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Original Research Article

Anti-inflammatory and bronchodilatory constituents of leaf extracts of *Anacardium occidentale* L. in animal modelsOluwakemi Josephine Awakan^{a,*}, Sylvia Omonirume Malomo^{a,b}, Abdullahi Adeyinka Adejare^c, Adedoyin Igunnu^b, Olubunmi Atolani^d, Abiodun Humphrey Adebayo^e, Bamidele Victor Owoyele^f^a Department of Biological Sciences, College of Science and Engineering, Landmark University, PMB 1001, Omu-Aran, Nigeria^b Department of Biochemistry, Faculty of Life Sciences, University of Ilorin, PMB 1515, Ilorin, Nigeria^c Department of Physiology, Faculty of Basic Medical Sciences, College of Medicine of the University of Lagos, PMB 12003, Lagos, Nigeria^d Department of Chemistry, Faculty of Physical Sciences, University of Ilorin, PMB 1515, Ilorin, Nigeria^e Department of Biochemistry, College of Science and Technology, Covenant University, PMB 1023, Ota, Nigeria^f Department of Physiology, Faculty of Basic Medical Sciences, College of Health Sciences, University of Ilorin, PMB 1515, Ilorin, Nigeria

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ABSTRACT

Objective: *Anacardium occidentale* L. leaf is useful in the treatment of inflammation and asthma, but the bioactive constituents responsible for these activities have not been characterized. Therefore, this study was aimed at identifying the bioactive constituent(s) of *A. occidentale* ethanolic leaf extract (AOEL) and its solvent-soluble portions, and evaluating their effects on histamine-induced paw edema and bronchoconstriction.

Methods: The bronchodilatory effect was determined by measuring the percentage protection provided by plant extracts in the histamine-induced bronchoconstriction model in guinea pigs. The anti-inflammatory effect of the extracts on histamine-induced paw edema in rats was determined by measuring the increase in paw diameter, after which the percent edema inhibition was calculated. The extracts were analyzed using gas chromatography-mass spectrometry to identify the bioactive constituents. Column chromatography and Fourier transform infrared spectroscopy were used respectively to isolate and characterize the constituents. The bronchodilatory and anti-inflammatory activities of the isolated bioactive constituent were evaluated.

Results: Histamine induced bronchoconstriction in the guinea pigs and edema in the rat paw. AOEL, hexane-soluble portion of AOEL, ethyl acetate-soluble portion of AOEL, and chloroform-soluble portion of AOEL significantly increased bronchodilatory and anti-inflammatory activities ($P < 0.05$). Oleamide (9-octadecenamide) was identified as the most abundant compound in the extracts and was isolated. Oleamide significantly increased bronchodilatory and anti-inflammatory activities by 32.97% and 98.41%, respectively ($P < 0.05$).

Conclusion: These results indicate that oleamide is one of the bioactive constituents responsible for the bronchodilatory and anti-inflammatory activity of *A. occidentale* leaf, and can therefore be employed in the management of bronchoconstriction and inflammation.

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1. Introduction

Inflammation is implicated in many degenerative diseases such as rheumatoid arthritis, shoulder tendonitis, cancer and asthma

[1,2]. It is a protective reaction of the cells or tissues of the body to irritation (allergic or chemical), infections or injury [3]. Inflammation is characterized by pain, heat, redness and swelling. It can lead to the loss of function due to dilation of the blood vessels and increased intercellular spaces, which result in the movement of leukocytes, proteins and fluids into the inflamed regions [3]. The inflammation of allergic asthma is characterized by exposure

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to allergens through IgE-dependent mechanisms [4]. This allergic inflammatory response is similar to what is seen in parasitic and helminthic infections, characterized by infiltration with eosinophils [4].

Exposure of the airways to chemicals (ozone, NO₂) and particulate matter in smoke (cigarette smoke, diesel exhaust), or biological agents (e.g., virus infections, allergens, pollens) can trigger a wide range of cellular, vascular and neural events, and also induce the generation of reactive oxygen species [5], which predisposes the system to inflammation and bronchoconstriction. Bronchoconstriction is a tightening of the smooth muscle surrounding the bronchi and bronchioles, with an accompanying wheezing and shortness of breath [5]. Its molecular mechanisms are underlined by a wide range of mediators that are generated by various cell types surrounding the smooth muscle layer of airway passages.

Medicinal plants have often been employed in traditional and folk medicine for the treatment of inflammation and bronchoconstriction, since antiquity. Often used plants include *Markhamia tomentosa* (Benth.) [6], *Ramalina farinacea* (L.) Ach. [7], *Humulus lupulus* L. [8], *Vitis vinifera* [9] and *Anacardium occidentale* L. [10].

A. occidentale, known as cashew, belongs to the family Anacardiaceae. It is a multipurpose tree from the Amazon and African rainforest that grows up to 15 m high, with a thick and twisting trunk, having branches so meandering that often reach the ground [11]. The leaves have been used for the treatment of bronchitis and inflammation, the hallmarks of asthma [12,13]. Despite studies on *A. occidentale* medicinal use, the bioactive constituents responsible for its anti-inflammatory and bronchodilatory activities have not been characterized. Therefore, this study aimed at evaluating and characterizing the anti-inflammatory and bronchodilatory constituent of leaf extracts of *A. occidentale* in animal models.

2. Materials and methods

2.1. Plant material and authentication

A. occidentale leaves were harvested from the campus of Landmark University, Omu-Aran, Nigeria. The leaves were identified and authenticated in the Herbarium Unit of the Department of Plant Biology, University of Ilorin, Ilorin, Nigeria, where a sample was deposited and a voucher number of UIH 001/835 was assigned.

2.2. Chemicals

Indomethacin was obtained from Sigma Aldrich, Inc., St Louis, USA. Other reagents used included salbutamol (Glaxo Smithkline, USA), hydrocortisone sodium succinate (Nitin Life Sciences Ltd, Haryana, India), oleamide (Ebay, Australia) and histamine (sc-204000A; Santa Cruz Biotechnology, Germany). Ethanol, n-hexane, trichloromethane, ethyl acetate and other chemical reagents were of analytical grade.

2.3. Experimental animals

A total of 63 guinea pigs and 95 Wistar rats were obtained from the National Veterinary Research Institute, Vom, Nigeria and the Animal House Holding Unit, Department of Biochemistry, University of Ilorin, Ilorin, Nigeria. They were housed in cages in the Animal Holding Unit of the Department of Biological Sciences, Landmark University, Omu-Aran, with a 12 h light/day cycle. The animals were allowed free access to rat feed and clean tap water. Ethical approval for the study was obtained from the University of Ilorin Ethical Review Committee.

2.4. Preparation of extracts

A. occidentale leaves were air dried and pulverized, after which 2 kg of the powder was subjected to cold extraction for 48 h with 10 L of absolute ethanol. It was thereafter filtered and concentrated over a rotary evaporator (RE 300, Staffordshire, UK) at 50 °C to give a percentage yield of 16% greenish syrup-like crude extract. The crude ethanolic extract (200 g) was subjected to fractionation through solvent-solvent extraction using distilled water and three solvents in the successive order: n-hexane, chloroform and ethyl acetate. The crude *A. occidentale* ethanolic leaf extract (AOEL) was first dissolved in 800 mL of distilled water and partitioned with 800 mL of n-hexane. The mixture was shaken thoroughly for about 4 h. Thereafter, the n-hexane portion was decanted with a separating funnel and concentrated using a rotary evaporator. The water-soluble portion was further subjected to successive extractions using the same process and the following solvents successively: chloroform and ethyl acetate. Extracts were obtained from each solvent and the remaining aqueous portion (after partitioning with the three solvents) was evaporated to dryness. The percentage yields of the n-hexane-soluble portion of AOEL (HAOEL), the chloroform-soluble portion of AOEL (CAOEL) and the ethyl acetate-soluble portion of AOEL (EAOEL) were 46.28, 20.00 and 15.00, respectively.

2.5. Bronchodilatory activity

2.5.1. Animal grouping and extract administration

Forty-two guinea pigs were assigned to 14 treatment groups as follows: Group 1 received normal saline, Group 2 received the reference drug salbutamol (0.2 mg/kg); Groups 3–6 received AOEL, HAOEL, CAOEL and EAOEL respectively at a dose of 250 mg/kg; Groups 7–10 received AOEL, HAOEL, CAOEL and EAOEL respectively at a dose of 375 mg/kg and Groups 11–14 received AOEL, HAOEL, CAOEL and EAOEL respectively at a dose of 500 mg/kg. All doses are represented in terms of body weight.

2.5.2. Evaluation of bronchodilatory activity

The bronchodilatory effect was determined by measuring the percentage protection of the extracts on histamine-induced bronchoconstriction in guinea pig according to the method described by Kumar et al. [14]. The guinea pigs were fasted overnight and then exposed to 0.2% histamine aerosol in an air-tight chamber. The pre-convulsion time (PCT), the time of aerosol exposure to the onset of dyspnea leading to appearance of convulsion in the animals, was recorded. The animals were immediately removed from the chamber and placed in fresh air for 24 h to recover. Thereafter, they were given their various treatments according to the indicated grouping. The animals were re-exposed to histamine 1, 4 and 24 h after treatment, and their PCTs were again recorded. The percentage (%) protection was calculated according to the formula: $(1 - C/T) \times 100$, where C = PCT prior drug administration, and T = PCT n hour (number of hours) after drug administration.

2.6. Anti-inflammatory activity

2.6.1. Animal grouping and extract administration

Seventy Wistar rats were assigned into 14 treatment groups as follows: Group 1 received normal saline, Group 2 received the reference indomethacin (10 mg/kg); Groups 3–6 received AOEL, HAOEL, CAOEL and EAOEL respectively at a dose of 250 mg/kg; Groups 7–10 received AOEL, HAOEL, CAOEL and EAOEL respectively at a dose of 375 mg/kg and Groups 11–14 received AOEL, HAOEL, CAOEL and EAOEL respectively at a dose of 500 mg/kg.

2.6.2. Evaluation of anti-inflammatory activity

The anti-inflammatory effect of the extracts on histamine-induced paw edema in rats was determined according to the method described by Okunrobo et al. [15]. The drugs and extracts were administered to the respective groups of rats. After one hour, paw edema was induced in the rats by subcutaneous injection of 0.1 mL of 1% histamine into the sub-plantar area of the experimental rats. The paw diameter was measured with a Vernier caliper at 0, 1, 2, 3, 4 and 5 h after the injection of histamine. The percentage edema inhibition was calculated according to the equation: percentage edema inhibition = $(1 - d_t/d_c) \times 100$, where d_t = difference in paw diameter in the treated group and d_c = difference in paw diameter of the control group.

2.7. Gas chromatography-mass spectrometry

Gas chromatography-mass spectrometry (GC-MS) analyses of AOEL, HAOEL, CAOEL and EAOEL were carried out on GC-MS QP 2010 Ultra Shimadzu Japan with a selective mass detector 5973 RTx 5MS column (30 m × 0.25 mm, 0.25 μm film thickness). The operating conditions of the column were as follows: oven temperature to ramp from 180 °C (hold for 3 min) to 280 °C at 8 °C/min, and held isothermally for 2 min. The injector temperature was maintained at 250 °C and the volume of injected sample was 1.00 μL. The MS ran in electron impact mode at 71 eV and mass spectral data were acquired in the mass range 40–550 m/z. The identification of compounds was performed by comparing their mass spectra with data from NIST 11 (National Institute of Standards and Technology, USA).

2.8. Column chromatography fractionation of ethyl acetate leaf extract from *A. occidentale*

Due to the lower complexity and prominence of major compounds in the ethyl acetate extract, it was selected for further examination by column chromatography fractionation to isolate the pure bioactive compound. Approximately 5.0 g of the ethyl acetate extract was subjected to gravity silica gel column chromatography, and eluted with solvent system of increasing polarity. Specifically, the column was eluted with hexane, and then with increasing concentration of dichloromethane (DCM) in hexane until only DCM was used. This was followed by elution with increasing concentration of methanol in DCM until only methanol was used. A total of 101 fractions of approximately 30 mL each were obtained and pooled to 11 groups (A–K), based on the thin-layer chromatography (TLC) profile viewed under ultraviolet light, after developing using appropriate solvent systems. The combined fractions were concentrated and weighed. Fraction A (50 mg), a yellowish powder, was a complex mixture and it contained numerous inseparable compounds, while B (70 mg) separated into two distinct layers (yellow upper layer and greenish lower layer). The greenish layer was discarded due to its complexity and deep coloration from the heavy presence of chlorophyll, while the yellow upper layer was purified on a short flash chromatography silica gel, yielding a pure yellow oil compound (30 mg) with R_f value 0.5 (Hexane: DCM = 1:2). Fraction C (90 mg) and fraction D (130 mg) were purified on preparative TLC (PTLC) to yield whitish brown compounds. Fraction E (130 mg), a whitish brown powder, showed only one prominent spot at R_f 0.5, when developed with hexane: DCM (1:2). Fractions F, H, I and K were ignored because they had negligible weight and complex mixtures. Fraction G (190 mg) afforded a pure brownish compound (Oleamide) which was purified using PTLC and stored for further analyses. Fraction J (35 mg) was subjected to PTLC, using the same method as fraction C to afford a light brown compound.

2.9. Fourier transform-infrared analysis

Infrared spectra of isolated bioactive compounds (i.e., fractions G and E) were measured on a Fourier transform-infrared (FTIR) spectrophotometer (model 8400S, Shimadzu Corporation, Kyoto, Japan) using a KBr pellet.

2.10. Anti-inflammatory and bronchodilatory activities of oleamide

2.10.1. Animal grouping and extract administration for the evaluation of the anti-inflammatory activity of oleamide

Twenty-one guinea pigs were further assigned to 7 treatment groups as follows: Group 1 received normal saline, Group 2 received the reference drug salbutamol (0.2 mg/kg); Groups 3 and 4 each received 70 mg/kg of AOG and AOE respectively. All doses are represented in terms of body weight. The evaluation of anti-inflammatory activity was carried out as stated in section 2.6.2.

2.10.2. Animal grouping and extract administration for the evaluation of the bronchodilatory activity of oleamide

Twenty-five Wistar rats were further assigned to 5 treatment groups as follows: Group 1 received normal saline; Group 2 received the reference drug hydrocortisone (4 mg/kg); Groups 3–5 received oleamide at 30, 60 and 80 mg/kg respectively. All doses are represented in terms of body weight. The evaluation of the bronchodilatory activity was carried out as stated in section 2.5.2.

2.11. Data analysis

Data were analyzed on GraphPad Prism 5 (GraphPad Software Inc., San Diego, California) with a one-way analysis of variance. Post-hoc tests were conducted with the Newman-Keuls multiple comparison test. Data are presented as means of animals in each group ± standard error of mean. Values were considered significant at $P < 0.05$.

3. Results

3.1. Bronchodilatory activity of AOEL

The bronchodilatory effect of AOEL was determined by measuring the percentage protection of the extracts on histamine-induced bronchoconstriction in guinea pigs.

At the 250 mg/kg dose of AOEL, all treatments offered significant ($P < 0.05$) protection against bronchoconstriction 1 and 24 h after administration, while only HAOEL, CAOEL and EAOEL, as well as salbutamol, offered significant ($P < 0.05$) protection 4 h after administration. At 1 h post-administration of treatment, salbutamol (0.2 mg/kg) and HAOEL offered significant protection at $P < 0.001$, while AOEL and EAOEL offered significant protection at $P < 0.01$. At 4 and 24 h post-administration of treatment, HAOEL offered significant protection at $P < 0.001$, while CAOEL and EAOEL, as well as salbutamol, offered significant protection at $P < 0.01$ (Fig. 1).

At the 375 mg/kg dose of leaf extracts of *A. occidentale*, all treatments offered significant protection ($P < 0.05$) against bronchoconstriction except HAOEL and CAOEL at 1 and 4 h after administration respectively. At 1 h post-administration of treatment, salbutamol (0.2 mg/kg) and HAOEL offered significant protection at $P < 0.001$, while CAOEL and EAOEL offered significant protection at $P < 0.01$. At 4 h post-administration of treatment, EAOEL offered significant protection ($P < 0.01$). At 24 h post-administration of treatment, HAOEL, CAOEL and EAOEL, as well

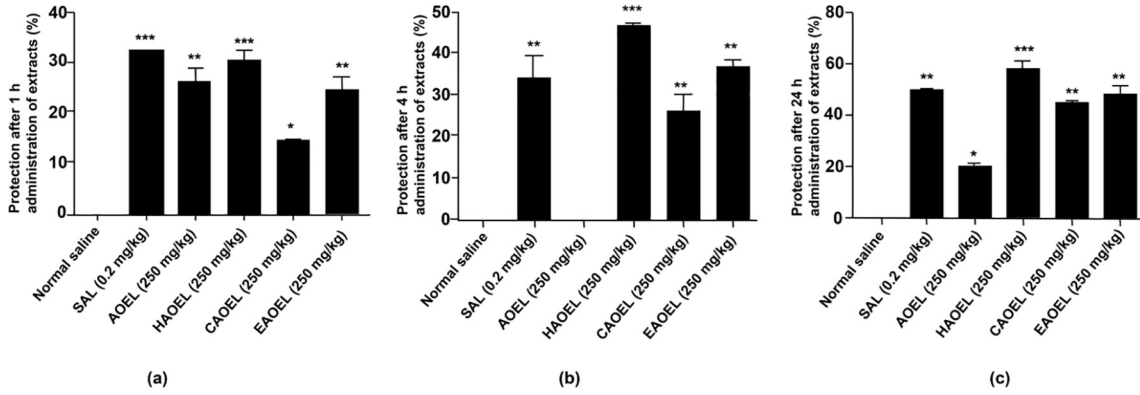


Fig. 1. Effects of administration of 250 mg/kg of *A. occidentale* ethanolic leaf extract. The bronchodilatory effect of *A. occidentale* ethanolic leaf extract, its solvent-soluble portions and salbutamol on histamine-induced bronchoconstriction in guinea pigs was determined; panels a, b and c represent measurements taken at 1, 4 and 24 h after drug administration, respectively. Values are represented as mean of three animals \pm standard error of mean. Asterisks denote level of statistical significance between each group and the normal saline control ($P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$). SAL: salbutamol; AOEL: *A. occidentale* ethanolic leaf extract; HAOEL: n-hexane-soluble portion of AOEL; CAOEL: chloroform-soluble portion of AOEL; EAOEL: ethyl acetate-soluble portion of AOEL.

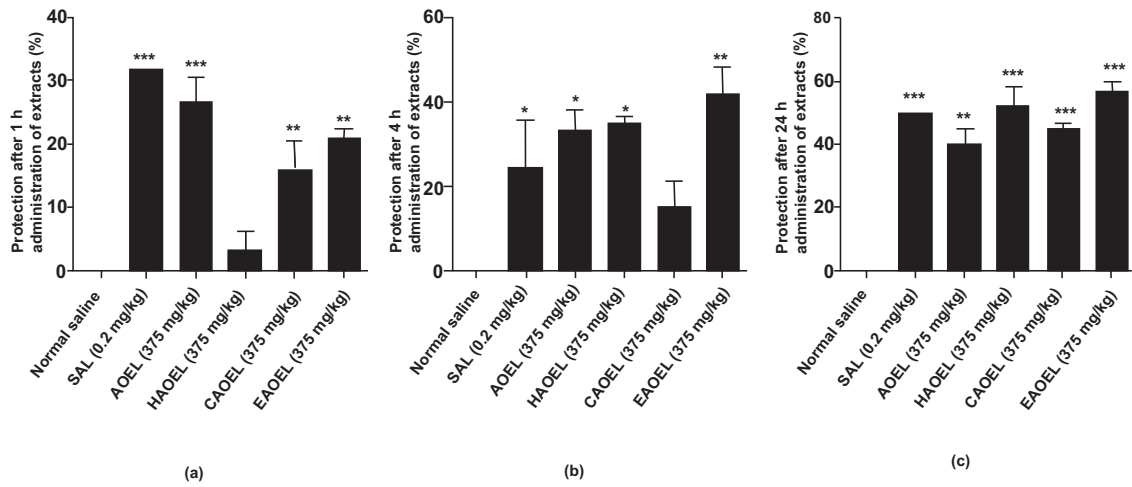


Fig. 2. Effects of administration of 375 mg/kg of *A. occidentale* ethanolic leaf extract. The bronchodilatory effect of *A. occidentale* ethanolic leaf extract, its solvent-soluble portions and salbutamol on histamine-induced bronchoconstriction in guinea pigs was determined; panels a, b and c represent measurements taken at 1, 4 and 24 h after drug administration, respectively. Values are represented as mean of three animals \pm standard error of mean. Asterisks denote level of statistical significance between each group and the normal saline control ($P < 0.05$; $^{*}P < 0.01$; $^{***}P < 0.001$). SAL: salbutamol; AOEL: *A. occidentale* ethanolic leaf extract; HAOEL: n-hexane-soluble portion of AOEL; CAOEL: chloroform-soluble portion of AOEL; EAOEL: ethyl acetate-soluble portion of AOEL.

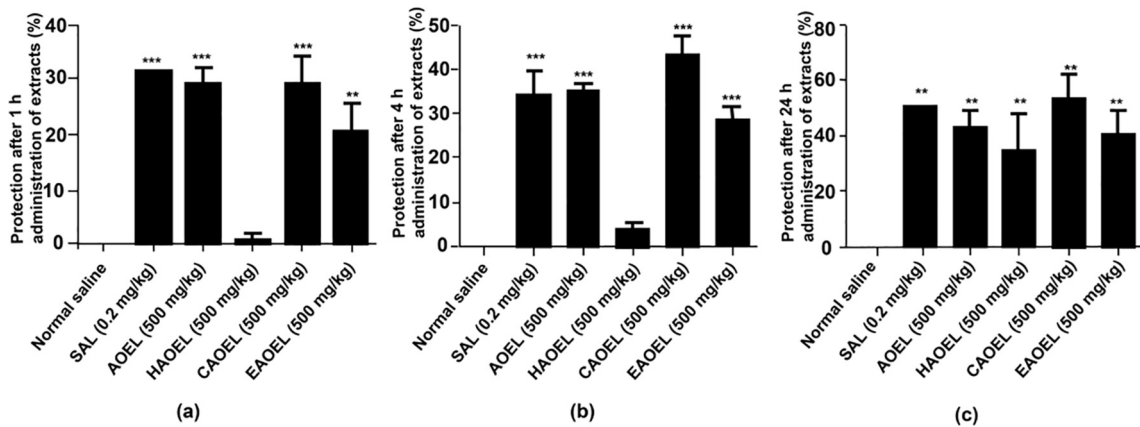


Fig. 3. Effects of administration of 500 mg/kg of *A. occidentale* ethanolic leaf extract. The bronchodilatory effect of *A. occidentale* ethanolic leaf extract, its solvent-soluble portions and salbutamol on histamine-induced bronchoconstriction in guinea pigs was determined; panels a, b and c represent measurements taken at 1, 4 and 24 h after drug administration, respectively. Values are represented as mean of three animals \pm standard error of mean. Asterisks denote level of statistical significance between each group and the normal saline control ($^{*}P < 0.01$; $^{***}P < 0.001$). SAL: salbutamol; AOEL: *A. occidentale* ethanolic leaf extract; HAOEL: n-hexane-soluble portion of AOEL; CAOEL: chloroform-soluble portion of AOEL; EAOEL: ethyl acetate-soluble portion of AOEL.

as salbutamol, offered significant protection at $P < 0.001$, while AOEL offered significant protection at $P < 0.01$ (Fig. 2).

At the 500 mg/kg dose of leaf extracts of *A. occidentale*, all treatments offered significant protection ($P < 0.05$) against bronchoconstriction except HAOEL at 1 h and 4 h after administration. At 1 h post-administration of treatment, salbutamol (0.2 mg/kg), AOEL and CAOEL offered significant protection at $P < 0.001$, while EAOEL offered significant protection at $P < 0.01$. At 4 h post-administration of treatment, salbutamol (0.2 mg/kg), AOEL, CAOEL and EAOEL offered significant protection ($P < 0.001$). At 24 h post-administration of treatment, AOEL, HAOEL, CAOEL and EAOEL, as well as salbutamol, offered significant protection ($P < 0.01$, Fig. 3).

3.2. Anti-inflammatory activity of leaf extracts of *A. occidentale*

The anti-inflammatory effect of the extracts against histamine-induced paw edema in rats was determined by measuring the increase in paw diameter and the percent edema inhibition of rats treated with test compounds, relative to untreated control.

At the 250 mg/kg dose, there was significant ($P < 0.05$) inhibition of paw edema 1 h after drug administration in all the treatment groups; indomethacin, HAOEL, CAOEL and EAOEL had a 100% paw edema inhibition, while AOEL had a 60% paw edema inhibition. AOEL and HAOEL significantly ($P < 0.05$) inhibited paw edema by 94% and 100% respectively 2 h after drug administration. All the treatment significantly ($P < 0.05$) inhibited paw edema 4 h after drug administration, HAOEL by 100%, indomethacin and AOEL by 85% and EAOEL and CAOEL by 73% and 68%, respectively. HAOEL had the highest paw edema inhibition at the 250 mg/kg dose. There was no significant ($P > 0.05$) difference in the inhibition of paw edema at the 3rd and 5th hours after drug administration (Table 1).

At the 375 mg/kg dose, AOEL, CAOEL and indomethacin significantly ($P < 0.05$) inhibited paw edema 1 h after drug administration by 100%. CAOEL, HAOEL, AOEL, indomethacin and EAOEL, at 2 h after drug administration, inhibited paw edema by 100%, 87%, 84%, 81% and 61% respectively. CAOEL, AOEL, indomethacin, EAOEL and HAOEL, at 4 h after drug administration, inhibited paw edema by 100%, 100%, 85%, 75% and 58% respectively. CAOEL had the highest paw edema inhibition at the 375 mg/kg dose. There was no significant ($P > 0.05$) inhibition of paw edema at the 3rd and 5th hours after drug administration (Table 2).

At the 500 mg/kg dose, there was a 100% inhibition of paw edema 1 h after drug administration by indomethacin and AOEL. Two hours after drug administration, AOEL, HAOEL, CAOEL and indomethacin inhibited paw edema by 100%, 100%, 81% and 81% respectively. At 4 h after drug administration, inhibition of paw edema was 100% by both HAOEL and CAOEL, and 90% and 85% by AOEL and indomethacin, respectively. At the 500 mg/kg dose, AOEL most effectively inhibited paw edema, closely followed by CAOEL. There was no significant ($P > 0.05$) inhibition of paw edema after the 3rd and 5th hours of drug administration (Table 3).

Table 1
Anti-inflammatory activity of 250 mg/kg of the ethanolic extract of *A. occidentale* leaf.

Treatment group	Increase in paw diameter (cm)/protection (%)				
	1 h	2 h	3 h	4 h	5 h
Histamine + normal saline (control)	0.06 ± 0.02	0.08 ± 0.02	0.08 ± 0.03	0.10 ± 0.03	0.06 ± 0.03
Indomethacin (10 mg/kg)	0.00 ± 0.00** (100)	0.02 ± 0.01 (81)	0.03 ± 0.02 (63)	0.02 ± 0.02* (85)	0.00 ± 0.00 (100)
AOEL (250 mg/kg)	0.03 ± 0.01** (60)	0.01 ± 0.01* (94)	0.02 ± 0.01 (78)	0.02 ± 0.02* (85)	0.00 ± 0.00 (100)
HAOEL (250 mg/kg)	0.00 ± 0.00** (100)	0.00 ± 0.00* (100)	0.02 ± 0.02 (75)	0.00 ± 0.00** (100)	0.00 ± 0.00 (100)
CAOEL (250 mg/kg)	0.00 ± 0.00** (100)	0.02 ± 0.02 (81)	0.00 ± 0.00 (100)	0.03 ± 0.02* (68)	0.00 ± 0.00 (100)
EAOEL (250 mg/kg)	0.00 ± 0.00** (100)	0.05 ± 0.03 (42)	0.11 ± 0.05 (0)	0.03 ± 0.02* (73)	0.02 ± 0.01 (70)

Values are represented as mean ± standard error of mean with $n = 3$. * $P < 0.05$, ** $P < 0.01$, vs control. AOEL: *A. occidentale* ethanolic leaf extract; HAOEL: n-hexane-soluble portion of AOEL; CAOEL: chloroform-soluble portion of AOEL; EAOEL: ethyl acetate-soluble portion of AOEL.

3.3. GC-MS profile of HAOEL and EAOEL

All extracts were also subjected to GC-MS analysis to identify their bioactive constituents. Five compounds (pentanoic acid, 4-methyl-2-pentanol, 3-butyn-1-ol, butanamide and hexanamide) were identified in AOEL. Hexanamide had the highest area percentage of 42.39 (Table 4).

In the HAOEL, 10 compounds were identified, and two of these had very large area percentages: 9-octadecenamide (59.94%) and 1,2,3-benzenetriol (17.03%, Table 5).

Twenty compounds were identified in CAOEL. Four of the compounds had relatively high area percentages between 12% and 20%: 1,2,3-benzenetriol (19.69%), glycerin (14.42%), 9-octadecenamide (13.06%) and cyclopentane (12.51%). The remaining 16 compounds had area percentages ranging between 0.23% and 8.49% (Table 6).

Six compounds were found in EAOEL; one was found to have high area percentage: 9-octadecenamide (82.56%) (Table 7). Oleamide (Supplemental Fig. 1, available from the corresponding author), also known as 9-octadecenamide, was the most abundant compound in the extracts and was isolated using column chromatography and purified using PTL. The MS analysis of compounds obtained from fractions G (Supplemental Fig. 2, available from the corresponding author) and E (Supplemental Fig. 3, available from the corresponding author) from EAOEL shows oleamide.

3.4. FTIR characterization of fractions G and E isolated from *A. occidentale* leaf extracts

The FTIR spectrum of fraction G (Supplemental Fig. 4, available from the corresponding author) indicated a prominent peak at ν_{\max} 3 373 cm^{-1} which corresponds to the stretching vibration of a N—H bond. The presence of moisture in the sample seems to submerge and broaden the peak. The C—H stretching of aliphatic alkane was observed at 2 926 and 2 850 cm^{-1} , while the peak at 1 650, with a shoulder at 1 700 cm^{-1} , corresponds to carbonyl stretching of an amide group (Supplemental Fig. 4, available from the corresponding author). The vibration of the C=C stretching of unsaturation was depicted at 1 612, while the C=C bending vibration was observed at 1 448 cm^{-1} (Supplemental Fig. 4, available from the corresponding author). The prominent vibrations of fraction G depicted in the spectrum correspond to what was observable in synthetic oleamide (Supplemental Fig. 5, available from the corresponding author) and literature [16]. The FTIR spectrum of fraction E (Supplemental Fig. 6, available from the corresponding author) was identical to fraction G (Supplemental Fig. 4, available from the corresponding author).

3.5. Bronchodilatory and anti-inflammatory activities of oleamide

The bronchodilatory and anti-inflammatory activities of the bioactive constituent oleamide were evaluated.

Table 2
Anti-inflammatory activity of 375 mg/kg of the ethanolic extract of *A. occidentale* leaf.

Treatment Group	Increase in paw diameter (cm)/protection (%)				
	1 h	2 h	3 h	4 h	5 h
Histamine + normal saline (control)	0.06 ± 0.02	0.08 ± 0.02	0.08 ± 0.03	0.10 ± 0.03	0.05 ± 0.03
Indomethacin (10 mg/kg)	0.00 ± 0.00* (100)	0.02 ± 0.01** (81)	0.03 ± 0.02 (63)	0.02 ± 0.02* (85)	0.00 ± 0.00 (100)
AOEL (375 mg/kg)	0.00 ± 0.00* (100)	0.01 ± 0.01* (84)	0.00 ± 0.00 (100)	0.00 ± 0.00* (100)	0.00 ± 0.00 (100)
HAOEL (375 mg/kg)	0.04 ± 0.02 (36)	0.01 ± 0.00* (87)	0.01 ± 0.01 (88)	0.04 ± 0.03* (58)	0.02 ± 0.01 (74)
CAOEL (375 mg/kg)	0.00 ± 0.00* (100)	0.00 ± 0.00** (100)	0.00 ± 0.00 (100)	0.00 ± 0.00** (100)	0.00 ± 0.00 (100)
EAOEL (375 mg/kg)	0.02 ± 0.01 (64)	0.03 ± 0.02* (61)	0.06 ± 0.02 (31)	0.03 ± 0.02* (75)	0.01 ± 0.01 (87)

Values are represented as mean ± standard error of mean with $n = 3$. * $P < 0.05$, ** $P < 0.01$, vs control. AOEL: *A. occidentale* ethanolic leaf extract; HAOEL: n-hexane-soluble portion of AOEL; CAOEL: chloroform-soluble portion of AOEL; EAOEL: ethyl acetate-soluble portion of AOEL.

Table 3
Anti-inflammatory activity of 500 mg/kg of the ethanolic extract of *A. occidentale* leaf.

Treatment Group	Increase in paw diameter (cm)/protection (%)				
	1 h	2 h	3 h	4 h	5 h
Histamine + normal saline (control)	0.06 ± 0.02	0.08 ± 0.02	0.08 ± 0.03	0.10 ± 0.03	0.05 ± 0.03
Indomethacin (10 mg/kg)	0.00 ± 0.00* (100)	0.02 ± 0.01* (81)	0.03 ± 0.02 (63)	0.02 ± 0.02** (85)	0.00 ± 0.00 (100)
AOEL (500 mg/kg)	0.00 ± 0.00* (100)	0.00 ± 0.00* (100)	0.02 ± 0.01 (75)	0.01 ± 0.01** (90)	0.01 ± 0.01 (87)
HAOEL (500 mg/kg)	0.03 ± 0.02 (50)	0.00 ± 0.00* (100)	0.00 ± 0.00 (100)	0.00 ± 0.00** (100)	0.00 ± 0.00 (100)
CAOEL (500 mg/kg)	0.00 ± 0.00* (100)	0.01 ± 0.01* (81)	0.02 ± 0.02 (78)	0.00 ± 0.00** (100)	0.00 ± 0.00 (100)
EAOEL (500 mg/kg)	0.11 ± 0.02 (0)	0.13 ± 0.00 (0)	0.07 ± 0.04 (13)	0.07 ± 0.02 (33)	0.02 ± 0.01 (70)

Values are represented as mean ± standard error of mean with $n = 3$. * $P < 0.05$, ** $P < 0.01$, vs control. AOEL: *A. occidentale* ethanolic leaf extract; HAOEL: n-hexane-soluble portion of AOEL; CAOEL: chloroform-soluble portion of AOEL; EAOEL: ethyl acetate-soluble portion of AOEL.

Table 4
Gas chromatography-mass spectrometry analysis of AOEL.

R. Time	Area	Area%	Height	Height%	A/H	Name
36.747	66 950	20.61	28 522	23.25	2.35	2-Methyl pentanoic acid
37.934	26 915	8.29	9 456	7.71	2.85	4-Methyl-2-pentanol, methyl ether
41.425	26 380	8.12	13 439	10.96	1.96	3-Butyn-1-ol
42.838	66 869	20.59	26 315	21.45	2.54	Butanamide
43.378	137 680	42.39	44 932	36.63	3.06	Hexanamide
Total	324 794	100.00	122 664	100.00		

R. Time: Retention Time; AOEL: *A. occidentale* ethanolic leaf extract; A/H: area/height.

Table 5
Gas chromatography-mass spectrometry analysis of HAOEL.

R. Time	Area	Area%	Height	Height%	A/H	Name
8.239	155 124	3.94	19 605	1.97	7.91	5-Amino-6-nitropyrimidine-2,4 (1H, 3H)-dione
10.439	74 772	1.90	22 514	2.26	3.32	4H-pyran-4-one,2,3-dihydro-3,5-dihydroxy-6
19.456	670 489	17.03	106 776	10.74	6.28	1,2,3-Benzenetriol
36.498	50 566	1.28	23 325	2.35	2.17	2-Methylpentanoic acid
36.670	72 384	1.84	27 65	2.78	2.62	Methyl n-decanoate
41.348	66 083	1.64	16 634	1.67	3.97	Oxalic acid, cyclobutylhexyl ester
43.270	115 236	2.93	29 011	2.92	3.97	Butanamide
47.661	2 359 121	59.94	644 734	64.85	3.66	(Z)-9-Octadecenamide
47.789	117 566	2.99	29 867	3.00	3.94	Butanamide
51.842	254 782	6.47	74 081	7.45	3.44	Di(2-ethylhexyl)phthalate
Total	3 936 123	100.00	994 212	100.00		

R. Time: Retention Time; HAOEL: n-hexane-soluble portion of *A. occidentale* ethanolic leaf extract; A/H: area/height.

One hour after the administration of the compounds, the isolated oleamide (fraction G) had the greatest effect, significantly increasing ($P < 0.05$) bronchodilatory activity by 15.11%, followed by the synthetic oleamide (60 mg/kg), which increased activity by 6.76% (Fig. 4a). Oleamide (60 mg/kg), salbutamol, fraction G and oleamide (80 mg/kg) significantly increased ($P < 0.05$) bronchodilatory activity by 20.35%, 20.01%, 17.05% and 13.79% respectively at 4 h post-administration of compounds (Fig. 4b). Salbutamol, oleamide (60 mg/kg) and fraction G significantly increased ($P < 0.05$) bronchodilatory activity by 33.45%, 32.97% and 27.62% respectively 24 h after administration (Fig. 4c).

Oleamide and the standard drug significantly increased ($P < 0.05$) anti-inflammatory activity from the 1st to the 5th hour after administration of treatments. A maximal anti-inflammatory activity of 98.41% was provided by oleamide (30 mg/kg) 5 h after administration of treatments (Table 8).

4. Discussion

In this study, we demonstrated that oleamide is a prominent anti-inflammatory and bronchodilatory constituent of leaf extracts of *A. occidentale* in animal models. Our results showed that AOEL,

Table 6
Gas chromatography-mass spectrometry analysis of CAOEL.

R. Time	Area	Area%	Height	Height%	A/H	Name
3.511	1 044 482	7.71	90 186	3.92	11.58	Dihydroxyacetone
5.461	1 953 437	14.42	201 560	8.76	9.69	Glycerin
8.465	1 693 873	12.51	196 785	8.55	8.61	1-acetyl-1,2-epoxy-cyclopentane
10.551	1 149 586	8.49	140 904	6.12	8.16	2,3-Dihydro-3,5-dihydroxy-6-methyl- 4H-pyran-4-one
13.565	665 883	4.92	110 597	4.81	6.02	5-Hydroxymethylfurfural
14.097	144 599	1.07	21 296	0.93	6.79	1,2,5-Oxadiazole
19.714	2 666 829	19.69	430 411	18.70	6.20	1,2,3-Benzenetriol
36.748	79 801	0.59	30 298	1.32	2.63	Cyclopropene
37.859	217 782	1.61	62 843	2.73	3.47	2,3-Epoxyhexanol
43.382	111 821	0.83	35 870	1.56	3.12	Hexadecenamide
47.759	1 768 921	13.06	506 987	22.03	3.49	(Z)-9-Octadecenamide
47.889	100 873	0.74	25 759	1.12	3.92	3-Butyn-1-ol
48.755	178 690	1.32	50 859	2.21	3.51	2,2,5-Trimethyl- 3,4-hexanedione
51.110	31 137	0.23	11 627	0.51	2.68	3-methylundecane
51.927	210 710	1.56	59 118	2.57	3.56	Di(2-ethylhexyl)phthalate
53.151	382 570	2.82	108 409	4.71	3.53	Eicosane
55.234	366 046	2.70	92 766	4.03	3.95	2-Bromo dodecane
57.239	222 983	1.65	63 730	2.77	3.50	2,4-Dimethyldecane
58.736	422 255	3.12	39 826	1.73	10.60	8,9,9,10,10,11-Hexafluoro-4,4-dimethyl-3,5-dioxatetracyclo[5.4.1.0(2,6).0(8,11)]dodecane
61.531	132 458	0.98	21 696	0.94	6.11	Dichloroacetic acid, 2,2-dimethylpropyl ester
Total	13 544 736	100.00	2 301 527	100.00		

R. Time: Retention Time; CAOEL: chloroform-soluble portion of *A. occidentale* ethanolic leaf extract; A/H: area/height.

Table 7
Gas chromatography-mass spectrometry analysis of EAOEL.

R. Time	Area	Area%	Height	Height%	A/H	Name
26.090	242 208	2.80	80 290	3.72	3.02	Cyclopropene
36.709	70 780	0.82	36 692	1.70	1.93	Propyne
43.320	383 604	4.43	105 076	4.86	3.65	Enanthamide
47.746	7 148 269	82.56	1 714 972	79.37	4.17	(Z)-9-Octadecenamide
47.846	408 660	4.72	106 123	4.91	3.85	3-Butyn-1-ol
51.881	404 318	4.67	117 688	5.45	3.44	Phthalic acid, 2-ethylhexyl ester
Total	8 657 839	100.00	2 160 841	100.00		

R. Time: Retention Time; EAOEL: ethyl acetate-soluble portion of *A. occidentale* ethanolic leaf extract; A/H: area/height.

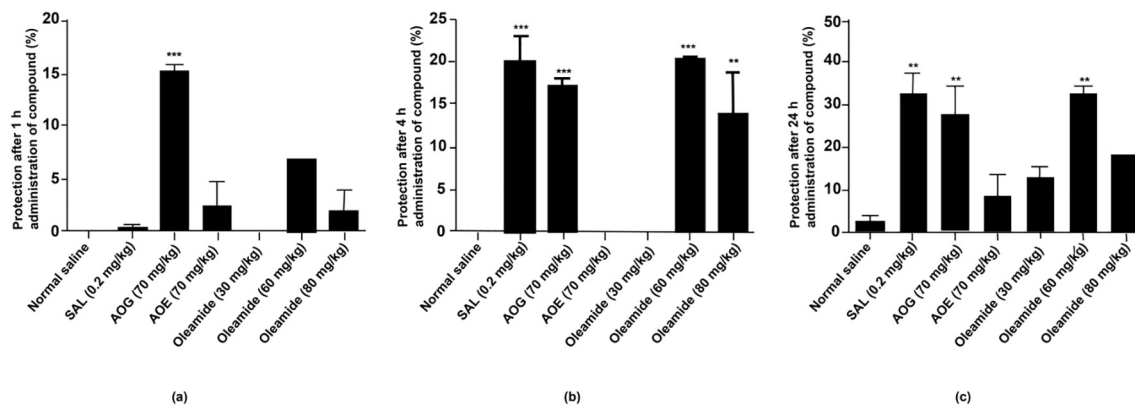


Fig. 4. Effects of administration of isolated fractions G and E, and synthetic oleamide and salbutamol on histamine-induced bronchoconstriction in guinea pigs 1, 4 and 24 h after drug administration. Data were analyzed by a one-way analysis of variance followed by the Newman–Keuls multiple comparison test. Values are represented as mean of three replicates \pm standard error of mean. Asterisks denote statistically significant ($^*P < 0.01$, $^{***}P < 0.001$) differences compared with normal saline control. AOG: fraction G; AOE: fraction E.

HAOEL, CAOEL and EAOEL have significant bronchodilatory and anti-inflammatory activities. Oleamide (9-octadecenamide) was identified as the most abundant compound in the extracts and was isolated. Oleamide significantly increased bronchodilatory and anti-inflammatory activities.

A traditional immunological model for the induction of airway obstruction by an antigen is the histamine-induced bronchoconstriction [17]. Histamine exposure in the airway leads to increased vascular permeability, mucus production and contraction of airway smooth muscle cells, all of which result in airway

hyper-responsiveness and obstruction, and as such, direct bronchoconstriction [18]. Our results showed that *A. occidentale* leaf extracts and salbutamol (a bronchodilator) expressed bronchodilatory activities in histamine-induced bronchoconstriction in guinea pigs (Figs. 1–3). This observation is similar to the work of Mensah et al. [19], where methanolic extract of *Abrus precatorius* leaves prolonged the PCT in guinea pigs following histamine-induced bronchospasm. The significant increase in the PCT and bronchodilatory activity by the extracts is an indication that the plant is acting through the stimulation of β_2 -adrenergic receptors.

Table 8

Effects of administration of oleamide and hydrocortisone against histamine-induced paw edema in Wistar rats.

Treatment group	Increase in paw diameter (cm)/protection (%)				
	1 h	2 h	3 h	4 h	5 h
Normal saline (control)	0.22 ± 0.01	0.21 ± 0.02	0.21 ± 0.02	0.20 ± 0.03	0.16 ± 0.01
Hydrocortisone (4 mg/kg)	0.13 ± 0.02** (39.66)	0.05 ± 0.01*** (75.88)	0.04 ± 0.01*** (82.74)	0.04 ± 0.01*** (81.88)	0.04 ± 0.02*** (76.98)
Oleamide (30 mg/kg)	0.06 ± 0.02*** (71.26)	0.06 ± 0.01*** (71.76)	0.04 ± 0.01*** (80.95)	0.01 ± 0.00*** (96.25)	0.00 ± 0.01*** (98.41)
Oleamide (60 mg/kg)	0.12 ± 0.01** (40.23)	0.09 ± 0.03*** (58.59)	0.06 ± 0.02*** (67.62)	0.04 ± 0.02*** (74.00)	0.03 ± 0.01*** (73.33)
Oleamide (80 mg/kg)	0.13 ± 0.01** (35.63)	0.12 ± 0.02*** (41.65)	0.10 ± 0.09*** (55.24)	0.05 ± 0.02*** (70.00)	0.03 ± 0.02*** (70.79)

Values are represented as mean ± standard error of mean with $n = 3$. ** $P < 0.01$, *** $P < 0.001$, vs control.

Stimulation of β_2 -adrenergic receptors causes relaxation of smooth muscle by increasing cAMP formation. Additionally, β_2 -adrenoreceptor activation enhances Ca^{2+} extrusion and intracellular Ca^{2+} binding, both effects acting to decrease free intracellular Ca^{2+} concentrations [20]. In addition, agents which increase cAMP in the cells inhibit histamine release/secretion [21].

Histamine is an important inflammatory mediator and potent vasodilatory substance which increases vascular permeability [22]. Stimulation of histaminic H_1 receptor on the blood vessels of rat paw by histamine brings about vasodilation leading to the exudation of fluid from the venular end of vessels, resulting in edema and increased vascular permeability, leading to extravasation of cells [23]. The events of inflammation involve the formation of a cycle of inflammatory mediators [24] which was interrupted by the extracts. This study showed that *A. occidentale* leaf extracts and indomethacin ameliorated inflammatory activity in histamine-induced paw edema (Tables 1–3). This implies that the extracts, like indomethacin, can improve, or prevent worsening of the life threatening inflammatory condition.

Usually, plant extracts exist as a combination of different types of bioactive compounds or phytochemicals with different polarities [25] whose combined effect may be either synergistic or antagonistic. Thus, there is often a need to isolate such compounds and investigate their singular effect, comparing it to their combined effect. In this study, the extracts were analyzed using GC–MS; oleamide was identified as the most abundant compound in the extracts (Tables 4–7). Oleamide was further isolated and characterized using FTIR spectroscopy (Figs. S3, S4 and S5, available from the corresponding author). Cis-9-octadecenamide is the prototype member of an emerging class of lipid-signaling molecules, the primary fatty acid amides. Other members of the class include *N*-arachidonoyl ethanolamine (anandamide; AEA), *N*-palmitoyl ethanolamine (PEA) and *N*-oleoyl ethanolamine (OEA) [26,27]. Oleamide has been shown to enhance microglial anti-inflammatory activity both *in vitro* and *in vivo* [28]. Evidence also abounds that oleamide exhibits some cannabimimetic and relaxation responses, and that rimonabant, a CB_1 cannabinoid receptor antagonist, significantly inhibited relaxation induced by oleamide [29]. Delta⁹ tetrahydrocannabinol is a cannabinoid agonist and the major psychoactive component of *Cannabis*, which has been shown to act like salbutamol to significantly improve ventilatory function. Maximal bronchodilation was achieved more rapidly with salbutamol in that study, but at 1 h both drugs were equally effective [30]. The observed potent bronchodilatory and anti-inflammatory properties exhibited by the extracts could be due to the presence of oleamide. Oleamide was therefore isolated in this study as fractions G and E from EAOEL with a 93% similarity index (Figs. S2 and S3, available from the corresponding author).

Medicinal plants may be potential sources of inhibitors of cyclooxygenase-2/lipoxygenase that may have fewer side effects than nonsteroidal anti-inflammatory drugs [31]. The lipoxygenase (LOX) pathway is active in leucocytes and many immune-competent cells, including mast cells, neutrophils, eosinophils, monocytes and basophils. When cells become activated, arachi-

donic acid is cleaved from cell membrane phospholipids by phospholipase A_2 and donated by LOX-activating protein to LOX, which then metabolizes arachidonic acids in a series of reactions to leukotrienes, a group of inflammatory mediators [32]. Leukotrienes act as phagocyte chemo-attractant, recruiting cells of the innate immune system to sites of inflammation. For instance, in an asthmatic attack, it is the production of leukotrienes by LOX that causes the constriction of bronchioles leading to bronchospasm [33]. Therefore, the selective inhibition of LOX is an important therapeutic strategy for asthma [31]. Findings from this study showed that oleamide significantly increased ($P < 0.05$) bronchodilatory and anti-inflammatory activities by 32.97% and 98.41%, respectively (Fig. 4, Table 8). This suggests that oleamide may be acting as an inhibitor of the activities of LOX, thus providing potential therapies to manage both bronchoconstriction and inflammation.

In conclusion, this study demonstrated that oleamide is a major bioactive constituent responsible for the bronchodilatory and anti-inflammatory activities of *A. occidentale* leaf. The isolated compound can therefore be further explored for the probable management of these conditions.

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Competing interest

The authors declare no conflict of interest.

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