Heliyon 7 (2021) e07910

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Fruits of *Vitex doniana* sweet: toxicity profile, anti-inflammatory and antioxidant activities, and quantification of one of its bioactive constituents oleanolic acid

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ARTICLE INFO

Keywords:
Acute inflammation
Subacute toxicity
DPPH antioxidant activity
HPLC method validation
Biomarker
Herbal drug quality control
Oleanolic acid
Vitex doniana sweet

ABSTRACT

Background: *Vitex doniana* Sweet fruit, an under-utilised crop specie of Ghana, has not been validated for its ethnomedical use in managing inflammatory conditions. Therefore, the study sought to investigate its anti-inflammatory and antioxidant activities as well as isolate and quantify one of its active constituents.

Materials and methods: *In-vivo* anti-inflammatory activity of the methanol fruit extract was evaluated using the carrageenan-induced oedema model in chicks. The *in-vitro* antioxidant property was also investigated using the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay. The acute and subacute toxicity studies of the fruit extract were evaluated in rodent models.

Results: No signs of autonomic and central nervous system stimulation/depression were recorded. The LD_{50} by oral route, was estimated to be beyond 3000 mg/kg. Subacute studies revealed an increase in red blood cell and lymphocyte counts. Liver enzymes, serum proteins and bilirubin levels did not significantly increase. The crude extracts at doses of 10, 30 and 100 mg/kg inhibited paw oedema considerably. The ethyl acetate fraction showed the highest antioxidant activity (IC_{50} = 99.35 ± 0.77 μg/mL). Oleanolic acid, isolated from the ethyl acetate extract, showed significant anti-inflammatory and antioxidant activities. A sensitive high-performance liquid chromatography method for the detection and estimation of oleanolic acid, as a biomarker compound for *V. doniana* fruit, was developed and validated for quality assurance purposes.

Conclusion: The extract of *V. doniana* fruits possesses considerable anti-inflammatory and antioxidant properties and was non-toxic under laboratory conditions.

1. Background

Inflammation is involved in the pathogenesis of several diseases, including rheumatoid arthritis, glomerulonephritis, hepatitis and vasculitis with their associated high debilitations and mortality [1]. The release of inflammatory mediators such as prostaglandins, leukotrienes and cytokines during the inflammation cascade, plays a critical role in the body's immune response to noxious stimuli. However, if these mediators are not regulated, the result is chronic inflammation.

During the inflammatory cascade, production and release of toxic reactive oxygen radicals such as peroxide and superoxide by polymorphonuclear leukocytes in oxidative burst as part of phagocytosis, is associated with oxidative stress. Oxidative stress is implicated in the complications of inflammatory conditions as these highly reactive chemicals reacts with membrane lipids, proteins, nucleic acids and enzymes, resulting in cellular damage [2]. If not deactivated or controlled, these reactive oxygen species may accumulate and overwhelm the body's defense mechanism and aggravate the inflammatory process, leading to chronic inflammation [3, 4].

The drugs of choice for the management of inflammatory disorders; steroidal and non-steroidal anti-inflammatory drugs, are documented to have a plethora of unwanted side effects, including immunosuppression,

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https://doi.org/10.1016/j.heliyon.2021.e07910
Received 9 June 2021; Received in revised form 6 August 2021; Accepted 30 August 2021

Cushing syndrome, hyperlipidaemia, gastric ulcerations, bleeding and renal dysfunction [5, 6]. Thus, the search for and development of less toxic and effective alternative agents for the management of inflammatory disorders is an excellent research strategy. The large repository of plants used in traditional medicine may provide important drug leads for the discovery of such novel agents.

Vitex doniana Sweet is commonly used in traditional settings for the management of a number of diseases. A decoction of the woody parts of the plant is used in the treatment of inflammatory disorders, stomach pains, diarrhoea, rheumatic pains and dysentery [7]. The plant is known for its fruit which serves as a delicacy for majority of people, especially in the Northern and Eastern part of Ghana. The use of the fruit is widespread; however, its biological activities have not been explored. Medicinal plants contain a number of secondary metabolites that may exhibit direct anti-inflammatory activity as well as mitigate the threat of reactive oxygen species in oxidative burst. For some others, their anti-inflammatory actions have been linked to their antioxidant activities. For example, the anti-inflammatory activities of some triterpenoids such as ursolic acid, betulinic acid and the flavonoids apigenin, oroxylin A, quercetin and myricetin have been associated with their antioxidant activities [5, 8].

Therefore, this study aimed at investigating the anti-inflammatory and antioxidant activities of the fruits of *V. doniana* and track down some of its bioactive metabolites. The study also sought to develop a high-performance liquid chromatography (HPLC) method for the detection and quantification of oleanolic acid (isolated from the plant) as a biomarker. Establishing such analytical parameters would help in crude drug evaluation (for quality control purposes). Its safety profile was also determined *in vivo*.

2. Materials and methods

2.1. Drugs and chemicals

Analytical grade solvents: chloroform, ethyl acetate, petroleum ether, methanol; anisaldehyde, ammonium molybdate, sulphuric acid, sodium hydroxide, trichloroacetic acid were obtained from BDH laboratory Limited (Poole, England), 2, 2-diphenyl-1-picrylhydrazyl (DPPH), disodium hydrogen phosphate, gallic acid, ascorbic acid, sodium dihydrogen phosphate. monohydrate, potassium ferricyanide, ferric chloride, polysorbate 80 were bought from Sigma Aldrich (St. Louis, MO. USA), diclofenac, dexamethasone (Troge Medical GmbH, Germany), normal saline (Amanta Healthcare Ltd., Gujarat, India), All solvent systems employed for the HPLC analyses were of HPLC grade.

2.2. Plant material collection and processing

The fresh fruits of *V. doniana* were harvested from a farmland in Tamale, in the Northern region of Ghana in 2017 and authenticated by Mr. Clifford Asare of the Department of Herbal Medicine, Faculty of Pharmacy and Pharmaceutical Sciences, Kwame Nkrumah University of Science and Technology (KNUST) where voucher specimen has been deposited (Specimen voucher number: KNUST/HM/2017/F001). The fresh fruits were then washed under running water and the seeds removed. The fleshy pericarp was then oven-dried at 60 °C for 48 h and pulverised into coarse powder.

2.3. General procedures

Column Chromatography (CC) was performed using silica gel 60 (70–230 mesh; AppliChem, GmbH, Darmstadt, Germany) or sephadex LH-20 (25–100 μm; Amersham Biosciences) as stationary phases. Thin Layer Chromatography (TLC) was carried out using pre-coated silica gel 60 plates (0.25 mm thickness) incorporated with fluorescent indicator GF254. 1D and 2D NMR spectra were recorded at 25 °C on a Bruker Avance 500 MHz NMR spectrophotometer. Chemical shifts (δ) were expressed in parts per million (ppm) using tetramethylsilane (TMS) as internal standard and coupling constants (*J*) were measured in Hertz (Hz). Functional groups were identified using the Bruker Fourier transform infrared (FT-IR) spectrometer scanned between 4000-400 cm^{-1} with a resolving power of 4 cm^{-1} and a cumulative scanning limitation of 24 times. Melting point was determined using the Stuart SMP10 digital melting point apparatus.

2.4. Extraction of plant material and isolation of VDF1

The powdered fruits of *V. doniana* (2.8 kg) was soxhlet-extracted using 10 L of methanol (MeOH) for 72 h and the filtrate concentrated under vacuum to a small volume using the rotary evaporator at 50 °C. The extract was then evaporated to dryness in a desiccator to afford a semi-solid extract (VDF, 145 g). Thereafter, 140 g of the VDF was successively partitioned with petroleum ether (Pet Ether), ethyl acetate (EtOAc) and MeOH to and concentrated to afford three major fractions: VDF-Pet Ether (9.61g), VDF-EtOAc (38 g) and VDF-MeOH (86 g) fractions [9].

VDF-EtOAc (35 g) was subjected to column chromatography on silica gel and gradiently eluted with 100% petroleum ether, then, 15%, 25%, 35%, 45%, 60%, 70%, 80% and 100% ethyl acetate, followed by 20%, 40%, 60%, 80% and 100% methanol to obtain sixty fractions (80 mL each) which were bulked to obtain 6 fractions: BF1 – BF6. Fraction three (BF3) (5.0 g) was further purified using silica and gradiently eluted with petroleum ether 90%, then 15%, 20%, 30%, 40%, 60% and 100% ethyl acetate to obtain forty-six fractions (30 mL each). The eluates were monitored with TLC and bulked into four [4] sub-fractions (BF3A – BF3D) based on their TLC profiles. Sub-fraction three (BF3C) was further column chromatographed on sephadex LH-20 eluting isocratically with chloroform and methanol, $CHCl_3$: MeOH (1:1) to yield compound VDF1 (1000 mg) as a white amorphous powder.

2.5. Experimental animals

Day old white leghorn cockerels (*Gallus gallus* domesticus) were obtained from Akate farms, Kumasi. They were fed with chick starter mash, from Maridav Ghana Limited, Tema and water *ad libitum*.

Sprague-Dawley rats (8–10 weeks old, 120–160 g) were obtained from the animal house of the Department of Pharmacology, KNUST. They were fed with commercial pellets from Agricare Limited, Kumasi.

The experimental animals were kept in standard aluminium laboratory cages (34 × 47 × 18 cm) with fine wood shavings as bedding and maintained in a 12-hour light and dark cycle, temperature of 25 ± 2 °C. All experimental procedures were carried out in accordance with the Declaration of Helsinki and the Guiding Principles in the Care and Use of Animals and were subsequently approved by Departmental ethics committee (PCOL/ETH/07022019).

2.6. Toxicity studies of V. doniana fruit extract (VDF)

2.6.1. Acute toxicity studies of VDF

Acute toxicity studies of VDF was evaluated according to the Organisation of Economic Co-operation and Development (OECD) guidelines (407) for testing of chemicals [10]. Sprague-Dawley rats of both sexes were randomly assigned to one of five groups (n = 5). Group I served as the vehicle control receiving only distilled water. Groups II–V received 100, 300, 1000 and 3000 mg/kg of extract by oral gavage respectively. Animals were observed for 0.5, 1, 2, 4 and 24 h after drug administration for general physiological and behavioural changes as well as mortality.

2.6.2. Subacute toxicity of VDF

Subacute oral toxicity study was carried out according to OECD guidelines (407) for testing of chemicals [10]. Sprague-Dawley rats were randomly assigned to one of four dose levels (n = 6). On the basis of the results obtained from the oral acute toxicity study, VDF was administered

at 100, 300 and 1000 mg/kg every 24 h for 14 days. Control animals received normal saline. On the day after the last dose, all animals were euthanised and blood samples were collected for haematological analysis using EDTA tubes and serum biochemical studies using gel-tubes [11]. Their kidneys, liver and spleen were removed, cleaned of excess tissues and the wet weight determined. Liver and kidney were preserved in 10% formalin for histopathological studies.

2.7. Anti-inflammatory activity of VDF, fractions and isolate

The carrageenan foot oedema model of inflammation in chicks, described by Roach and Sufka [12] with modification by Wood et al., [13], was employed in evaluating the anti-inflammatory activities of the extracts using diclofenac and dexamethasone as reference drugs. Carrageenan (10 μL of a 2% suspension in normal saline) was injected sub-plantar into the left footpads of the chicks. The foot volume was measured before injection and at hourly intervals for 5 h, after injection, by means of a vernier caliper. The experimental chicks received the extracts dosed at 30, 100 and 300 mg/kg, diclofenac (10, 30 and 100 mg/kg, *p.o*), dexamethasone (0.1, 1.0 and 3.0 mg/kg, *p.o*) whereas the control animals received only normal saline which served as the vehicle. The oedema component of inflammation was determined by measuring the difference in foot volume before carrageenan injection and at the various time intervals. Similarly, the isolated compound was evaluated for anti-inflammatory activity as described, dosing orally at 10, 30 and 100 mg/kg body weight.

2.8. Antioxidant activity of extracts of V. doniana fruit and oleanolic acid

The extracts of *V. doniana* (2000–62.5 μg/mL in methanol), oleanolic acid and the reference drug, gallic acid, at concentrations of 100–0.78 μg/mL were evaluated for their DPPH free radical scavenging activity using standard methods described by Govindarajan et al., [14].

2.9. Liquid chromatographic method development and validation for oleanolic acid estimation

An isocratic and sensitive HPLC method was developed and validated for both the qualitative and quantitative determination of oleanolic acid as either isolate of oleanolic acid or in the extracts obtained from the fruits of *V. doniana*.

2.9.1. HPLC system employed in the method development

The HPLC system consisted of an Agilent, Model No. 1260 containing a binary pump, autosampler, degasser and a variable wavelength detector (Agilent Technologies Inc., Santa Clara, USA). Optimised chromatographic conditions included the use of a reverse phase stationary support material (Phenomenex, C8; 5μ, 150 mm×4.6 mm, USA), a mobile phase composition of acetonitrile: 0.1 % orthophosphoric acid (90:10; v/v, Sigma Aldrich (St. Louis, MO. USA). Elution of the compound of interest was in isocratic mode and the compound detected at a wavelength of 210 nm with a flow rate of 1 mL/min. All data were acquired using the chemstation software.

2.9.2. Preparation of standard solution of oleanolic acid (0.001% $^{w}/_{v}$)

An amount (1 mg) of adequately dried isolate of oleanolic acid was accurately weighed and transferred into a 100 mL volumetric flask containing 50 mL of the mobile phase (as diluent). The solution was sonicated for 10 min and finally made up to volume with mobile phase to obtain a stock solution of 0.01 mg/mL. Serial dilutions were then prepared by accurately pipetting 0.625 mL, 1.25 mL, 2.5 mL, 5.0 mL, 10.0 mL and 20.0 mL of the 0.01 mg/mL solution into a 50 mL volumetric flask separately and made up to volume with the mobile phase. Solutions of different concentrations were then analysed with the optimised HPLC conditions and responses obtained for the individual concentrations recorded. A standard curve was then prepared.

2.9.3. Validation of the HPLC method

The developed analytical method was validated to establish its ability to provide reliable data needed for quality assurance purposes. This was done according to the International Conference on Harmonization (ICH) guidelines [15] with parameters such as linearity and range, limit of detection (LOD) and limit of quantification (LOQ), precision (intra-day and inter-day), accuracy, robustness and stability of solution investigated. Furthermore, the optimisation of certain performance parameters such as asymmetry factor and resolution factor of peaks obtained for the developed analytical method were also investigated.

2.9.4. Specificity

The analytical method was evaluated to establish unequivocally that oleanolic acid in the presence of other components or impurities, both in the solvent systems or in the extracts, could be determined and quantified without significant interference from such components or impurities. The blank or mobile phase was analysed first, followed by a 0.0005 mg/mL concentration of the standard solution (blank solution spiked with standard solution) and finally the solution of the crude sample.

2.9.5. Linearity and range

Linearity and range of the method were assessed by testing different concentrations of oleanolic acid (0.000125 mg/mL to 0.004 mg/mL) in the mobile phase. Triplicate determinations were carried out for each test concentration. A plot of the mean peak areas obtained from the analyses of the respective concentrations and statistical analysis was performed by the method of least.

2.9.6. Limit of detection (LOD) and quantification (LOQ)

The LOD and LOQ for oleanolic acid was determined from the linearity data using Eqs. (1) and (2).

$$y = 3.3\ SD/S \text{ and} \quad (1)$$

$$y = 10\ SD/S \text{ respectively.} \quad (2)$$

where SD - standard deviation of the responses, S - slope of the calibration curve.

2.9.7. Precision

2.9.7.1. Intra-day precision. A volume (2.5 mL) of the 0.01 mg/mL standard stock solution of oleanolic acid was accurately measured into a 50 mL volumetric flask and made up to volume with the mobile phase to obtain the working concentration of 0.0005 mg/mL. This solution was analysed using the described analytical method six times and the intra-day precision determined using the relative standard deviation (RSD) of the results obtained.

2.9.7.2. Inter-day precision. A solution of concentration 0.0005 mg/mL was accurately prepared and analysed using the described analytical method. All analyses were done in triplicates and the inter-day precision determined using the RSD of the results obtained. Here, the solution was analysed by two different analysts under the same experimental conditions.

2.9.8. Accuracy/recovery

This parameter was investigated at three concentration levels of 0.0004, 0.0005 and 0.0006 mg/mL representing 80%, 100% and 120% levels of the working concentration. The recoveries of oleanolic acid by the method of standard addition were determined.

2.9.9. Robustness

Investigation for the robustness of the method was done by analysing a solution of concentration 0.0005 mg/mL under two conditions (original conditions as described above and a varied condition by using a C8 column from a different manufacturer). All analyses were done in

triplicate and the robustness determined using the RSD of the results obtained.

2.9.10. Stability of oleanolic acid solution

The stability of the oleanolic acid solution (0.0005 mg/mL) was tested over a 48-h period to access its stability during the period of analysis. Triplicate sample injections were carried out at predetermined time intervals and the mean peak areas were analysed afterwards.

2.10. Determination of oleanolic acid content in the fruit extract of *V. doniana* (VDF)

About 10 mg of the methanolic crude extract of the fruits of *V. doniana* was accurately weighed and transferred into a 50 mL beaker and dissolved in the mobile phase. The solution was sonicated for 10 min to completely dissolve the extract. This solution was accurately transferred into a 100 mL volumetric flask and made up to volume with the mobile phase. This final solution was filtered prior to HPLC analyses using a membrane filter. About 20 uL of the crude extract solution was then analysed using the optimised conditions described. The chromatograms (triplicate determination) obtained were used for the analyses. The quantity in milligram (mg) of oleanolic acid per gram of the crude extract were then determined.

2.11. Statistical analysis

GraphPad Prism 5 for Windows (version 5.01, 2007; GraphPad Prism Software, San Diego, USA) was used to perform validation related-calculations, which included standard deviations, relative standard deviations, standard errors of means, regression analysis, construction of calibration plots for plant isolates, among others.

All results for ED_{50} in the anti-inflammatory assay were presented as mean ± standard error of mean, (SEM). Statistical analysis was done using Dunnet Multiple Comparison Test. P value <0.05, was considered significant.

3. Results

3.1. Toxicity profile

Administration of *V. doniana* fruit extract (VDF) (100–3000 mg/kg) to Sprague-Dawley rats in the acute toxicity studies did not result in mortality and its lethal dose (LD_{50}) was estimated to be above 3000 mg/kg body weight. There were no signs of neuro-behavioural changes or alteration in the autonomic nervous system of treated animals.

Subacute studies revealed an increase in haematological parameters; red blood cells, haemoglobin, packed cell volume as well as lymphocyte counts at all doses. This effect was statistically significant at 100 mg/kg for haemoglobin and haematocrit. Treatment of Sprague-Dawley rats didn't affect the mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration and the mean corpuscular volume. Neutrophils decreased significantly at 100 mg/kg and 300 mg/kg. Platelet count was however, not affected. Liver enzymes (alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase and gamma-glutamyl transferase) as well as serum proteins and bilirubin levels were not significantly affected by *V. doniana* treatment. Renal function was also not significantly affected (Table 1). Morphological study of organs as well as histopathological studies did not reveal any significant changes with highly perfused organs such as the liver, kidney, heart and lungs. The relative organ to body ratios of spleen, thymus and adrenals were not significantly affected by treatment (Table 2).

Table 1. Effect of VDF on haematological and biochemical parameters in rodents.

Haematological Parameters	*V. doniana* extract (mg/kg body weight)			
	0	100	300	1000
WBC (10^9L)	25.4 ± 1.52	38.0 ± 5.99	26.7 ± 5.03	19.5 ± 2.23
RBC (10^{12}L)	6.9 ± 0.09	7.9 ± 0.43	7.6 ± 0.65	7.1 ± 0.17
HB (g/dl)	14.2 ± 0.17	17.0 ± 0.79*	16.3 ± 1.19	15.2 ± 0.27
HCT (%)	39.9 ± 0.73	47.5 ± 1.53*	44.2 ± 2.92	41.6 ± 0.90
MCV (fL)	57.9 ± 0.25	56.8 ± 1.11	58.4 ± 1.28	58.8 ± 1.42
MCH (pg)	21.9 ± 0.11	21.5 ± 0.29	21.5 ± 0.37	21.4 ± 0.38
MCHC (%)	37.6 ± 0.20	35.9 ± 2.01	36.9 ± 0.57	36.5 ± 0.23
PLATELET (109L)	562.1 ± 21.3	421.8 ± 42.4	584.8 ± 54.8	630.6 ± 68.5
LYMPHOCYTE (%)	81.8 ± 0.72	86.8 ± 1.29	86.2 ± 0.83	84.5 ± 2.01
NEUTROPHIL (%)	8.3 ± 0.54	5.0 ± 0.77*	5.1 ± 0.34*	5.8 ± 1.12
Serum Biochemistry				
ALT (U/L)	133.8 ± 5.1	116.2 ± 8.50	132.8 ± 31.2	95.58 ± 9.2
AST (U/L)	249.7 ± 9.0	203.0 ± 13.8	210.4 ± 22.6	265.0 ± 18.8
ALP (IU/L)	212.0 ± 7.1	233.4 ± 23.5	186.3 ± 27.6	206.8 ± 28.1
GGT (IU/L)	5.0 ± 0.38	6.8 ± 0.64	4.3 ± 0.46	3.7 ± 0.55
TOTAL PROTEIN (g/L)	73.0 ± 0.99	69.1 ± 5.73	72.4 ± 4.57	68.7 ± 3.73
ALBUMIN (g/L)	30.5 ± 0.63	29.2 ± 1.77	35.3 ± 3.90	29.6 ± 2.58
GLOBULIN (g/L)	42.4 ± 1.09	39.9 ± 4.49	37.0 ± 6.56	39.2 ± 1.63
BILIRUBIN (μmol/L)	6.3 ± 0.19	6.7 ± 0.73	7.4 ± 1.04	5.7 ± 0.47
DIRECT BILIRUBIN (μmol/L)	2.8 ± 0.14	2.9 ± 0.52	3.8 ± 0.29	2.1 ± 0.29
INDIRECT BILIRUBIN (μmol/L)	3.4 ± 0.18	3.8 ± 0.41	3.6 ± 0.92	3.6 ± 0.48
UREA (mmol/L)	11.3 ± 0.34	9.6 ± 1.45	10.1 ± 1.51	10.1 ± 1.29
CREATININE (mmol/L)	67.2 ± 1.88	79.1 ± 5.07	75.7 ± 5.49	68.0 ± 4.86

Values are presented as mean ± SEM. $P < 0.05$ (statistically significant). VDF:MeOH extract of *V. doniana fruits*.

Table 2. Effect of VDF on the relative organ weight of rodents.

Parameter (g)	*V. doniana* extract (mg/kg body weight)			
	0	100	300	1000
Initial weight	121.8 ± 11.00	127.2 ± 13.5	104.0 ± 0.05	108.0 ± 1.00
Final weight	142.1 ± 12.3	130.2 ± 8.35	122.0 ± 4.00	117.0 ± 3.99
Liver	4.8 ± 0.26	5.5 ± 0.26	4.6 ± 0.44	3.6 ± 1.99
Kidney	0.96 ± 0.06	0.91 ± 0.20	0.83 ± 0.11	0.69 ± 0.05
Spleen	0.29 ± 0.05	0.21 ± 0.03	0.23 ± 0.05	0.24 ± 0.03
Thymus	0.25 ± 0.02	0.28 ± 0.03	0.26 ± 0.02	0.25 ± 0.04
Adrenals	0.07 ± 0.05	0.08 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
Rel. liver	0.03 ± 0.00	0.04 ± 0.00	0.04 ± 0.00	0.03 ± 0.00
Rel. Kidney	0.007 ± 0.0	0.006 ± 0.0	0.006 ± 0.0	0.006 ± 0.0
Rel. Spleen	0.002 ± 0.0	0.002 ± 0.0	0.002 ± 0.0	0.002 ± 0.0
Rel. Thymus	0.002 ± 0.0	0.002 ± 0.0	0.002 ± 0.0	0.002 ± 0.0
Rel Adrenal	0.0004 ± 0.0	0.0005 ± 0.0	0.0002 ± 0.0	0.0002 ± 0.0

Values presented as mean ± SEM in grams for all weight measurements (n = 6). Rel. represents relative organ.

3.2. Anti-inflammatory activity of VDF, fraction and isolate

The anti-inflammatory activity of the crude MeOH extract of *V. doniana* fruit (VDF), solvent fractions and isolated compound, oleanolic acid, were assessed using carrageenan induced oedema model in chicks. VDF, oleanolic acid and diclofenac exhibited a significant ($P < 0.05$) reduction of inflammation induced by carrageenan in the chicks with the observed effect occurring 2 h after carrageenan injection (Figure 1). The overall anti-inflammatory activity was expressed as the median effective dose, ED_{50} (mg/kg). Dexamethasone demonstrated the greatest activity, followed by diclofenac, methanol soluble fraction, ethyl acetate fraction, oleanolic acid, petroleum ether fraction and the crude methanol extract, respectively (Table 3).

During the inflammatory cascade, the unregulated surge of free radicals released by phagocytic leukocytes, has been linked to chronic inflammatory conditions [16]. Hence the present study explored the

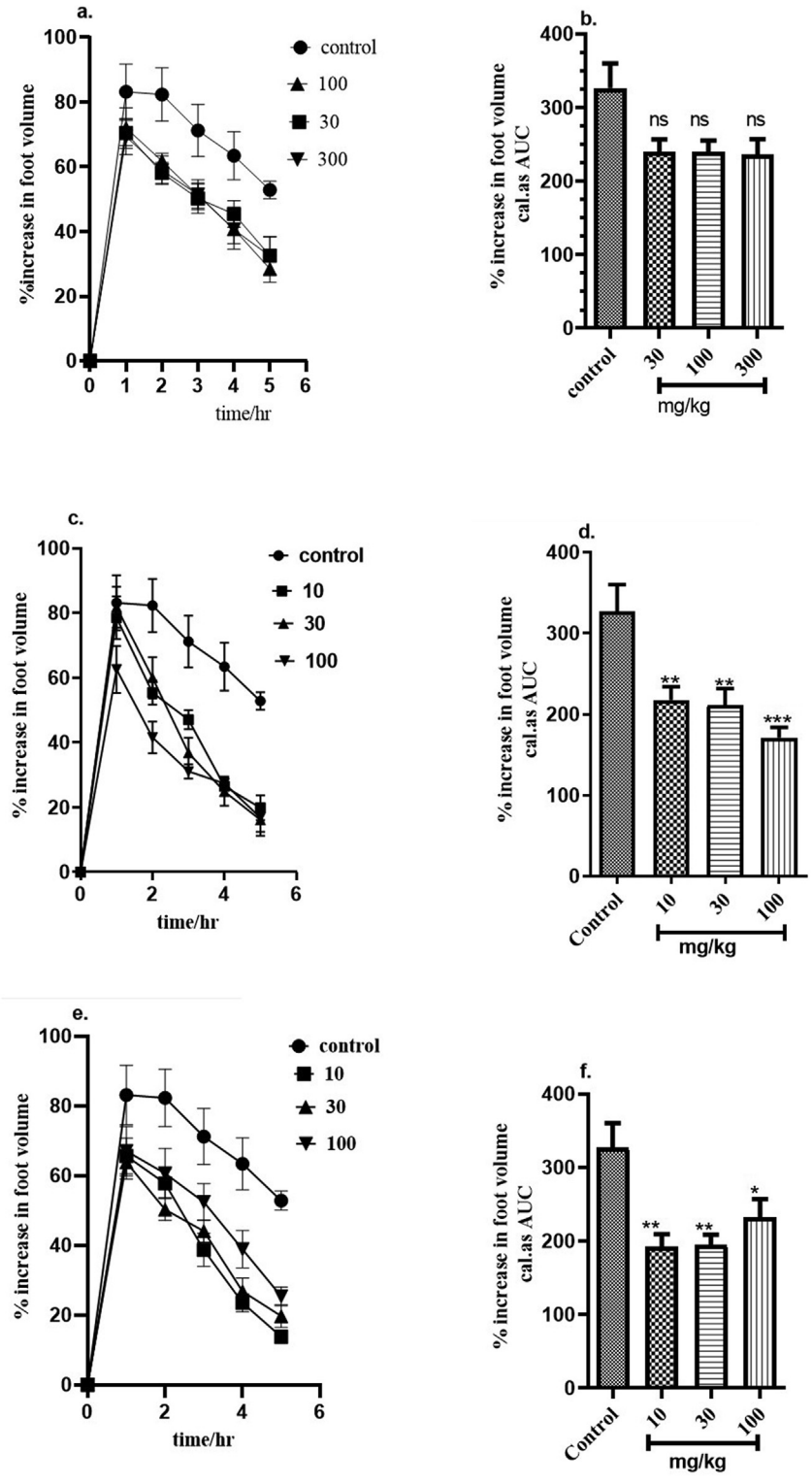

Figure 1. Effects of crude extract of *V. doniana* (30–300 mg/kg, oral), diclofenac (10–100 mg/kg; *p. o*) and compound (oleanolic acid) (10–100 mg/kg, oral) on the time course curve (a, c and e respectively) and the percentage increase in foot volume (oedema) expressed as area under the curve, (AUC) (b, d and f respectively) for 5 h in carrageenan-induced paw oedema in chicks. Values are indicated as mean ± SEM (n = 5) ***P < 0.001 **P < 0.01; *P < 0.05, ns = not significant compared to vehicle treated group (one way ANOVA) followed by Dunnet's *post hoc* test.

Table 3. Effect of the extracts of *V. doniana* fruits, oleanolic acid and standard drugs on carrageenan-induced oedema.

Plant extract/drug	ED_{50} (mg/kg) ± SEM
VDF	67 ± 0.65
VDF-Pet ether	46.21 ± 0.67
VDF-EtOAc	19.17 ± 2.89
VDF-MeOH	16.52 ± 4.65
Dexamethasone	7.19 ± 4.91
Diclofenac	7.55 ± 1.09
Oleanolic acid	19.24 ± 0.87

Values are mean ± SEM. VDF: Crude methanol extract; VDF-Pet ether, VDF-EtOAc and VDF-MeOH: petroleum ether, ethyl acetate and methanol fractions respectively of the crude extract.

Table 4. DPPH scavenging activity of extracts of *V. doniana* fruits, oleanolic acid and gallic acid.

Extract/compound	IC_{50} (μg/mL) ± SD
VDF	116 ± 0.57
VDF-Pet ether	117 ± 0.54
VDF-EtOAc	99.35 ± 0.77
VDF-MeOH	104.4 ± 0.65
Gallic acid	0.71 ± 0.62
Oleanolic acid	2.80 ± 0.56

VDF: Methanol extract of *V. doniana* fruits; VDF-Pet ether, VDF-EtOAc and VDF-MeOH: petroleum ether, ethyl acetate and methanol fractions respectively of the crude extract.

antioxidant capacity of *V. doniana* fruit extracts in mitigating the damaging effects of free radicals using the DPPH radical scavenging activity. The reference drug used in this assay was gallic acid. The extracts, oleanolic acid and gallic acid demonstrated varying degrees of DPPH radical scavenging activity. The ethyl acetate extract showed the highest activity followed by the methanol, total crude and pet-ether extracts, respectively (Table 4).

In an attempt to track down the bioactive constituents, the ethyl acetate fraction was column chromatographed to afford the triterpenoid, oleanolic acid (Figure 2). The compound demonstrated significant anti-inflammatory activity (Table 3 and Figure 1).

3.3. Characterisation of VDF1 as oleanolic acid

Compound **VDF1** was obtained as a white amorphous powder whose melting point was determined to be 298–300 °C. The IR spectrum (Figure S1) revealed characteristic signals for hydroxyl, carbonyl and olefinic groups at *Vmax* of 3457 cm^{-1}, 2924 cm^{-1}, 2852 cm^{-1} and 1687 cm^{-1}. The molecular formula was determined as $C_{30}H_{48}O_3$ suggesting that VDF1 is a triterpenoid.

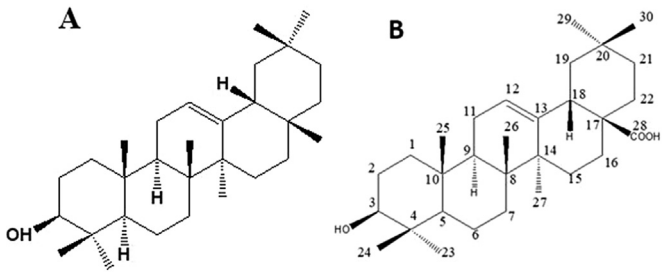

Figure 2. a. Oleanane-type triterpenoid nucleus b. Oleanolic acid.

The ^1H NMR spectrum of VDF1 (Figure S2 and S3) indicated the presence of seven aliphatic methyl singlets at $^{\delta}H$ 0.75 (H-26), $^{\delta}H$ 0.77 (H-24), $^{\delta}H$ 0.90 (H-29), $^{\delta}H$ 0.91 (H-25), $^{\delta}H$ 0.92 (H-30), $^{\delta}H$ 0.98 (H-23) and $^{\delta}H$ 1.13 (H-27); and an olefinic proton at $^{\delta}H$ 5.28 (H-12, b, s). The spectrum also revealed signals at $^{\delta}H$ 3.21 (1H, d) and $^{\delta}H$ 3.23 (1H, m) which were assigned to methine protons on oxygenated carbons. The DEPT 135 spectrum (Figure S4, S5 and S6) revealed thirty (30) carbon signals that included seven [7] methyl carbons $^{\delta}C$ 28.10 (C-23), $^{\delta}C$ 15.55 (C-24), $^{\delta}C$ 15.32 (C-25), $^{\delta}C$ 17.14 (C-26), $^{\delta}C$ 25.96 (C-27), $^{\delta}C$ 33.09 (C-29) and $^{\delta}C$ 23.60 (C-30) as well as an acidic carbon at $^{\delta}C$ 183.47 (C-28). It also showed ten methylene, five methine and seven quaternary carbons. A broad singlet at $^{\delta}H$ 5.28 in the ^1H NMR was corroborated by resonances at $^{\delta}C$ 122.67 and $^{\delta}C$ 143.61 in the DEPT spectrum. These were assigned to the carbon-carbon double olefinic bond at C-12 and C-13 respectively. The spectrum also revealed a signal for one [1] oxymethine carbon at $^{\delta}C$ 79.04 (C-3). The carboxylic acid functionality at C-17 was confirmed based on the HMBC spectrum (Figure S7, S8 and S9) between the resonances at $^{\delta}C$ 2.83 (H-18) with $^{\delta}C$ 46.53 (C-17) and the carboxyl carbon signal at $^{\delta}C$ 183.47 (C-28). These data indicated that VDF1 has an olean-12-en-28-oic acid nucleus with the hydroxyl group in its structure [17, 18]. The NMR data for VDF1 agreed with that published for oleanolic acid [18] (Table 5).

3.4. HPLC quantification and validation method for oleanolic acid

A C8 reversed phase stationary support material successfully ensured the efficient separation and resolution of the isolated oleanolic acid at a retention time of 4.108 ±0.15min (Figure 3). Three [3] other unknown components within the crude extract were also separated and resolved at 2.158 ± 0.1 min, 2.502 ± 0.05 min and 2.919 ± 0.04 min respectively (Figure 3).

Details of all the parameters validated are shown in the sections below:

3.4.1. Specificity

The specificity of the method for oleanolic acid was determined by analysing the chromatograms obtained from analysing the mobile phase only, followed by a solution of oleanolic acid (mobile phase spiked with oleanolic acid standard solution) and a solution of the crude extract of *V. doniana*. No peak was observed from the mobile phase only. The retention time of oleanolic acid was comparable to the peak of the same compound as identified in the extract. It was observed that a pronounced peak at 4.106 min (mean of triplicate determinations) in the chromatogram of the solution of the crude extract, attributable to oleanolic acid was obtained. This was comparable to the peak observed at 4.108 min (mean of triplicate determinations) in the chromatogram for the oleanolic acid in the standard solution. Also, the peaks of the other compounds in the extract as well as that from unretained compounds in the mobile phase eluted at retention times that were significantly different from that of oleanolic acid ($F_{[3,8]} = 370.8$, $p < 0.0001$). Thus, there was no interference observed in the chromatograms. Hence the developed method was specific (Table 6).

3.4.2. Linearity and range

A dynamic linear range of 0.000125 mg/mL to 0.004 mg/mL with a correlation coefficient (r^2) of 0.9988 provided the needed evidence of a good correlation between various concentrations of isolated oleanolic acid with peak areas obtained (Figure 4).

3.4.3. Precision

3.4.3.1. Intra-day and inter-day precision. The intra-day and inter-day precision obtained (Table 7) were all less than 2.0 % as specified per ICH guidelines. This shows the repeatability of the developed HPLC method.

Table 5. ^{1}H and Carbon 13 (^{13}C) NMR data of VDF1 and oleanolic acid in deuterated chloroform ($CDCl_3$).

Position	Oleanolic acid [18]		VDF1	
	$^{\delta}$ H (mult)	$^{\delta}$ C	$^{\delta}$ H (mult)	$^{\delta}$ C
1		38.40		38.38 (CH_2)
2		27.20		27.17 (CH_2)
3	3.22, d	79.0	3.21, d	79.04 (CH)
4	-	38.70	-	38.75 (C)
5	0.74	55.20	0.73, d	55.19 (CH)
6		18.30		18.29 (CH_2)
7		32.60		32.60 (CH_2)
8	-	39.30	-	39.25 (C)
9	1.54	47.60	1.55	47.62 (CH)
10	-	37.10	-	37.08 (C)
11		22.90		22.91 (CH_2)
12	5.29, br, s	122.60	5.28, br, s	122.67 (CH)
13	-	143.60	-	143.61 (C)
14	-	41.60	-	41.57 (C)
15		27.70		27.67 (CH_2)
16		23.4		23.39 (CH_2)
17	-	46.50	-	46.53 (C)
18	2.83, dd	41.00	2.83, dd	40.96 (CH)
19		45.90		45.88 (CH_2)
20	-	30.60	-	30.69 (C)
21		33.80		33.79 (CH_2)
22		32.40		32.44 (CH_2)
23	0.99, s	28.10	0.98, s	28.10 (CH_3)
24	0.74, s	15.30	0.77, s	15.55 (CH_3)
25	0.91, s	15.50	0.91, s	15.32 (CH_3)
26	0.77, s	17.10	0.75, s	17.14 (CH_3)
27	1.14, s	25.90	1.13, s	25.96 (CH_3)
28	-	182.30	-	183.47 (C)
29	0.91, s	33.00	0.90, s	33.09 (CH_3)
30	0.93, s	23.50	0.92, s	23.60 (CH_3)

3.4.4. Accuracy/recovery and robustness

Other validation parameters like recovery and robustness provided reliable data that can enhance the qualitative and quantitative determination of pure oleanolic acid or when present in crude extracts. The mean amounts of recovered oleanolic acid obtained, with an RSD of less than 2.0 % in the robustness studies, validates the developed method as accurate and robust (Tables 8 and 9).

3.4.5. Limit of detection and quantification (LOD and LOQ)

A sensitive reverse phase method with LOD and LOQ of 0.000106 mg/mL and 0.00032 mg/mL respectively were determined. This provides evidence for the highly sensitive method developed.

3.4.6. Stability of solutions

The % RSD for oleanolic acid in solution determined over the 48-hour period of investigation, ranged from 0.22 to 2.91% (Table 10).

3.4.7. Optimised system suitability parameters

The performance of a developed analytical chromatographic technique requires optimisation of certain critical parameters, to ensure reproducibility of reliable data is assured, irrespective of slight variabilities that could be encountered from one laboratory to another. Performance parameters such as peak symmetry as well as resolution of the peaks of oleanolic acid were thus investigated and established.

Optimised performance parameters of the developed and validated HPLC method had the following characteristics;

Specificity: Specific for oleanolic acid as per method developed.

Linearity: > 0.98.

Peak symmetry: $0.9 \leq As \leq 1.2$ (where As is asymmetry factor).

Resolution (using oleanolic acid peak as the biomarker): $Rs \geq 1$.

3.5. Determination of the amount of oleanolic acid in methanolic extract of the fruits of V. doniana

A mean amount of 90.24 mg oleanolic acid was determined per gram of *V. doniana* fruit extract (Table 11).

4. Discussion

Sub-Saharan Africa abound in agro-biodiversity but regrettably, it is the region with the highest malnutrition and food scarcity [19]. Neglected and under-utilised food resources constitute the bedrock of the diversity in traditional and indigenous food systems of communities in developing countries. Indigenous people living in rural areas possess food resources that can be harnessed by the agriculture and health sectors. These neglected and under-utilised traditional crop species rely on biological functioning of the ecosystem and thus require low input of synthetic fertilisers, pesticides and irrigation methods [19]. One such under-utilised crop is *V. doniana*, a tree highly valued by people in various parts of Africa for its edible pulp and seeds as well as the leaves used as vegetables. Scientific validation of its numerous folklore uses, towards the promotion of this under-utilised specie, is not widespread. The present study explored the anti-inflammatory, antioxidant and assessed the toxicity profile of the methanol fruit extract.

The fruit extract of *V. doniana* was found to be safe for consumption even at 3000 mg/kg body weight and showed some beneficial effects on haematological and biochemical parameters (Table 1). It significantly increased the number of red blood cells, pack cell volume and their haemoglobin content. The increase in lymphocyte count at all doses coupled with the mild increase in white blood cells especially at low doses of the extract (Table 1) is suggestive of a possible immune-stimulatory effect. It however did not affect platelet count. The LD_{50} value of the methanol stem bark extract in a similar study was found to be greater than 5000 mg/kg body weight [20]. Thus, the non-toxic nature of *V. doniana* fruits, highlighted in this study, is corroborated by results of other studies on different parts of the plant.

The reduction of oedema in the first hour, by the extract (Figure 1), is suggestive of the possible blocking of the synthesis and release of prostaglandins, lysozyme and proteases [21] responsible for initiating inflammation in the first phase of carrageenan induced oedema. The methanol and ethyl acetate fractions showed the highest anti-inflammatory activities among the plant extracts. Thus, the anti-inflammatory compounds in *V. doniana* may be polar to medium polar in nature (Table 3). The highest anti-inflammatory activity recorded by the methanol soluble fraction is in agreement with the traditional mode of preparation using polar (or aqueous) solvents. In a similar study by Ochieng *et al.* [20], the methanol extract of the stem bark of this plant was shown to exhibit considerable anti-inflammatory activity and thus lends support to the potential use of various parts of *V. doniana* to mitigate inflammatory conditions. The observed anti-inflammatory activity of the extracts (Table 3) gives credence to the use of the plant in treating inflammatory disorders as suggested by folklore medicine. The compound, oleanolic acid, isolated from the fruit, was more active than the crude extract (Table 3). Thus, purification improves the anti-inflammatory activity of *V. doniana* fruit.

During the inflammatory cascade, the unregulated surge of free radicals released by phagocytic leukocytes, has been linked to chronic inflammatory conditions [16]. The observed higher antioxidant activity of the isolated constituent highlights the importance of purification of some medicinal plant extracts to afford higher therapeutic activities. The triterpenoid, oleanolic acid and its isomers occur abundantly in nature either as aglycones or free acids. Oleanolic acid has been isolated from

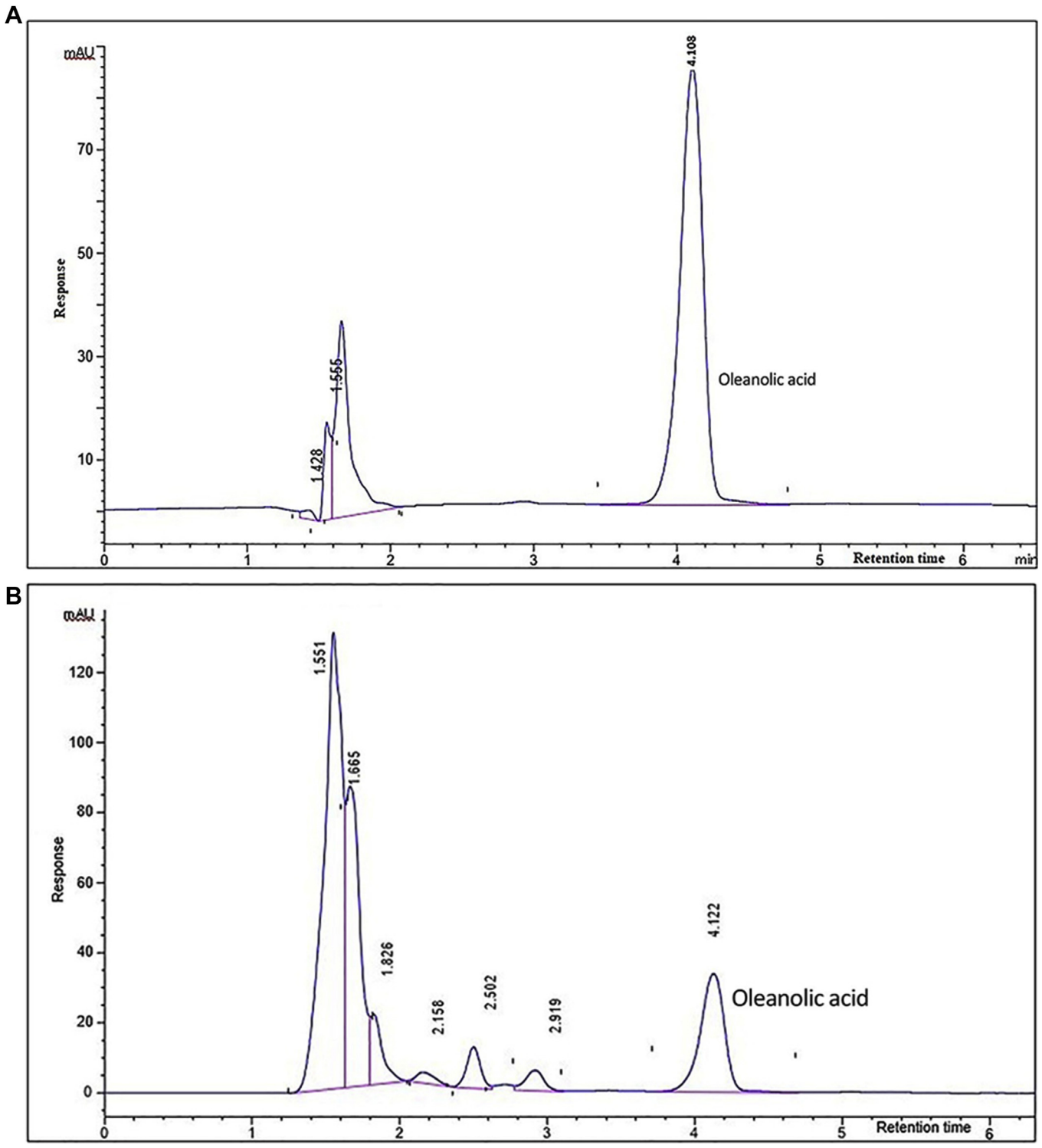

Figure 3. A. Chromatogram of isolated oleanolic acid (0.00050 mg/mL), B. Chromatogram of crude extract of *V. doniana* (0.00019 mg/mL).

many plants including *Olea europeae* Linn., *Syzygium aromaticum* L. Baill., *Calendula officinalis* Linn., *Eucalyptus globulus* Labill, *Salvia officinalis* L., *Satureja montana* L., *Rosmarinus officinalis* L. and *Ocimum basilicum* L. [22]. The compound has been reported to exhibit a dose-dependent inhibition of croton-oil induced ear oedema in mice. The basic carbon nucleus is thought not to have any significant influence on the activity. However, a carboxyl or alcoholic group at carbon-20 and 30 increased the activity [23]. It also selectively inhibited COX-2 catalysed prostaglandin biosynthesis with an IC_{50} of 295 μM and a selectivity ratio of 0.8. The *meta* position of the carboxyl group is reported to essentially influence the inhibitory activity of the complement system [24]. The inhibition of nitric oxide (NO) production by oleanolic acid has also been reported. Studies have shown that oleanolic acid possess antitumour activity (IC_{50} = 26.74 μg/mL) by inhibiting the growth of human leukaemia and lymphoma cells specifically, P3HRI [25]. Elsewhere, its hepatoprotective, antidiabetic and antihypertensive activities have also been reported [26, 27]. The observed activity in this study and that reported in literature indicate that oleanolic acid exhibit considerable anti-inflammatory and antioxidant activities and may be partly responsible for the bioactivity of the fruit extract of *V. doniana*.

Table 6. Results of specificity.

Sample solution	Retention times (mean ± SD)				
	Solvent front	Unknown Cpd 1	Unknown Cpd 2	Unknown Cpd 3	Oleanolic acid
Sample A – Mobile phase only	-	-	-	-	-
Sample B – Mobile phase + oleanolic acid	1.555 ± 0.03	-	-	-	4.108 ± 0.15
Sample C – Mobile phase + extract	1.551 ± 0.06	2.158 ± 0.1	2.502 ± 0.05	2.919 ± 0.04	4.106 ± 0.02
One-way ANOVA analysis of retention times	Retention times are significantly different from each other ($F_{[3,8]} = 370.8$, $p < 0.0001$)				

Table 7. Results for intra-day and inter-day precision.

Precision parameters		% content	%RSD
Intra-day precision	Replicate determinations (n = 6)	100.01 ± 0.02	0.02
Inter-day precision	Analyst 1 (n = 3)	100.11 ± 0.18	0.18
	Analyst 2 (n = 3)	100.09 ± 0.10	0.10
Acceptance criteria			**RSD < 2%**

Quality control of herbal products present with challenges due to the myriads of phytochemicals present. The compound approach in the standardisation of herbal or natural products is reportedly one of the best ways of ensuring their quality and purity [28]. This informed the development of an isocratic HPLC method for the detection and quantification of one of the bioactive components (oleanolic acid) to serve as a quality assurance parameter for the fruit of *V. doniana*. The results obtained indicates that the sensitive, precise, accurate and robust method developed could be used to identify and quantify oleanolic acid even in the presence of other components (Figure 3). All validated parameters yielded results within acceptable criteria. The results obtained for the stability studies clearly indicates that solution of oleanolic acid was stable in the applied and reported solvent system for 24 h after preparation where the RSD determined were not more than 2.0% (Table 10). Thus, reproducible results can be obtained with solutions of oleanolic acid stored at room temperature for 24 h. This indicates that the solution(s) of oleanolic acid cannot be used after 24 h where significant levels of variability are observed. The optimised system suitability parameters established will also ensure that the application of this method for both quantitative as well as qualitative determinations will result in reliable and reproducible

Table 8. Oleanolic acid recovery studies.

Level	Amount taken (mg/mL)	Amount added (mg/mL)	% Recovery*
80%	0.00050	0.00040	100.02 ± 0.10
100%	0.00050	0.00050	100.11 ± 0.09
120%	0.00050	0.00060	101.22 ± 0.11

* indicate the mean of triplicate determinations.

Table 9. Results for robustness studies.

Test	Original Condition	Varied Condition I
	% Content*	% Content*
At 100% concentration level (0.00050 mg/mL)	100.09 ± 0.09	100.72 ± 0.22
%RSD	**0.09**	**0.22**

* Indicate the mean of triplicate determinations.

Table 10. Results for stability of solutions studies.

Time/hours	Mean concentration*	%RSD
0	99.89	0.27
2	99.67	0.34
4	99.60	0.22
6	99.72	0.28
8	99.61	0.92
12	99.41	0.74
18	98.72	0.98
24	98.09	2.91
36	97.33	2.28
48	96.25	2.54

* Values at respective time points are the mean of triplicate determinations.

Table 11. Amount of oleanolic acid in *V. doniana* fruit extract.

Number of Determinations	Amount of Oleanolic acid (mg/g)
1	89.92
2	90.83
3	89.97
MEAN ± SD	**90.24 ± 0.51**

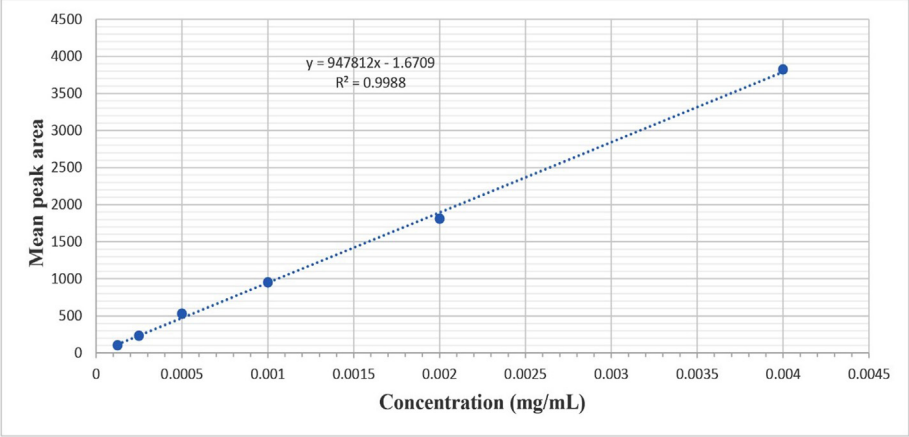

Figure 4. Calibration curve for isolated oleanolic acid.

scientific data for quality assurance purposes. This validated HPLC method was applied in the identification (using the retention times) and the quantification of oleanolic acid (isolated) and in the crude extract.

The method could be employed for routine quality control purposes as all the parameters investigated under the method validation, produced sensitive, reliable and reproducible data.

5. Conclusion

This study has demonstrated that methanol extract of the fruits of *V. doniana* shows considerable anti-inflammatory and antioxidant activities and this was linked to its oleanolic acid content (reported in the plant for the first time). Isolation of compounds in the active methanol soluble fraction could be considered in future research. A HPLC method for the detection and quantification of oleanolic acid has been developed and validated for routine quality control of both isolated oleanolic acid and the fruit methanol extract. The present study has also shown the fruits of *V. doniana* to be safe for consumption.

Declarations

Author contribution statement

Silas Adjei; Isaac Kingsley Amponsah; Samuel Oppong Bekoe; Benjamin Kingsley Harley; Kwesi Boadu Mensah: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Abraham Yeboah Mensah; Gabriel Fosu-Mensah:Performed the experiments; Contributed reagents, materials, analysis tools or data.

Michael Kwesi Baah: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Funding statement

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Data availability statement

Data will be made available on request.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

Supplementary content related to this article has been published online at https://doi.org/10.1016/j.heliyon.2021.e07910.

Acknowledgements

We are grateful to the technical staff of the Faculty of Pharmacy and Pharmaceutical Sciences and Central laboratory, KNUST, Kumasi-Ghana.

References

[1] C.A. Dinarello, Anti-inflammatory agents: present and future, Cell 140 (6) (2010) 935–950.

[2] H. Sies, C. Berndt, D.P. Jones, Oxidative stress, Annu. Rev. Biochem. 86 (1) (2017) 715–748.

[3] M. Nita, A. Grzybowski, The role of the reactive oxygen species and oxidative stress in the pathomechanism of the age-related ocular diseases and other pathologies of the anterior and posterior eye segments in adults, Oxid. Med. Cell Longev. (2016) 1–23.

[4] N. Vergnolle, The inflammatory response, Drug Dev. Res. 59 (4) (2003) 375–381.

[5] A. Azab, A. Nassar, A.N. Azab, Anti-inflammatory activity of natural products, Molecules 21 (10) (2016) 1321.

[6] P.J. Barnes, Anti-inflammatory actions of glucocorticoids: molecular mechanisms, Clin. Sci. 94 (6) (1998) 557–572.

[7] C.N. Ezekwesili, H.A. Ogbunugafor, J.O. Ezekwesili-Ofili, Anti-diabetic activity of aqueous extracts of *Vitex doniana* leaves and *Cinchona calisaya* bark in alloxan–induced diabetic rats, Int. J. Trop. Dis. 2 (4) (2012) 290–300.

[8] C.S. Nworu, P.A. Akah, Anti-inflammatory medicinal plants and the molecular mechanisms underlying their activities, Afr. J. Tradit., Complementary Altern. Med. 12 (suppl) (2015) 52–61.

[9] B.K. Harley, R.A. Dickson, I.K. Amponsah, I.O. Ben, D.W. Adongo, T.C. Fleischer, S. Habtemariam, Flavanols and triterpenoids from *Myrianthus arboreus* ameliorate hyperglycaemia in streptozotocin-induced diabetic rats possibly via glucose uptake enhancement and α-amylase inhibition, Biomed. Pharmacother. 132 (2020) 110847.

[10] OECD, Test No. 407: Repeated Dose 28-day Oral Toxicity Study in Rodents, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris, 2008.

[11] A. Gautam, D. Donohue, A. Hoke, S.A. Miller, S. Srinivasan, B. Sowe, et al., Investigating gene expression profiles of whole blood and peripheral blood mononuclear cells using multiple collection and processing methods, PloS One 14 (12) (2019), e022517.

[12] J.T. Roach, K.J. Sufka, Characterization of the chick carrageenan response, Brain Res. 994 (2) (2003) 216–225.

[13] E. Woode, R.A. Poku, G.K. Ainooson, E. Boakye-Gyasi, W.K.M. Abotsi, T.L. Mensah, et al., An evaluation of the anti-inflammatory, antipyretic and antinociceptive effects of *Ficus exasperata* (Vahl) leaf extract, J. Pharmacol. Toxicol. 4 (4) (2009) 138–157.

[14] R. Govindarajan, S. Rastogi, M. Vijayakumar, A. Shirwaikar, A.K. Rawat, S. Mehrotra, et al., Studies on the antioxidant activities of *Desmodium gangeticum*, Biol. Pharm. Bull. 26 (10) (2003) 1424–1427.

[15] Guideline, ICH Harmonised Tripartite, Q2 (R1) international conference on harmonisation of technical requirements for registration of pharmaceuticals for human use, validation of analytical procedures: text and methodology, International Conference on Harmonization, Switzerland, 2005.

[16] P. Arulselvan, M.T. Fard, W.S. Tan, S. Gothai, S. Fakurazi, M.E. Norhaizan, et al., Role of antioxidants and natural products in inflammation, Oxid. Med. Cell Longev. 2016 (2016) 1–15.

[17] G. Vlahov, G. Rinaldi, P. Del Re, A.A. Giuliani, ^{13}C NMR spectroscopy for determining different components of epicuticular wax of olive fruit (*Olea europaea*) Dritta cultivar, Anal. Chim. Acta 2 (7) (2008) 916.

[18] S.B. Mahato, A.P. Kundu, ^{13}C NMR spectroscopy of pentacyclic triterpenes - a compilation and some salient features, Phytochemistry 37 (6) (1994) 1517–1575.

[19] W.J. Van Rensburg, W. Van Averbeke, R. Slabbert, M. Faber, P. Van Jaarsveld, I. Van Heerden, et al., African leafy vegetables in South Africa, Water SA 33 (3) (2007) 317–326.

[20] C.O. Ochieng, I.O. Ishola, S.A. Opiyo, L.A.O. Manguro, P.O. Owuor, K.C. Wong, Phytoecdysteroids from the stem bark of Vitex doniana and their anti-inflammatory effects, Planta Med. 79 (1) (2013) 52–59.

[21] V. Suba, T. Murugesan, R. Kumaravelrajan, S.C. Mandal, B.P. Saha, Antiinflammatory, analgesic and antiperoxidative efficacy of Barleria lupulina Lindl. extract, Phytother Res. 19 (8) (2005) 695–699.

[22] S. Jäger, H. Trojan, T. Kopp, M.N. Laszczyk, A. Scheffler, Pentacyclic triterpene distribution in various plants–rich sources for a new group of multi-potent plant extracts, Molecules 14 (6) (2009) 2016–2031.

[23] M. Del Carmen Recio, R.M. Giner, S. Máñez, J.L. Ríos, Structural requirements for the anti-inflammatory activity of natural triterpenoids, Planta Med. 61 (2) (1995) 182–185.

[24] H. Assefa, A. Nimrod, L. Walker, R. Sindelar, Enantioselective synthesis and complement inhibitory assay of A/B-ring partial analogues of oleanolic acid, Bioorg. Med. Chem. Lett 11 (13) (2001) 1619–1623.

[25] L.C. Chiang, W. Chiang, M.Y. Chang, L.T. Ng, C.C. Lin, Antileukemic activity of selected natural products in Taiwan, Am. J. Chin. Med. 31 (1) (2003) 37–46.

[26] J. Liu, Y.P. Liu, Q. Mao, C.D. Klaassen, The effects of 10 triterpenoid compounds on experimental liver injury in mice, Fund. Appl. Toxicol. 22 (1) (1994) 34–40.

[27] M.S. Ali, M. Jahangir, S.S. Hussan, M.I. Choudhary, Inhibition of α-glucosidase by oleanolic acid and its synthetic derivatives, Phytochemistry 60 (3) (2002) 295–299.

[28] O.F. Kunle, H.O. Egharevba, P.O. Ahmadu, Standardization of herbal medicines-A review, Int. J. Biodivers. Conserv. 4 (3) (2012) 101–112.