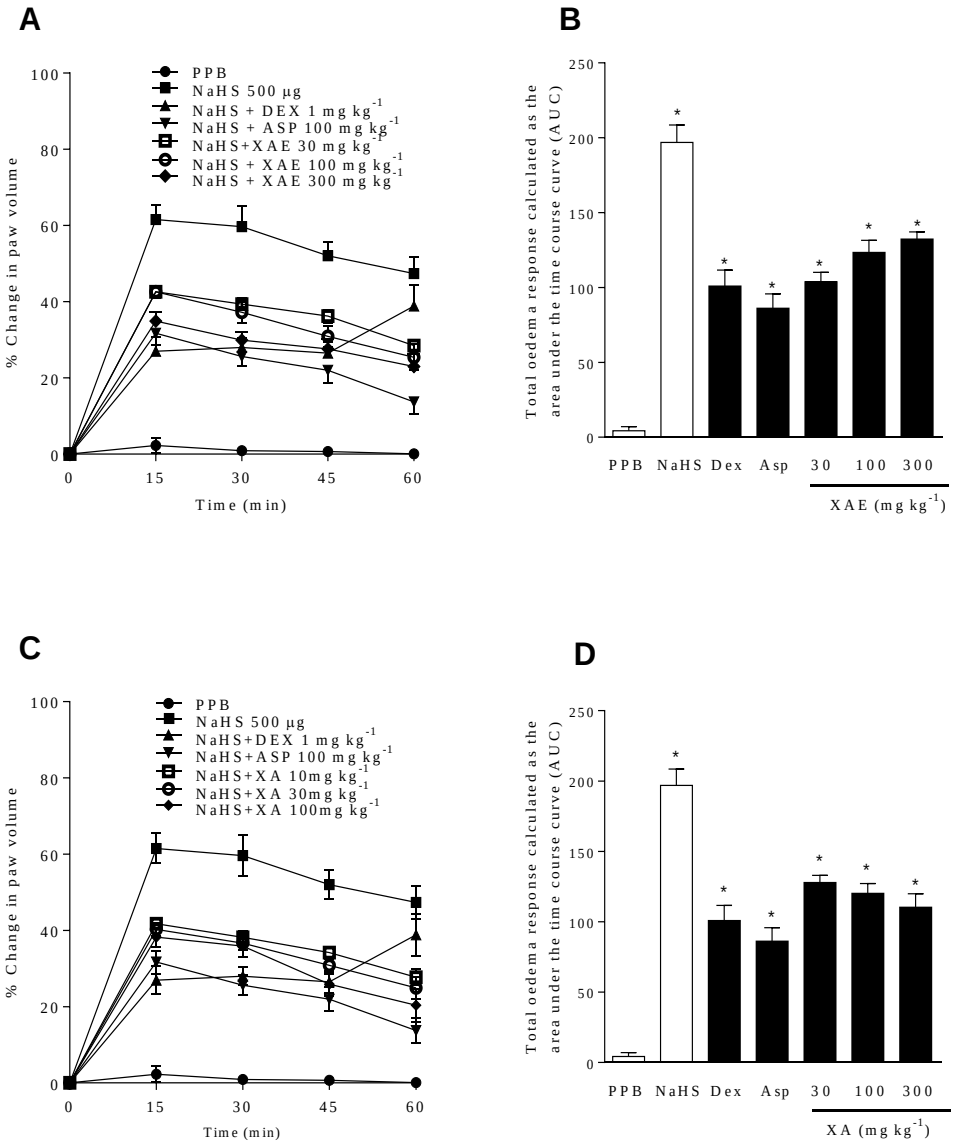

Fig 1: Effect of *Xylopia aethiopica* extract and xylopic acid on H_2S-induced paw oedema in mice

PPS (0.5 ml kg^{-1}), dexamethasone (1 mg kg^{-1}), aspirin (100 mg kg^{-1}), XAE (30, 100, 300 mg kg^{-1} *p.o.*) or XA (10, 30, 100 mg kg^{-1} *p.o.*) was given 1 h before oedema induction, (n = 5). Oedema was induced by injection of 500 µg of NaHS and monitored at 15 min intervals over 1 h as percentage increase in paw thickness (A, C). Total oedema induced during the 1 h was calculated as area under the time course curves, AUC (B, D. Data presented as Mean ± SEM. *P < 0.0001.

3.2 Histology

Paws of potassium phosphate buffer-treated control mice showed intact cell architecture (Plate 1A) with no signs of cytoplasmic vacuolation, inflammation, cellular degeneration, loss of tissue organisation and necrotic tissues (Fig 2A-E). The NaHS-treated mice showed significant loss of tissue organization (Plate 1B) with significant presence of inflamed, necrotic tissue, cytoplasmic vacuolation and cellular degeneration (Fig 2A-E). Aspirin significantly suppressed the inflammation and cellular degeneration with nearly no necrotic cells present (Plate 1C). Dexamethasone treatment also significantly reduced inflammation and loss of tissue architecture (Plate 1D) with reduced cytoplasmic vacuolation and cellular degenerative activity when compared with the NaHS-treated mice (Fig 2A-E). When mice were treated with XAE (300 mg kg^{-1}), there was reduced inflammation and cellular degeneration with no loss of tissue organization (Plate 1E). This presented with a significantly reduced necrosis, cytoplasmic

vacuolation and loss of tissue organization (Fig 2A-E). Similarly, mice treated with XA (100 mg kg^{-1}) showed reduced cellular degeneration and inflammation in the absence of necrotic tissues (Plate 1F). Furthermore, there was reduced cytoplasmic vacuolation, necrosis and loss of organization (Fig 2A-E).

4 Discussions

Inflammation is a highly orchestrated tissue response to trauma, infection, toxin or autoimmune injury[6]. The function of hydrogen sulphide in inflammation is well documented by many studies, and our research established the inhibitory potential of the aqueous ethanol dried fruit extract of *X. aethiopica* and xylopic acid on the H_2S-induced inflammation. Beck-Speier *et al*. (1993) observed that H_2S experiences oxidation into its responsive sulphite (HS-) through the activity of reactive oxygen species (ROS)[15]. HS^- then upregulates leukocyte adhesion and neutrophil functions, through activation of Mac-1 β2-integrin (CD11b/CD18) and protein kinase C (PKC)/Ca^{2+}-calmodulin pathway, respectively[16-18]. Hereafter, inhibitory impact of the fruit extract and xylopic acid avoids leukocyte attachment and neutrophilic activity which, generally enhanced by exogenous exposure to NaHS, are integral in the development of the inflammatory response. Again, inhalation of H_2S gas is known to induce functional pulmonary oedema, this is an indication that endogenous H_2S could also promote tissue swelling which was alleviated by the extract and xylopic acid in our study[19].

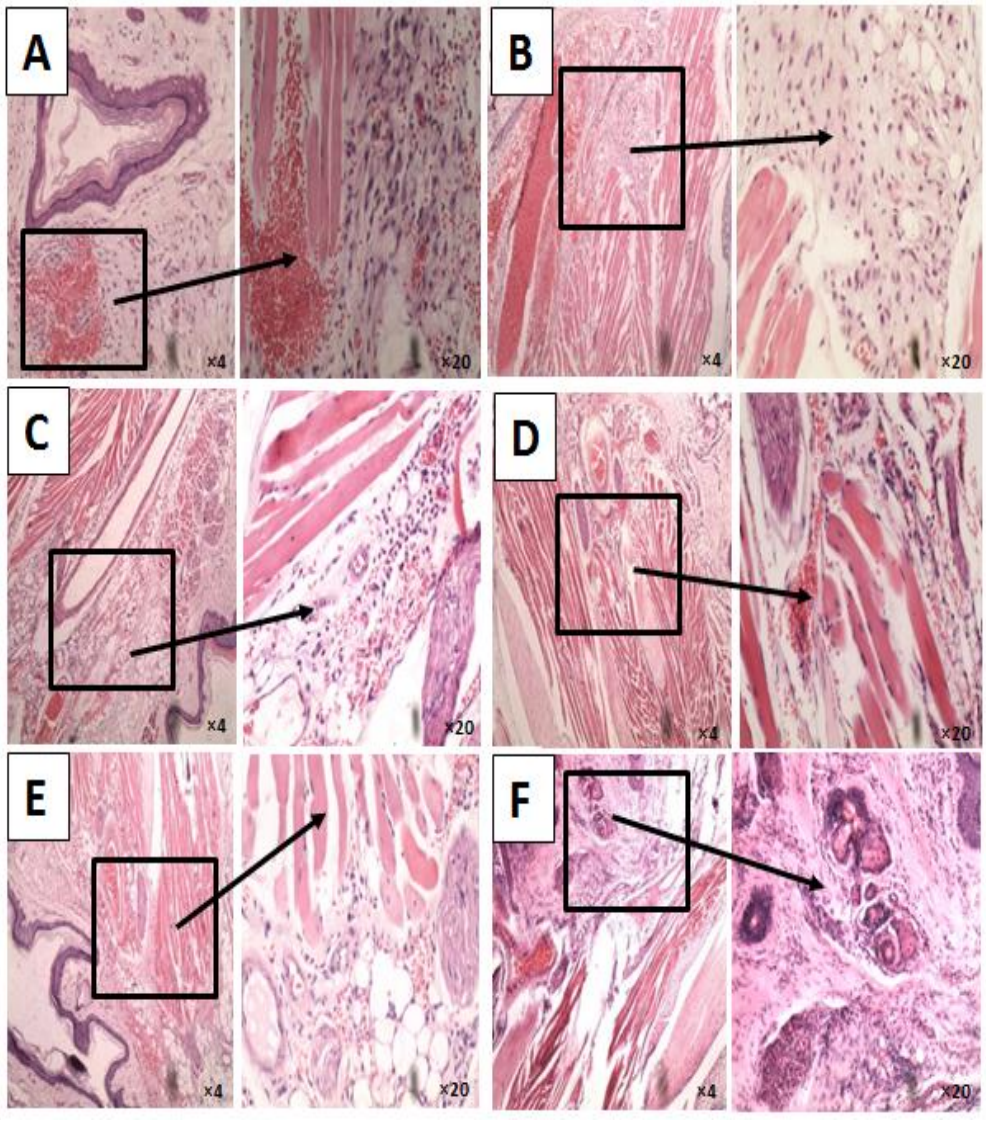

Plate 1: Histology of H_2S-induced paw oedema in mice

Mice were treated with PPB, aspirin (100 mg kg^{-1}), dexamethasone (1 mg kg^{-1}), XAE (300 mg kg^{-1}) or XA (100 mg kg^{-1}) and animals were killed by cervical dislocation 30 min after NaHS (500 mg per paw) or vehicle intra-plantar injection. Mice paws were fixed in neutral buffered formalin before being embedded in paraffin. Sections (4 mm) were stained with haematoxylin-eosin and analysed under light microscopy. (A) PPB; (B) NaHS; (C) NaHS+Asp; (D) NaHS+Dex; (E) NaHS+XAE; (F) NaHS+XA. Scoring was done under ×20 magnification.

Administration of H_2S donors, such as NaHS to animals either alone or in combination with LPS, induce marked hypotension, liver, lung and kidney inflammation and aggravated multiple organ and tissue injury through upregulation of tissue NF-κB, p38 and ERK1/2 signaling[20-22]. It is therefore, not surprising that prophylactic administration of the extract and xylopic acid

alleviated the tissue damage, oedema, necrosis, cytoplasmic vacuolation coupled with reduced inflammatory cell infiltration associated with the sub-plantar injection of NaHS in mice.

X. aethiopica and xylopic acid inhibition of H_2S-induced inflammation suggested the possible effect on secreted phospholipase A_2 ($sPLA_2$) activation, informed by H_2S role in sPLA2 activation.

The extract and xylopic acid, therefore, inhibit the linear and cyclic pathways of arachidonic acid metabolism, and it is in agreement with findings by Landucci *et al*. (2000) and Thimmegowda *et al*. (2007) which reported that H_2S-induced oedema has a similar profile to that of PLA_2-induced oedema, and its isoform $sPLA_2$ plays a major role in the inflammatory process[11,12].

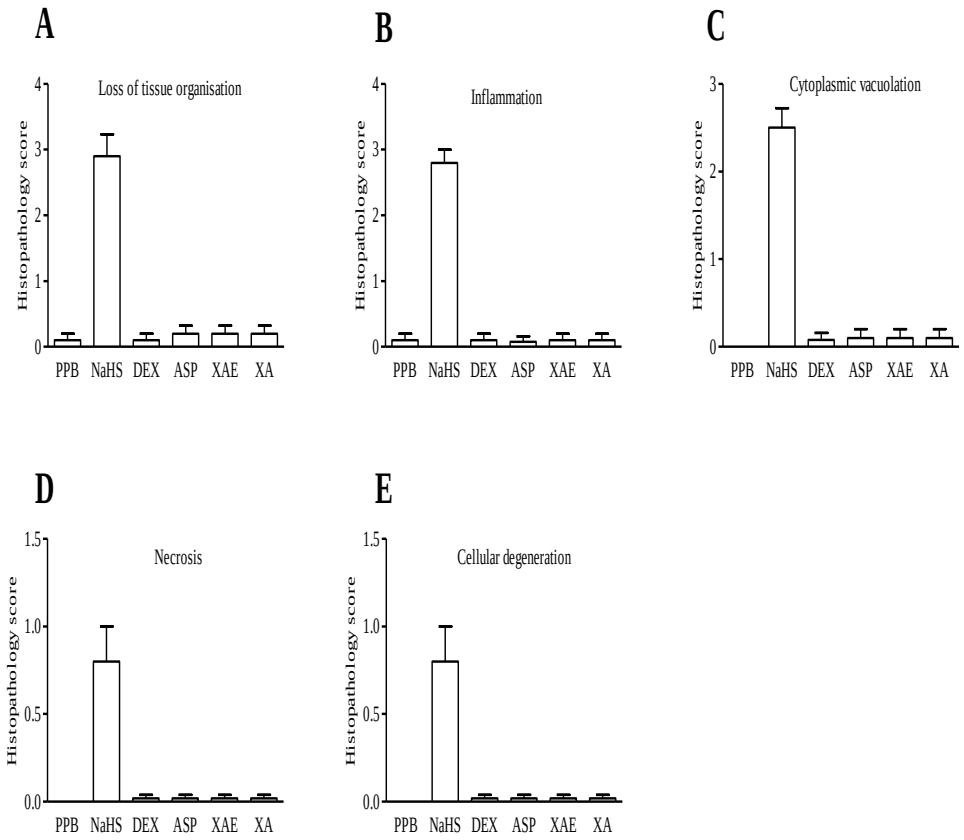

Fig 2: Histological score in H_2S-induced paw oedema in mice

Mice were treated with PPB, aspirin (100 mg kg^{-1}), dexamethasone (1 mg kg^{-1}), XAE (300 mg kg^{-1}) or XA (100 mg kg^{-1}) and animals were killed by cervical dislocation 30 min after NaHS (500 mg per paw) or vehicle intra-plantar injection. Mice paws were fixed in neutral buffered formalin before being embedded in paraffin. Sections (4 mm) were stained with haematoxylin-eosin and analysed under light microscopy. Histological slides were scored according to these parameters: loss of tissue organization, inflammation, necrosis, cellular degradation and cytoplasmic vacuolation. The degree of the disorganization was scored on a scale of 1–4. (i.e. 0 – Not present, 1 – Very mild, 2 – Mild, 3 – Moderate, 4 – Extensive). $P < 0.0001$ (compared with NaHS in all parameters of assessment).

5 Conclusion

This study has established that the anti-inflammatory role of the aqueous ethanol fruit extract of *Xylopia aethiopica* and xylopic acid involve inhibitory effect on hydrogen sulphide-induced inflammation and subsequently has an inhibitory role on phospholipase A_2 activation and also prevents tissue damage in mice.

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7 Conflicts of Interests

The authors hereby declare that there are no conflicts of interests.

8 Author's contributions

NO, RPB and DDO carried out data analysis/interpretation and manuscript preparation. NO and DDO worked on the final document approval. NO carried out the research conception/design and data acquisition.

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