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RESEARCH ARTICLE

Antiinflammatory properties of betulinic acid and xylopic acid in the carrageenan-induced pleurisy model of lung inflammation in mice

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This study investigated the antiinflammatory properties of betulinic acid (BA) and xylopic acid (XA) extracted from *Margaritaria discoidea* and *Xylopia aethiopica*, respectively. *M. discoidea* and *X. aethiopica* are plants native in Ghana and the West-African region and used traditionally to treat different pathologies including inflammatory conditions. The antiinflammatory effect of BA and XA was established by an *in vivo* assay using the carrageenan-induced pleural inflammation model in mice. Also, the ability of BA and XA to increase catalase, superoxide dismutase, glutathione levels and decrease lipid peroxidation level in reactive oxidative assays was assessed. In addition, the ability of XA and BA to prevent potential lung tissue damage was quantified. Pretreatment with BA and XA reduced significantly, signs of inflammation: neutrophil infiltration, oedema, and alveoli septal thickening in carrageenan-treated lung tissue. Additionally, BA or XA pretreatment lowered the degree of lipid peroxidation in the lung tissue while increasing the levels of catalase, superoxide dismutase, and glutathione *in vivo*. Comparatively, XA was more efficacious than BA in the prevention of lung tissue damage. BA and XA derived from *X. aethiopica* and *M. discoidea* possess antiinflammatory and *in vivo* antioxidant activities in mice pleurisy model. The effect of these compounds gives credence to the traditional use in the management of inflammatory conditions of the airway.

KEYWORDS

antioxidant, diclofenac, malondialdehyde, oxidative stress, pleural inflammation

1 | INTRODUCTION

Carrageenan-induced pleurisy model is considered an excellent acute inflammatory model in which leukocyte migration and other relevant biochemical parameters involved in the inflammatory response with the lungs can be quantified easily in the pleural exudates obtained (Dhalendra, Satapathy, & Roy, 2013). In this model of inflammation, vascular events lead to vasodilation followed by increased vascular permeability and cellular events during which inflammatory cells move by chemotaxis, get activated, and then phagocytize offending materials (Gilroy, Lawrence, Perretti, & Rossi, 2004). Mounting evidence suggests that reactive oxygen species play important role in the pathogenesis of inflammatory disorders (Kruidenier et al., 2003). A tilted balance in the levels of free radicals induced by carrageenan injection can lead to the initiation of cell and tissue damage (Impellizzeri et al., 2011), and agents that tend to reduce the inflammatory and oxidative stress will better preserve neighbouring tissue from irreversible damage.

The fruits of *Xylopia aethiopica*, a local spice widely distributed in Ghana and other West-African countries, are used to treat arthritis and other inflammatory conditions (Biney, Benneh, Ameyaw, Boakye-Gyasi, & Woode, 2016; Woode, Ameyaw, Abotsi, & Boakye-Gyasi, 2015). Previous studies have shown that the major phytochemical constituent in the fruits and seeds is kaurene diterpenoid, xylopic acid (Figure 1; Biney et al., 2016). Some authors have attributed the effects of the crude extract to this acid component with comparative studies, corroborating this hypothesis (Woode et al., 2016; Woode, Ameyaw, Boakye-Gyasi, & Abotsi, 2012). In other pharmacological studies, the ethanolic fruit extract has been shown to reduce signs of inflammation in adjuvant-induced arthritis and lipopolysaccharide-induced systemic anaphylaxis (Obiri & Osafo,

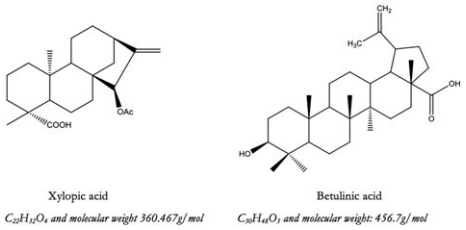


FIGURE 1 Chemical structures of xylopic acid and Betulinic acid

2013; Obiri, Osafo, Oppong-Sarfo, & Prah, 2014a). A recent study hints at the inhibition of phospholipase A_2 as one possible pathway for the antiinflammatory effects of xylopic acid (Osafo, Biney, & Obiri, 2016).

The stem bark of *Margaritaria discoidea* (Euphorbiaceae) is also used traditionally in Ghana for the treatment of arthritis, skin infection, and as a wound healing agent (Irvine, 1961). The stem bark extract has been shown to possess antiinflammatory effects (Dickson et al., 2010; Obiri, Osafo, Ayande, & Antwi, 2014b). Betulinic acid (Figure 1), a ubiquitous pentacyclic triterpenoid isolated from *Margaritaria discoidea*, has also shown significant antiinflammatory activity (Costa et al., 2014; del Carmen Recio et al., 1995; Mukherjee, Saha, Das, Pal, & Saha, 1997). The antiinflammatory effects of betulinic acid are in part the result of its interaction with glucocorticoid receptors (Mukherjee et al., 1997) and the inhibition of protein kinase C (Huguet, del Carmen Recio, Máñez, Giner, & Rıos, 2000). However, the possible contribution of xylopic and betulinic acids to their antiinflammatory activities via the inhibition of free radical generation has not been evaluated. Also, even though the antiinflammatory properties of the two agents have been evaluated by different groups, their specific effect on lung inflammation and their ability to modulate oxidative challenge under such circumstances is yet to be reported. It is possible for agents to show significant generalized antiinflammatory activity yet not be effective in specific inflammatory conditions such as occurs in lung inflammation or neuroinflammation.

To fill this knowledge gap, in this study, we examined the effect of the two plant-derived acids in lung inflammation with the carrageenan-induced pleurisy model and further elucidate selected downstream mechanism(s) of this antiinflammatory effect with specific focus on pleural exudate formation, *in vivo* oxidative stress markers, and histopathological changes in lung tissue.

2 | MATERIALS AND METHODS

2.1 | Isolation of compounds from plant material

2.1.1 | Isolation of xylopic acid

Pure isolate of xylopic acid was obtained from the dried pulverized fruit of *Xylopia aethiopica* by ethanolic extraction (Woode et al., 2012; Figure 2). Exactly 0.0256 g of xylopic acid was weighed and used to prepare an emulsion of xylopic acid with tween 80 *q.s.*

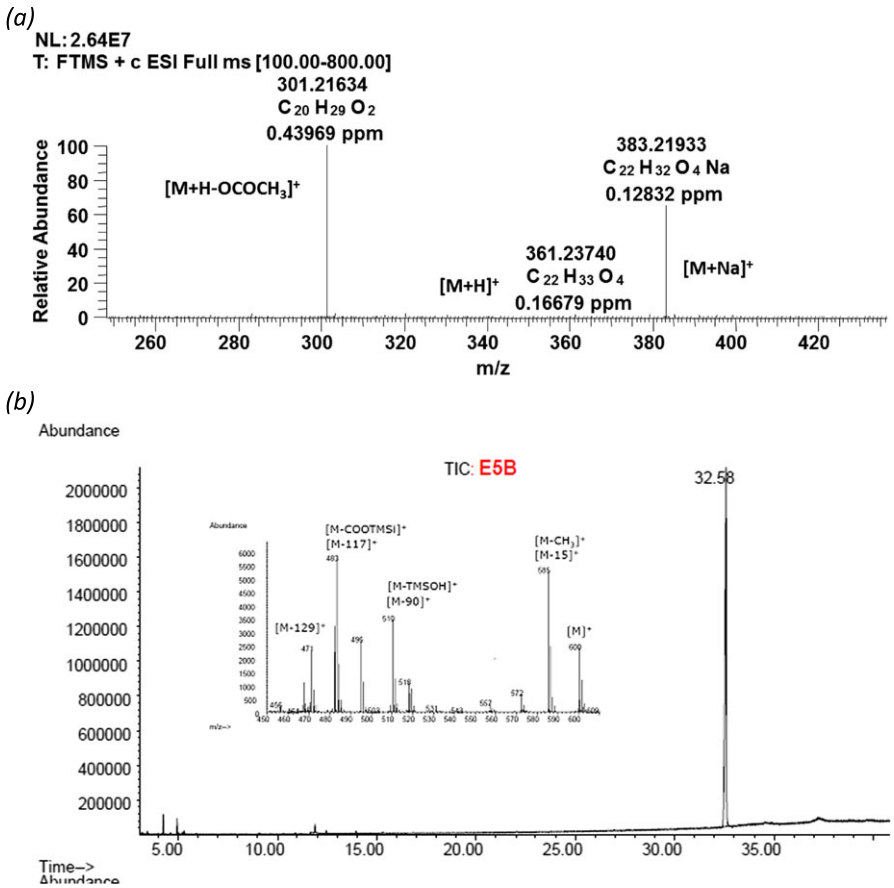


FIGURE 2 (a) Liquid chromatography mass spectrometry (LCMS) chromatogram of xylopic acid and the (b) Gas chromatography mass spectrometry (GCMS) chromatogram of the trimethylsilyl ether derivative of betulinic acid isolated from *X. aethiopica* and *M. discoidea*, respectively [Colour figure can be viewed at wileyonlinelibrary.com]

2.1.2 | Isolation of betulinic acid

Pure betulinic acid was isolated from dry powdered stem bark of *Margaritaria discoidea* by 70% ethanolic extraction (Ekuadzi, 2013; Figure 2). Exactly 0.0256 g of betulinic acid was used to prepare an emulsion of betulinic acid with tween 80 *q.s*.

2.2 | Drugs and chemicals used

Diclofenac sodium (TROGE, Hamburg, Germany); hydrogen peroxide (Bell's Healthcare, Cheshire, England); chloroform, glacial acetic acid, phosphate buffer potassium dichromate, trichloroacetic acid (Amresco®, Solon, USA), *o*-dianisidine, adrenaline, carrageenan sodium, Tris buffer, 5,5′ – dithio-*bis*-2-nitro benzoic acid, thiobarbituric acid and bicarbonate buffer (Sigma-Aldrich Chemical Co, St Louis, USA). In all experiments, BA and XA were administered orally as emulsion using pharmacologically insert tween 80 as emulsifying agent (1% $^{v}/_{v}$). Animals in carrageenan control group received only the vehicle (tween [1% $^{v}/_{v}$] in distilled water) whereas naïve animals received just distilled water.

2.3 | Animals

Fifty Imprint Control Region (ICR) mice 20–25 g were housed in the animal facility at the Department of Pharmacology, Kwame Nkrumah University of Science and Technology. The animals were housed in stainless steel cages (34 × 47 × 18 cm^3; n = 5) with soft wood shavings as bedding, fed with normal mice chow, given water *ad libitum*, and maintained under normal laboratory conditions (temperature 24–25 °C, relative humidity 60–70%, and 12 hr light–dark cycle). Prior to carrageenan-induced pleural and lung inflammation, the mice were randomly assigned to various groups and treated via the oral route as follows:

Group I	carrageenan control
Group II	naïve control
Group III–V	xylopic acid (10, 30, or 100 mg/kg)
Group VI–VIII	betulinic acid (10, 30, or 100 mg/kg)
Group IX–XI	diclofenac (10, 30, or 100 mg/kg)

2.4 | Carrageenan-induced pleurisy

Mice were pretreated with test compounds or solvent 1 hr before induction of pleural inflammation. Pleurisy was induced by injection with 0.1 ml carrageenan (1% $^{w}/_{v}$) via the intrapleural route on the right side of the chest (Ahmad et al., 2014). Four hours postcarrageenan injection, mice were sacrificed by overdose inhalation of diethyl ether. The chest was opened, and the pleural cavity was rinsed with 1 ml normal saline solution. The resulting pleural exudate was aspirated, and the total volume was introduced into separate Eppendorf tubes. The exudate was stored in a refrigerator at −80 °C for subsequent analysis. The lung tissue was also isolated and fixed in buffered formaldehyde solution (40% in phosphate-buffered saline) for subsequent histological examination (Cuzzocrea et al., 2000).

2.4.1 | Total protein (Biuret method)

To determine quantitatively the total protein in serum or plasma, the Biuret method was employed using the specific quantities of blank (B), standard (S), and test (T) specified in Table 1. Each test tube was mixed well and incubated at 37 °C for 5 min. Absorbance was read of standard (S) and test (T) against blank (B) at 555 nm.

$$\text{Total protein (g/dl)} = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times 5.5\ (\text{Concentration}^{n}\ \text{of standard}).$$

2.4.2 | Activity assays for oxidative stress markers elevated in inflammation

a. Quantification of catalase activity

The activity of catalase was measured colorimetrically by the method of Aebi (1984) based on the enzyme's ability to hydrolyse H_2O_2 with some modifications. Briefly, 100 µl of pleural exudate was added to 1 ml 0.01 M phosphate buffer (pH 7.0) and 400 µl of 1.18 M H_2O_2. The reaction admixture was incubated at room temperature for 5 min before the reaction was stopped by adding 2 ml of dichromate-acetic acid mixture (5% potassium dichromate + glacial acetic acid, 1:3). Absorbance of the coloured product was measured at 620 nm with a spectrophotometer. The activity of catalase was expressed as unit per milligram protein using molar extinction coefficient of catalase 39.4 M^{-1} cm^{-1}, where one-unit catalase activity is defined as the amount of enzyme that degrades 1 mmol H_2O_2 per minute at 25 °C (pH 7.0)

$$\text{mUnit of CAT activity/mg protein} = \frac{\text{Absorbance}_{620\ \text{nm}}}{3.94 \times \text{weight of protein}} \times 1000$$

b. Quantification of reduced glutathione levels

Reduced glutathione (GSH) levels in treated animals were assayed as previously described by Ellman (1958). One hundred microliter of the supernatant of the pleural exudate was added to 2.4 ml 0.02 M Ethylenediamine tetraacetic acid (EDTA) and kept at 4 °C for 10 min. Thereafter, 2 ml distilled water and 0.5 ml Trichloroacetic acid (TCA) (50% $^{w}/_{v}$) were subsequently added and the resultant mixture centrifuged at 3,000×*g* for 5 min. One milliliter of the supernatant was then added to 2 ml of 0.4 M Tris HCl (pH 8.9) and 0.05 ml 10 mM 5, 5′ – dithio-*bis*-2-nitro benzoic acid solution, mixed thoroughly, and incubated at room temperature for 5 min. A blank containing all reagents except the tissue extract was prepared alongside. Absorbance was read with a spectrophotometer at 412 nm. The total glutathione in the sample solution was used to determine the standard curve. Reduced glutathione concentration was interpolated from the standard curve, $y = 0.0004x + 0.0026$ and expressed as micromole GSH per milligram protein.

c. Quantification of lipid peroxidation

TABLE 1 Reagent and sample volumes used in total protein assay

Reagent	B	S	T
Biuret reagent	1.0 ml	1.0 ml	1.0 ml
Total protein standard (concentration = 5.5 g/dl)	—	50 µl	—
Specimen	—	—	50 µl

The chromogenic product malondialdehyde forms with thiobarbituric acid has been assayed extensively as a product of polyunsaturated fatty acid peroxidation and marker of oxidative stress (Scheme 1).

Malondialdehyde levels in the pleural exudates were evaluated using a previously described method (Heath & Packer, 1968) with minor modifications. One milliliter aliquot of the pleural exudate supernatant was added to 3 ml of 20% trichloroacetic acid containing 0.5% thiobarbituric acid in test tubes. The mixture was heated at 95 °C for 30 min and then rapidly cooled in an ice bath. The tube was centrifuged at 5,000×*g* for 10 min to precipitate cellular products. The resulting supernatant (250 µl) was pipetted into 96-well plate and the absorbance read at 532 nm with plate reader (Synergy H1 Multi-mode Reader, BioTek Technologies, Winooski, VT, USA). Non-specific absorbance was eliminated by reading absorbance at 600 nm subtracting from the 532 nm reading. The concentration of malondialdehyde was calculated using its molar extinction coefficient of $1.56 \times 10^{-5}\ M^{-1}\ cm^{-1}$.

$$_{nmol}\ \text{MDA per mg protein} = \frac{\text{Absorbance}_{532\ nm} - \text{AAbsorbance}_{600\ nm}}{1.56 \times 10^5 \times \text{total protein}} \times 10^9$$

d. Quantification of superoxide dismutase (SOD) activity

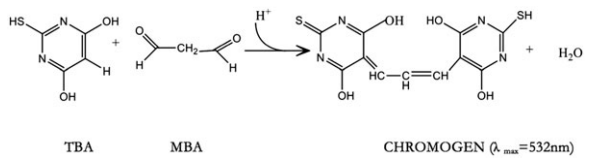
Superoxide dismutase activity was measured based on the ability of SOD to inhibit autoxidation of adrenaline to adrenochrome. To 0.5 ml of pleural fluid, 0.75 ml of ethanol (96%$^{v}/_{v}$) and 0.15 ml of chilled chloroform were added. The resultant solution was centrifuged at 2,000×*g* for 20 min to obtain a clear supernatant. EDTA (0.5 ml, 0.6 mM) was added to the supernatant that contained 1 ml of bicarbonate buffer (0.1 M) at pH 10.2. Fifty microliter of adrenaline (1.3 mM) was added to the mixture to initiate the reaction, and the absorbance of the adrenochrome formed was measured at 480 nm at 25 °C. A blank containing all reagents except the tissue extract was prepared alongside. Absorbance was read with a spectrophotometer at 480 nm.

$$\%\text{inhibition} = \frac{\text{Absorbance}_{\text{test}} - \text{Absorbance}_{\text{blank}}}{\text{Absorbance}_{\text{test}}} \times 100$$

$$\text{Units of SOD activity/mg protein} = \frac{\%\text{inhibition}}{50 \times \text{weight of protein}}$$

e. Statistical analysis on oxidative stress enzymes assays

All data are presented as mean ± SEM. The results were analyzed using one-way analysis of variance. When analysis of variance was significant, multiple comparisons between treatments were done using Holm–Sidak *post hoc test*. GraphPad Prism for Windows version 6.01 (GraphPad® Software, San Diego, CA, USA) was employed for all statistical analysis. The level of statistical significance was set at $p < .05$ for differences between compared groups.



SCHEME 1 Reaction scheme sowing how the chromogenic product is generated in the assay

3 | RESULTS

3.1 | Oxidative stress enzymes and markers

a. Catalase activity

In the catalase assay, betulinic acid, xylopic acid, and diclofenac showed a significant increase in catalase activity compared to the saline-treated group. Significantly higher levels of catalase was observed at 10–100 mg/kg of xylopic acid and 100 mg/kg of betulinic acid and diclofenac (Figure 3).

b. Glutathione (GSH)

Xylopic acid and diclofenac showed increased glutathione level at dose of 100 mg/kg, respectively, whereas betulinic acid exhibited no significant increase in glutathione levels at all tested doses (Figure 4).

c. Malondialdehyde level: Lipid peroxidation

In the malondialdehyde assay, all the tested compounds (xylopic acid, betulinic acid, and diclofenac) reduced malondialdehyde levels, with a pronounced reduction observed at all tested doses of xylopic acid and diclofenac (Figure 5).

d. Superoxide dismutase activity

Xylopic acid, betulinic acid, and diclofenac significantly increased SOD level compared to control groups within a dose range of 10–100 mg/kg (Figure 6).

3.2 | Analysis of lung tissue histopathology

3.2.1 | Effect of xylopic acid on whole lung tissue

The results revealed that xylopic acid protects against carrageenan-induced tissue damage compared to tissue excised from the solvent-treated mice (Figure 7). Increasing dose concentrations (10 mg/kg, 30 mg/kg, and 100 mg/kg) increased the protective function against tissue damage, respectively. The protective ability of betulinic acid was dose dependent. Increasing dose concentrations (10 mg/kg, 30 mg/kg, and 100 mg/kg) increased the protective function against tissue damage, respectively, with similar observations being seen in lung sections of diclofenac treated-mice.

4 | DISCUSSION

Data obtained in this study show that acute administration of xylopic acid or betulinic acid reduces neutrophil migration into the pleural space of mice 4 hr after injection of carrageenan. This inhibition of cell

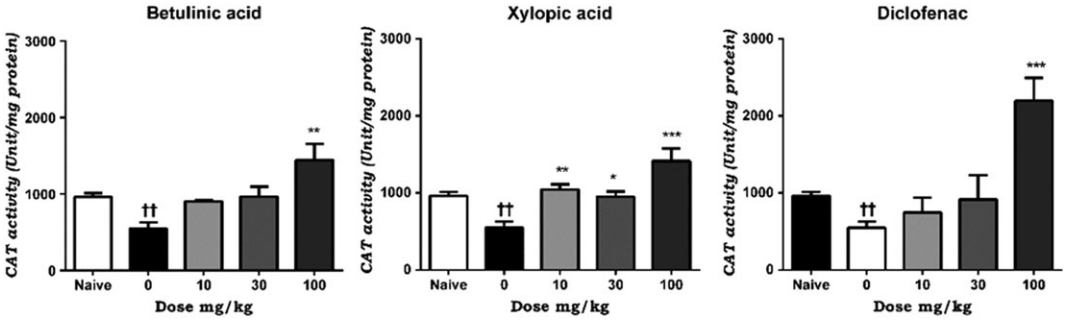


FIGURE 3 Effect of betulinic acid and xylopic acid and diclofenac on catalase level in mice at four after the induction of pleurisy via Cg (carrageenan) injection that served as the control group. Data show mean ± SEM (n = 5–6). Statistical analysis was performed using a one-way analysis of variance; *p < .05, **p < .01, and ***p < .001 all compared to the control group; †† p < .01 compared to naïve group

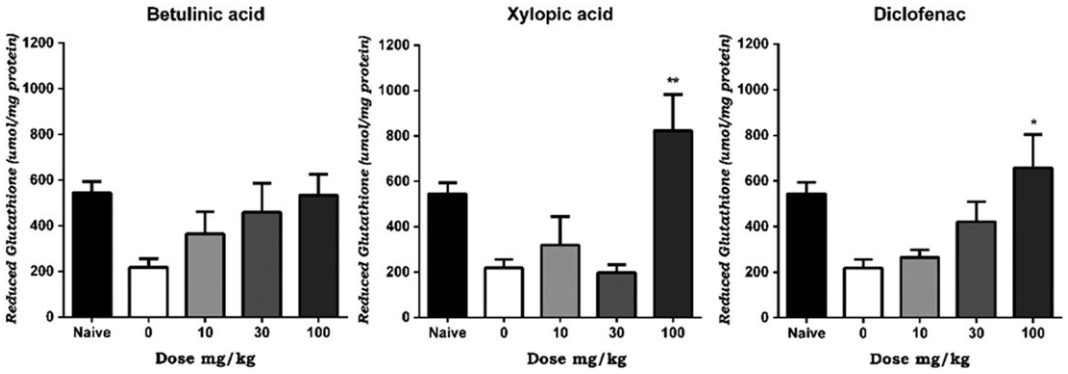


FIGURE 4 Effect of betulinic acid, xylopic acid, and diclofenac on glutathione levels in mice at 4 hr after the induction of pleurisy via Cg (carrageenan) injection that served as the control group. Data show mean ± SEM (n = 5–6). One-way analysis of variance; *p < .05 and ** p < .01 all compared to the control group

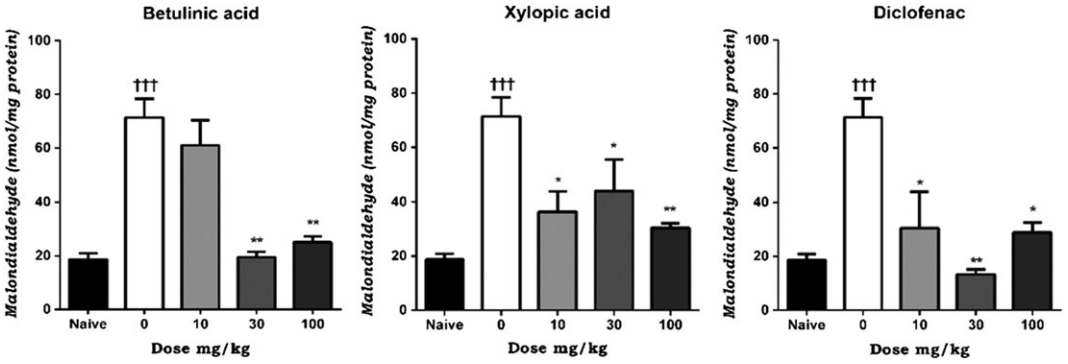


FIGURE 5 Effect of betulinic acid, xylopic acid, and diclofenac on malondialdehyde levels in mice 4 hr after the induction of pleurisy via Cg (carrageenan) injection that served as the control group. Data show mean ± SEM (n = 5–6). One-way analysis of variance; *p < .05 and **p < .01 all compared to the control group; ††† p < .001 compared to naïve group

influx into the pleural cavity was associated with a marked reduction in oxidative stress markers and an elevation of antioxidative enzymes.

Lung inflammation is associated with enhanced expression of pro-inflammatory cytokines that serves as intercellular signals that recruit cells and modulate cell function (Ahmad et al., 2014; Marzocco et al., 2004). Pleurisy induced with carrageenan in rodents is an important marker to measure the antiinflammatory effects of compounds and isolates and the possible effects on inflammatory mediators *in vivo* (Ahmad et al., 2014; Di Paola et al., 2005). In the cascade of events, introduction of carrageenan into the pleural cavity of mice, as was performed in this study, leads to an increased vascular permeability that is coupled with an initiation of an inflammatory reaction (Marzocco et al., 2004; Rosa & Willoughby, 1971). The latter is associated with lung inflammation, exudation of fluids into the pleural space accompanied by an elevated influx of polymorphonuclear leukocytes (Marzocco et al., 2004).

Steroidal and non-steroidal antiinflammatory agents have been shown to reduce several components of the inflammatory events, including but not limited to, neutrophil infiltration, pleural effusion volume, and damage to lung tissue (Mikami & Miyasaka, 1983; Nakatsugi, Terada, Yoshimura, Horie, & Furukawa, 1996).

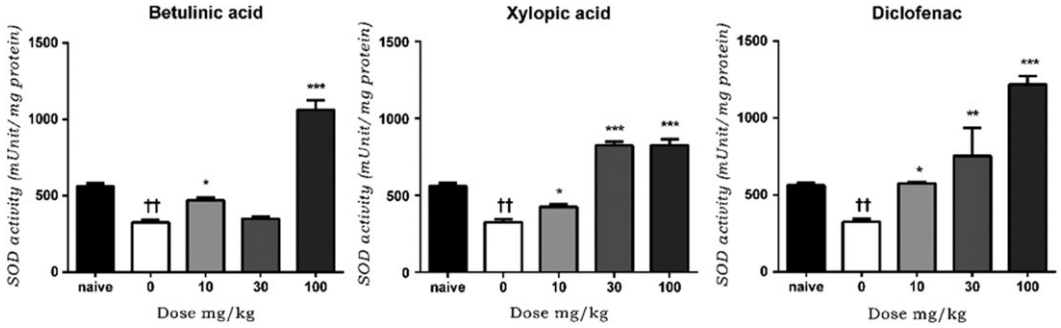


FIGURE 6 Effect of betulinic acid, xylopic acid, and diclofenac on superoxide dismutase levels in mice at four after the induction of pleurisy via Cg (carrageenan) injection that served as the control group. Data show mean ± SEM (n = 5–6). One-way analysis of variance; $p < .05$ is accepted as the level of significance; $^{*}p < .05$ $^{**}p < .01$, and $^{***}p < .001$ all compared to the control group; †† $p < .01$ compared to naïve group

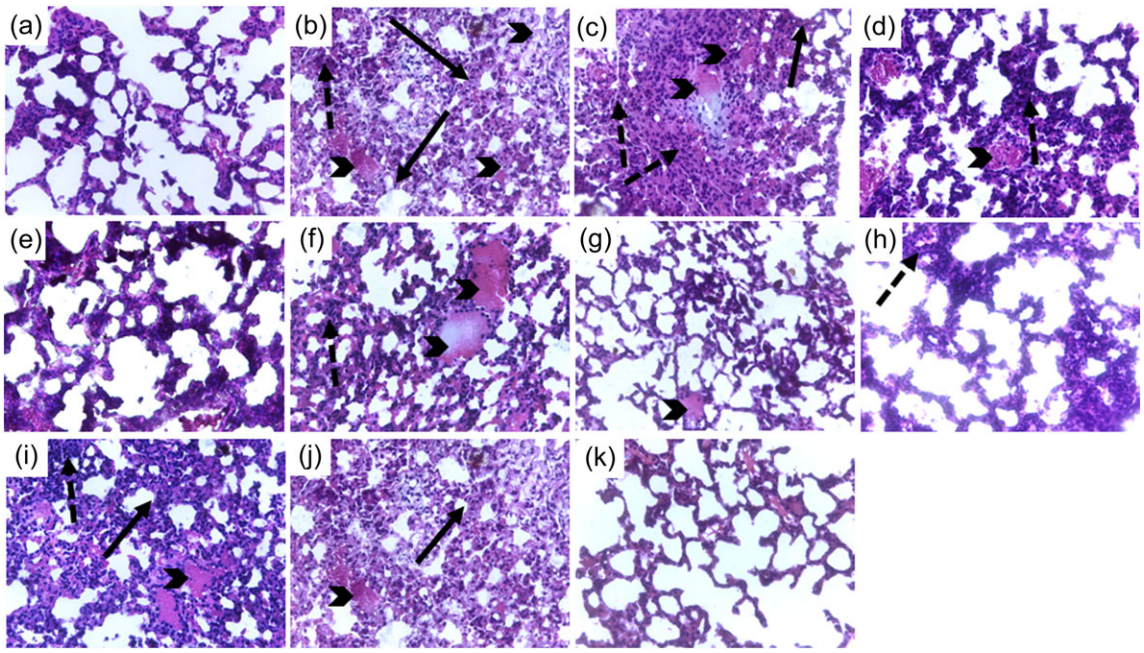


FIGURE 7 Representative sections of lung tissues of betulinic acid, xylopic acid, and diclofenac-treated mice in comparison to carrageenan treated (b) and naïve mice (a) in the mice pleurisy model of lung inflammation (n = 5). (Key: Arrow heads = oedema, bold arrows = aveoli septal thickening, broken arrows = neutrophil filtration. M = ×40) Top panel L-R: Naïve (a), Cg-treated (b), BA 10 mg/kg (c), BA 30 mg/kg (d). Middle panel L-R: Betulinic acid 100 mg/kg (e), xylopic acid 10 mg/kg (f), xylopic acid 30 mg/kg (g) xylopic acid 100 mg/kg (h). Bottom panel L-R: Diclofenac (DIC) 10 mg/kg (i), DIC 30 mg/kg (j), DIC 10 mg/kg (k). [Colour figure can be viewed at wileyonlinelibrary.com]

The antiinflammatory activities of xylopic acid and betulinic acid is in agreement with earlier works done with the crude extracts of *Xylopia aethiopica* (Obiri et al., 2014a; Obiri & Osafo, 2013) and *Magaritaria discoidea* (Dickson et al., 2010; Obiri et al., 2014b). Additionally, both plant extracts have shown significant *in vitro* antioxidant activities (Dickson et al., 2010; Karioti, Hadjipavlou-Litina, Mensah, Fleischer, & Skaltsa, 2004). The generation of reactive oxidative species, which exert their effects both directly and indirectly, is an important contributor to inflammatory injury. When cellular oxidants are generated in excess of the antioxidant defense capacity of cells, it results in deleterious changes to tissues (Halliwell, 2007; Mittal et al., 2014). Reactive Oxygen Species (ROS) as well as reactive nitrogen species also initiate, sustain, or intensify the inflammatory process by upregulating genes that activate the transcription of pro-inflammatory cytokines and adhesion molecules (Halliwell, 2007). Apart from their ability to enhance the inflammatory response, ROS also stimulate enrolment of more neutrophils and macrophages (Conforti et al., 2008). Hence, compounds that aid in the mop up or neutralization of ROS can lead to a relatively better resolution of inflammation.

Although we tested the ability of xylopic acid and betulinic acid in an acute model of inflammation, the enhancement of the antioxidative enzymes in the presence of ROS suggests the possible use in halting events that sustain chronic inflammatory conditions. This is not surprising as Obiri et al., 2014band Obiri et al. (2014a) have demonstrated that the extract of the plant *Xylopia aethiopica* has comparable antiinflammatory activity in a chronic model of arthritis in rats.

Histopathology can offer a pronounced structural peculiarity as a pragmatic, univocal, and decisively characteristic sign of an inflammatory process (Soren, Cooper, & Waugh, 1987). Hence, histopathological studies on lung sections after carrageenan challenge were carried out that showed the two acids were able to preserve normal alveolar architecture with reduced influx of neutrophils and oedema formation.

The lung histopathology supports the antiinflammatory activity of xylopic acid and betulinic acid. Preservation of alveolar tissue integrity in xylopic acid and betulinic acid pretreated mice suggests significant antiinflammatory activity. It was also observed that increased reduction in tissue damage was dose dependent. Comparing the two test drugs, xylopic acid showed a better protective function against tissue damage than betulinic acid within a dose range of 10–100 mg/kg.

In summary, this study provides the evidence that pretreatment of mice with xylopic acid or betulinic acid attenuates (a) the development of carrageenan-induced pleurisy, (b) carrageenan-induced oxidative stress, (c) neutrophil infiltration in the lung, and (d) the degree of lung injury (histology) caused by injection of carrageenan.

5 | CONCLUSION

Data generated from this study indicate that betulinic acid and xylopic acid cause a substantial reduction of lung inflammation induced by carrageenan in the mice. These findings support the traditional use of the plant extracts of *Magaritaria discoidea* and *Xylopia aethiopica* as therapeutic agents in conditions associated with acute inflammation and some respiratory disorders.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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