The potential of *Clausena anisata* (Willd.) Hook.f. ex Benth against *Propionibacterium acnes*

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Highlights

- Antibacterial activity of southern African plants traditionally used for the treatment of acne and its associated symptoms.
- Anti-inflammatory activity of *C. anisata* by reduction in IL-8 levels in U937 cells.
- The first report of the compound trans-4-Hydroxy-1-methyl-L-proline from *C. anisata.*

Abstract

Acne vulgaris (AV) or acne is characterised as a chronic inflammatory disorder affecting the pilosebaceous unit (sebaceous gland associated with hair follicle) where the causative microorganism *Propionibacterium acnes* has been identified. The present study was conducted to investigate the potential of 21 southern African plants against *P. acnes* based on antimicrobial, antioxidant and anti-inflammatory activity. Two species, namely, *Clausena anisata* and *Helichryssum kraussii* were able to inhibit *P. acnes* growth with MICs at 31.25 and 62.5 µg/ml, respectively. The antioxidant activity for the 21 extracts ranged from 1.10–109.10 µg/ml. Active antibacterial species *C. anisata* and *H. kraussii* also showed good antioxidant activity with IC_{50} of 34.46 and 4.24 µg/ml, respectively. These two lead extracts were further investigated for their cytotoxicity on human leukaemic monocyte lymphoma

(U937) cells. *Clausena anisata* showed less toxicity with an IC_{50} of 74.46 μg/ml and a selectivity index of 2.38. *Clausena anisata* was hence further selected for investigation of anti-inflammatory activity and mechanism of action studies on the lipase and hyaluronidase enzyme. The concentration of Interleukin-8 (IL-8) in cells treated with heat-killed *P. acnes* alone was 840.52 pg/ml. Cells stimulated with *P. acnes* which were co-treated with 50, 25, 12.5 and 6.25 μg/ml of *C. anisata* extract, showed decreased IL-8 production with concentrations of 322.48 ± 0.07, 365.98 ± 0.24, 383.62 ± 0.08 and 409.52 ± 0.13 pg/ml, respectively. The statistical analysis was done using the Graph Pad Prism 4. These results indicated the potential of *C. anisata* for a treatment against AV. Lipase activity was inhibited by 21.93% at 500 μg/ml by the *C. anisata* extract which indicated the potential of this extract to reduce sebum production associated with AV progression. Hyaluronidase activity was inhibited by 49.02% at 500 μg/ml and could potentially deter the spread of *P. acnes* cells to neighbouring cells during pathogenesis. The compound trans-4-hydroxy-1-methyl-L-proline was isolated for the first time from *C. anisata.* Anti-acne treatments often require multiple activities to function efficiently. The antimicrobial activity, antioxidant activity and anti-inflammatory activity of *C. anisata* make it a great candidate for further investigation against AV..

Keywords: Clausena anisata, Propionibacterium acnes, Antibacterial, Anti-inflammatory, Acne vulgaris

1. Introduction

According to the World Health Organization (WHO), approximately 70-95% of the world's population rely on plants as a form of primary health care (Carmona and Pereira, 2013). Medicinal compounds isolated from plants and other natural resources have been used for thousands of years. They have an important role in medicine and human health. For our human predecessors, the use of medicinal plants was most likely the only form of treatment for disease and injury (Ji et al., 2009).

South Africa possesses a large floral diversity and is estimated to have 30,000 species of higher plants, many of which are endemic. This species richness can be attributed to diverse climatic zones and habitat types. The country has 3000 species of plants which are used medicinally, many of these are sold at informal medicinal plant markets (Light et al., 2005). The 21 plants used in this study were from 16 different plant families. The selected species have traditional uses for a number of ailments which were either directly related to acne and

its symptoms as well as their use in the treatment of other skin conditions. Some popular medicinal plant families included the Asteraceae family which has 500-600 species, many of which are used medicinally. Many of the plant families in the current study were also found in other investigations of traditional uses of plants for skin disorders (Mabona & Van Vuuren, 2013).

Clausena anisata (Willd.) Hook. f. ex Benth is commonly known as the Horsewood. It belongs to the Rutaceae family and is indigenous to Zimbabwe. It is found throughout South Africa in the Eastern Cape, Free State, KwaZulu-Natal Limpopo, Mpumalanga and Western Cape provinces. It is used traditionally by the Rasta community (Western Cape cultural group) for treating body pains and for strength. Other medicinal uses include the treatment of parasites, worms, rheumatic fever, arthritis, toothache, constipation and its ability to deter mosquito (Philander, 2011). Various parts of the plant are used to treat eye problems, respiratory problems, heart disorders, high blood pressure, abdominal cramps, gastroenteritis, boils, swollen gums, arthritis and other inflammatory conditions (Kenechukwu et al., 2012).

Propionibacterium acnes has been identified as a key factor in acne pathogenesis for over 100 years. Its traces go back as early as the year 1896. In a more recent study, it was found that keratinocyte cysts injected with *P. acnes* resulted in cystic rupture. This indicates a link between the bacterium and the inflammatory processes associated with the disorder (Dessinioti & Katsambas, 2010). Acne vulgaris (AV) or acne is characterised as a chronic inflammatory disorder affecting the pilosebaceous unit (sebaceous gland associated with hair follicle) where the causative agent *Propionibacterium acnes* resides. The disorder is commonly observed between the ages of 12-24 years (White, 1998). The pathogenesis of *P. acnes* is a culmination of four main events; an increase in sebum levels, hyperkeratinisation within the follicle, colonisation of the pilosebaceous unit with *P. acnes* and ultimately stimulation of the host immune response (Dessinioti & Katsambas, 2010; Williams et al., 2012).

There are various forms of conventional treatments available for AV. These include topical treatments, oral treatments, laser therapy and hormonal therapies (Layton, 2005; Williams et al., 2012). The treatment regimens for acne are dependent on the level of severity and are chosen according to the number and type of visible lesions (Shaw & Kennedy, 2007). The use of these treatments often comes with some side-effects, which include dryness, redness, and irritation of the skin. Antibiotic resistance by *P. acnes* is enhanced because acne treatments generally have minimal duration times which last several months. It is a common practice for

combinations of antibiotics to be used. The major problem with this is the emergence of multi-drug resistant strains of *Propionibacteria* (Oprica et al., 2004). Therefore, the present study was conducted to investigate the potential of 21 southern African plants for AV based on their antimicrobial, antioxidant and anti-inflammatory activity.

2. Materials and methods

2.1. Chemicals and reagents

2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT), *Candida rugosa* lipase and *Streptococcus pyogenes* hyaluronidase, 5,5'-dithiobis(2-nitro benzoic acid) (DTNB), 2,3-dimercapto-1-propanol tributyrate (DMPTB), cetyltrimethylammonium bromide (CTAB), Ethylenediaminetetraacetic acid (EDTA) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich (Johannesburg, SA). PrestoBlue (PB) was purchased from Life Technologies (Johannesburg, SA). The Cell Proliferation Kit II (XTT) was purchased from Roche Diagnostics (Pty) Ltd. (Randburg, Johannesburg, South Africa). Reagent set B, BD and Human IL-8 ELISA kit were purchased from BD Biosciences (San Diego, CA, USA). The microbial strain of *P. acnes* (ATCC 11287) was purchased from Anatech Analytical Technology (Johannesburg, SA). The U937 cell line (CRL1593.2) was supplied by Highveld Biological (Pty) Ltd. (Modderfontein, Johannesburg, South Africa). Both culture media Brain Heart Infusion (BHI) and nutrient broth were purchased from Merck (Pty) Ltd. Foetal Bovine Serum (FBS) was purchased from Separations (Pty) Ltd. (Randburg, Johannesburg, South Africa). Roswell Park Memorial Institute 1640 medium (RPMI 1640), potassium phosphate buffer saline (PBS) and gentamycin were supplied by Highveld Biological (Pty) Ltd. (Modderfontein, Johannesburg, South Africa).

2.2. Plant material

Plant material was collected from the University of Pretoria and Walter Sisulu Botanical Gardens, Ranchi University and some were purchased. These were identified at the H. G. W. J. Schweickerdt Herbarium at the University of Pretoria and at the Ranchi University Herbarium and voucher specimens were then deposited (Table 1.).

Table 1. Selected southern African species with traditional usage for symptoms associated with acne and its symptoms.

Identified species name	Plant part used	Voucher specimen number	Usage or activity related to acne or it's symptoms	References
1. *Acacia karroo* Hayne	Leaves	120017	Leaves and gum are used as wound dressings. Gum mixed with *Capsicum* sp. to treat pus-filled wounds caused by inflammation	Grace et al., 2003, Adedapo et al., 2008
2. *Buddleja saligna* (Willd.)	Leaves	120009	The root decoctions used to treat anasarca	Hutchings et al., 1996
3. *Buddleja salviifolia* (L.) Lam.	Leaves	120008	Flowers used as wash for wounds	Hutchings et al., 1996
4. *Cheilanthes viridis* (Forssk.) Swartz	Leaves and stems	120011	Used to treat sores and other skin conditions. Dried leaf paste used to treat skin burns.	Hutchings et al., 1996
5. *Clausena anisata* (Willd.) Hook.f. ex Benth	Leaves and stems	118954	Various parts of the plant are said to treat boils and inflammatory conditions	Kenechukwu et al., 2012
6. *Clematis branchiata* Thunb.	Roots, leaves and stems	120016	Crushed leaves are mixed with red sand and applied to the skin to treat rashes in children	Hutchings et al., 1996
7. *Daucus carota* L.	Seeds	EBLRU/5/31/15	Infusions of the seeds have been used to treat oedema	Plants for a Future, 2012
8. *Euclea undulata* Thunb. Var. myrtina	Root bark	95254		
9. *Faurea saligna* Harv.	Leaves	118700	An decoction of the bark has been used to treat sores	Mthethwa, 2009
10. *Gunnera perpensa* L.	Leaves and stems	120010	Root infusions are used to treat swelling, cancerous sores and as wound dressings by some farmers in the Cape	Hutchings et al., 1996
11. *Gymnosporia buxifolia* (L.) Szyszyl	Leaves and aerial parts	119357	The leaves and roots are used as an anti-inflammatory agent in West Africa	Hutchings et al., 1996
12. *Helichrysum aureonitens* Sch. Bip.	Leaves	NEPM	Extracts of this shrub are used against *Herpes zoster* and *Herpes simplex* skin infections	Lourens et al., 2008

Identified species name	Plant part used	Voucher specimen number	Usage or activity related to acne or it's symptoms	References
13. *Helichrysum kraussii* Sch. Bip.	Flowers, stems and leaves	96694	The leaves are used to treat keloid scarring of the skin	Lourens et al., 2008
14. *Helichrysum splendidum* (Thunb.) Less	Leaves and stems	120012	A mixture of this plant with some *Senecio* sp. are applied to pimples	Lourens et al., 2008
15. *Heteromorpha arborescens* (Spreng.) Cham. & Schltdl.	Leaves	120022	Activity against *S. epidermidis* also associated with acne lesions	Nkomo & Kambizi, 2009
16. *Leucas martinicensis* (Jacq.) R. Br.	Seeds and leaves	96690	Used as an anti-inflammatory agent for ear inflammation	Santos et al., 2012
17. *Nigella sativa* L.	Seeds	CNH/78/2013/Tech II/42	Seed tinctures are used for skin eruptions and seed oil is used as a topical antiseptic	Ahmad et al., 2013
18. *Parthenium hysterphorus* L.	Leaves	EBLRU 3/27/13	Use as a traditional medicine for ulcers and wounds	Kumar et al., 2014
19. *Scadoxus puniceus* (L.) Friis & Nordal	Leaves	120023	Skin poultices are prepared from this species in southern Africa	Hutchings et al., 1996
20. *Tabarnaemontana elegans* Stapf.	Leaves and stems	96692	Latex used as an astringent and the extracts used against Gram-positive microorganisms	Hutchings et al., 1996
21. *Urtica dioica* (L.)	Leaves	NEPM	Topical application of the roots and leaves used for skin and joint inflammation but also for wounds	Globinmed, 2010

NEPM – Not Enough Plant Material.

2.3. Preparation of plant extracts

Plant parts were collected and shade dried at room temperature, out of direct sunlight. Dried plant material was ground into a fine powder using an IKA MF 10 Basic grinder (4 mm sieve). Powdered material was extracted at room temperature (25 °C) with 99% ethanol for 48 h, with constant agitation. The extracted material was then filtered using vacuum filtration and concentrated to complete dryness using a rotary evaporator. Prepared extracts were stored at 4 °C until for use in different bioassays.

2.4. Biological activity

2.4.1. Antimicrobial activity

Pure cultures of *Propionibacterium acnes* (ATCC 11827) were maintained on sterile Brain Heart Infusion agar plates. The antimicrobial activity of the extracts was performed according to the methods described by Lall et al. (2013). *Propionibacterium acnes* subcultures were inoculated in sterile nutrient broth and prepared to a density of 1.5×10^8 colony forming units per ml (CFU/ml) corresponding with the 0.5 McFarland Standard. Tetracycline was used as the positive control. Extracts were tested at concentrations from 3.91 to 500 µg/ml and tetracycline from 0.39 to 50 µg/ml, in triplicate. INT and PrestoBlue® were added as indicators of bacterial growth. The MIC was determined after the addition of the growth indicators and interpreted as the lowest concentration that inhibited bacterial growth. The recorded MICs were compared using PrestoBlue® and INT as growth indicators.

2.4.2. DPPH radical scavenging activity

The DPPH antioxidant assay was performed using the methods of Du Toit et al (2001) with minor modifications. For each extract, a dilution series was prepared in a 96-well ELISA plate. The plant extracts were tested from 3.91 to 500 µg/ml. Each concentration was tested in triplicate. Vitamin C was used as the positive control and ethanol was used as the solvent control. Colour controls for each extract were also used. The DPPH (0.04 M) was added to each test plate. After incubation for 30 min at room temperature, the radical scavenging capacity of the extracts was determined using a BIO-TEK Power-Wave XS multi-well reader (A.D.P., Weltevreden Park, South Africa) at 515 nm and KC Junior software (Highland Park, Winooski, Vermont, USA). The radical scavenging activity was measured as the amount of antioxidant necessary to decrease the initial absorbance of DPPH by 50%. This is known as the 50% inhibitory concentration (IC_{50}). The IC_{50} for each extract was determined using GraphPad Prism Version 4.0 (San Diego, California, USA). The results were then expressed as Vitamin C equivalents.

2.4.3. Cytotoxicity assay

The extracts which showed antibacterial activity were tested for their cytotoxicity. The cytotoxicity assay was performed according to the methods of Zheng et al (2001). Sterile 96-well micro-titre plates were used to seed the U937 cells at the required concentration 1×10^5 cells/ml with an added final concentration of 0.1 µg/ml, phorbol-12-myristate-13-

acetate (PMA). PMA was used to differentiate the monocyte cells to macrophages. The plates were then incubated for 24 h to allow the cells to attach. Plant extracts were diluted in sterile medium then added to the test plate from 3.13 to 400.00 µg/ml. Pentoxyfilline and PMA were also tested. Medium and solvent controls (2% (v/v) DMSO) were also included in the assay. Actinomycin D was used as the positive cytotoxic inducer and its concentration ranged from 0.002 to 0.5 µg/ml. Plates were then incubated for a further 72 h at 37 °C in 5% CO_2. After 72 h, 50 µl of XTT ((2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide)) reagent was added to each test plate. The plates were then read using a BIO-TEK Power-Wave XS multi-well reader (A.D.P., Weltevreden Park, South Africa) at 490 nm and a reference wavelength of 690 nm. Statistical analysis was done using GraphPad Prism Version 4.0 (San Diego, California, USA) in order to obtain the IC_{50} for each extract and all the controls.

2.4.4. IL-8 inhibition assay

The potential anti-inflammatory effect of *C. anisata* against IL-8 production by U937 cells was performed using an enzyme-linked immunosorbent assay (ELISA) technique (Tsai et al., 2010). This extract was selected as it exhibited the highest selectivity index based on cytotoxicity and antibacterial activity. The protocol for the assay was included in the reagent set B and human IL-8 antibody kits. Log phase cultures of *P. acnes* were harvested and washed three times with PBS. Cultures were then incubated at 80 °C for 30 min. The heat-killed bacteria were then stored at 4 °C until required. U937 cells were seeded at a cell density of 10^5 cells/well. Cells were stimulated with 100 µg/ml of heat-killed *P. acnes*. The effects of the addition of the *C. anisata* extract, at non-lethal concentrations (6.25-50 µg/ml) in stimulated U937 cells, was then investigated. Pentoxyfilline was used as the positive control. One percent DMSO was used as a solvent control. After 24 h the plates were centrifuged and cell-free supernatants were collected. Supernatants were stored at -72 °C until required. IL-8 was quantified for each of the tested concentrations using a BIO-TEK Power-Wave XS multi-well reader (A.D.P., Weltevreden Park, South Africa) at 450 nm with a reference wavelength of 570 nm. Calibration curves were constructed from a serial dilution series of IL-8 standards for calculation of IL-8 production. Results were then analysed using an online program ReaderFit.

2.5. Identification of constituents from *Clausena anisata* using step gradient isolation

2.5.1. Identification of trans-4-Hydroxy-1-methyl-L-proline

The ethanol extract of *C. anisata* (200 g) was subjected to column chromatography using silica gel 60 (70-230 mesh). The solvent gradient consisted of hexane, ethyl acetate, methanol and water. A total of 300 fractions were collected. Similar fractions were combined according to their TLC profiles. The active fraction (210-213) which eluted with 100% methanol (60 mg) exhibited an MIC of 31.25 µg/ml, similar to that of the ethanol extract. The active fraction was then re-columned on silica gel G and eluted in aqueous methanol (10% (v/v)). The compound was then recrystallised as an amorphous to light brown powder (15 mg). The ^{1}H-NMR and ^{13}C-NMR data corresponded with known compound trans-4-hydroxy-1-methyl-L-proline, with similar chemical shifts as those reported by Winkler (2006). (Fig 1.)

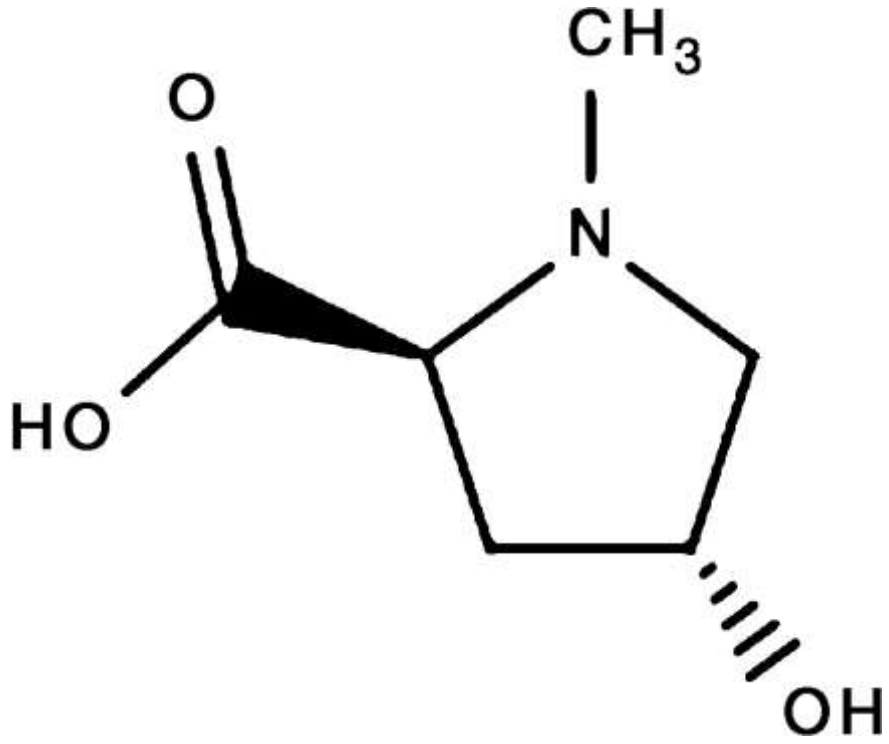

Fig. 1. Structure of isolated compound trans-4-hydroxy-1-methyl-L-proline.

2.5.2. GC-MS of oil fraction of *Clausena anisata*

The oil fraction (5-7) was selected for GC-MS analysis as it contained a number of volatile constituents that were not able to be separated using thin layer chromatography. GC-MS analysis of this eluted fraction from step gradient column chromatography was performed using an Agilent 6890 N GC (gas chromatograph) equipped with a ZB-5MS GUARDIAN (30 m, 0.25 mm ID, 0.25 µm film thickness) column, CTC CombiPAL Autosampler, and Agilent 5975B MS (mass spectrometer). The temperature of the injector and the interface were kept at

280 °C. The following temperature program was used for analysis: temperature was kept at 70 °C for 0.5 min, then increased to 100 °C at a rate of 5 °C min^{-1}, kept at 100 °C for 0.5 min and finally ramped at a rate of 5 °C min^{-1} to 240 °C and kept at that final temperature for 1 min. Helium was used as a carrier gas at a constant flow rate of 1 $ml.min^{-1}$. An injection volume of 1 µl was injected with a split ratio of 20:1. The first 5 min of the analysis was considered as solvent delay and omitted from the final chromatograms. The MS transfer line temperature was set at 280 ºC. The mass spectrometer was operated in electron impact ionisation mode (EI +) at 70 eV. The scan range was set at 25-650 m/z. Peaks were identified by mass matching to the NIST05 database.

2.6. Enzyme inhibition studies

2.6.1. Molecular docking of trans-4-hydroxy-1-methyl-L-proline

Molecular docking was performed using the docking program GOLD to indicate the potential activity of the isolated compound (trans-4-Hydroxy-1-methyl-L-proline) on enzyme activity against two pathogenic enzymes involved in sebum degradation and spread of *P. acnes*, namely lipase and hyaluronidase (Verdonk et al., 2003). The program optimises protein flexibility, as loop and active site residues play an important role in the ligand binding site of enzymes (Arvind., 2012; Kumar & Sobhia 2013, 2014, 2015 and 2016). It uses a genetic algorithm which considers ligand conformational flexibility along with partial protein flexibility i.e. side chain residues. The default docking parameters were employed for this docking study. It includes 100,000 genetic operations on a population size of 100 individuals and mutation rate of 95 as used by various other studies (Gautam et al., 2011; Grover et al., 2014a, 2014b, 2015, Lall et al., 2016a, 2016b). The crystal structures of *Candida rugosa* lipase (PDB ID: 3RAR) and *Streptococcus pyogenes* hyaluronidase (PDB ID: 3EKA) were obtained from the Protein Data Bank (Bernstein et al., 1978). The lipase enzyme had a crystal structure resolution of 2.19 Å and complexed with compound methyl-hydrogen-(R)-[(R)-methoxy-(phenyl)-methyl]-phosphonate while the hyaluronidase enzyme has a resolution of 3.1 Å and was complexed with ascorbic acid. The structure of the identified compound, trans-4-hydroxy-N-methylproline was sketched using Chemdraw3D and minimised considering an RMSD cut-off of 0.1 Å. The docking protocol was set by extracting and re-docking co-crystal ligands in lipase and hyaluronidase crystal structure with RMSD ≤ 1 Å. This was followed by docking of the compound trans-4-hydroxy-N-methylproline into the active site defined as 6 Å regions around the co-crystal ligand in the enzymes. Furthermore, the docked compound was evaluated for

possible molecular interactions within active site residues using PyMol Molecular Graphics System (Delano, 2002).

2.6.2. Lipase inhibitory activity of Clausena anisata extract and trans-4-hydroxy-N-methylproline compound

The lipase inhibition of the *C. anisata* extract and trans-4-hydroxy-N-methylproline were tested using the colorimetric assay described by Choi et al (2003). Ellman's reagent (DTNB) was dissolved in isobutanol to prepare a stock concentration of 40 mM. A stock concentration 10 mM of DMPTB was then prepared. These were then stored at -20 °C until the assay was performed. The lipase buffer was then prepared with 10 mM KCl and 10 mM Tris-HCl to pH 7.5 and was used to dissolve the lipase enzyme from *Candida rugosa* (Sigma Aldrich, Johannesburg, South Africa). A stock solution of 0.5 M EDTA, 10% (v/v) Triton X-100 and 1 M Tris-HCl were also prepared and formed part of the reaction mixture. The reaction mixture consisted of the following volumes 20 µl of 10 mM DMPTB, 20 µl of 40 mM DTNB, 2 µl of 0.5 M EDTA, 5 µl of 10% (v/v) Triton X-100 and 50 µl of 1M Tris-HCl, pH 7.5 were added in a 15 ml centrifuge tube. The volume was then adjusted to 900 µl. For the enzyme inhibition assay 180 µl of the reaction mixture was added to a 96 well plate and 10 µl of inhibitor (test sample) with 10 µl of enzyme (1 U/ml). For tests with no inhibitor 20 µl of enzyme was added. The plates were then incubated for 30 min at 37 °C, after which lipase inhibitory detection was quantified by reading the optical density at 405 nm.

2.6.3. Hyaluronidase inhibitory activity of Clausena anisata extract and trans-4-hydroxy-N-methylproline compound

The *C. anisata* extract and trans-4-hydroxy-N-methylproline were tested for their inhibitory activity against bacterial hyaluronidase activity, as this enzyme is involved in the spread of *P. acnes*. The turbidometric assay described by Hofinger et al. (2007) was used to determine the hyaluronidase inhibitory activity of the lead extract, with slight modifications. A citrate-phosphate buffer was prepared by mixing a solution of 0.1 M Na_2HPO_4 with 0.1 M NaCl and 0.1 M citric acid with 0.1 M NaCl to pH 5.0. The hyaluronic acid substrate stock solution was prepared to 2 mg/ml (in distilled water). The hyaluronidase from *Streptococcus pyogenes* was optimised to 1.0 U/ml. The enzyme was diluted in a 0.2 mg/ml stock solution of Bovine Serum Albumin (BSA) in distilled water. In a 96-well plate 66 µl of citrate-phosphate buffer, 10 µl BSA, 10 µl hyaluronidase enzyme and 4 µl of inhibitor in DMSO. The negative control was

DMSO without sample. This was incubated for 15 min at 37 °C. The reaction was then initiated by the addition of 10 µl of hyaluronic acid and further incubation for 30 min at 37 °C. The reaction was then stopped with the addition of 200 µl of 2.5% (w/v) CTAB in 0.5 M NaOH. The plates were then incubated at room temperature for 20 min. The turbidity was then measured by obtaining the optical density at 580 nm. Inhibition was compared to that of enzyme and substrate without any inhibitor.

3. Results and discussion

3.1. Antimicrobial activity

INT is a tetrazolium salt, which is reduced in the presence of viable bacterial cells to a red formazan dye. The reduction is an indication of functional mitochondrial dehydrogenase enzymes and bacterial cellular respiration (Hatzinger et al., 2003). PrestoBlue® is a cell viability reagent that is reduced from a blue to a pink colour in the presence of viable bacterial cells. This is due to the reduction of resazurin to resorufin by functional cellular oxidoreductase enzyme (Lall et al., 2013). The MICs for all the extracts using both PrestoBlue® and INT as bacterial growth indicators have been listed in Table 2. Most of the extracts tested for their activity against *P. acnes* showed moderate antibacterial activity against this microorganism. The characterisation of the active and non-active extracts was performed using the concentration ranges described by Holetz et al. (2002). The extract showing significant antibacterial activity using INT (MIC < 100µg/ml) was that of *Clausena anisata* with an MIC of 31.25 µg/ml. Most of the extracts showed moderate antibacterial activity with MICs between 100 to 500 µg/ml. In a study conducted by Chakravarthi et al. (2017), the ethanol extract of *C. excavata* had an MIC of 1000 µg/ml against *P. acnes*. It is interesting to note that the hexane extract of *C. excavata* had an MIC of 500 µg/ml, which was the same as that of the solvent gradient hexane essential oil obtained during isolation. The antibacterial activity of the oil can therefore be explained by the compounds isolated with hexane. The extracts of *D. carota* and the *N. sativa* extracts showed no activity (MIC > 500 µg/ml) against *P. acnes* as there was no inhibition observed at the highest tested concentration of 500 µg/ml. When using PrestoBlue®, both *H. kraussii* and *C. anisata* showed noteworthy antibacterial activity with MICs of 62.5 µg/ml and 31.25 µg/ml, respectively. The results showed a similar trend to that of INT except when determining the susceptibility of *P. acnes* to tetracycline. The bacterial cells tend to adhere at the bottom of the 96-well plate during incubation and the INT crystals were reduced predominantly at the bottom of the plate where aggregated bacterial cells were observed.

PrestoBlue® provides an advantage as all the media in the test wells is coloured, making it easier to determine the MIC visually. The extracts tested in this study compared well with some plants that are used as traditional remedies for acne. A study by Vora et al. (2017) showed that Rosemary (*Rosmarinus officinalis*) and German chamomile (*Matricaria chamomilla*) both inhibited *P. acnes* growth with an MIC of 156 µg/ml. Bacterial inoculums treated with tetracycline in the presence of INT showed almost no colour change, indicating an MIC < 0.39 µg/ml. However, with the use of PrestoBlue®, there was a clear distinction between metabolically active bacterial cells and those which showed inhibition of growth. Tetracycline exhibited an MIC at 3.13 µg/ml when using PrestoBlue®. Although the colorimetric reaction takes longer with PrestoBlue® there is a clear indication of the concentrations which were lethal to bacterial proliferation (Lall et al., 2013).

Table 2. Antibacterial and anti-oxidant activity of selected southern African plant species.

Extract	Minimum inhibitory concentration against *P. acnes* (µg/ml) using PrestoBlue and INT		Anti-oxidant activity against DPPH (µg/ml)
***1. Acacia karroo* Hayne**	500	500	2.32 ± 0.01
2. *Buddleja saligna* (Willd.)	500	500	19.30 ± 0.15
3. *Buddleja salviifolia* (L.) Lam.	500	500	32.29 ± 0.65
4. *Cheilanthes viridis* (Forssk.) Swartz	500	500	7.83 ± 0.04
5. *Clausena anisata* (Willd.) Hook.f. ex Benth	31.25	31.25	34.46 ± 0.12
6. *Clematis branchiata* Thunb.	500	500	44.18 ± 1.395
7. *Daucus carota* L.	> 500	> 500	b
8. *Euclea undulata* Thunb. Var. myrtina	500	500	11.82 ± 0.10
9. *Faurea saligna* Harv.	500	500	1.17 ± 0.04
10. *Gunnera perpensa* L.	500	500	1.10 ± 0.01
11. *Gymnosporia buxifolia* (L.) Szyszyl	500	500	5.22 ± 0.09
12. *Helichrysum aureonitens* Sch. Bip.	500	500	6.71 ± 0.18

Extract	Minimum inhibitory concentration against *P. acnes* (µg/ml) using PrestoBlue and INT		Anti-oxidant activity against DPPH (µg/ml)
13. *Helichrysum kraussii* Sch. Bip.	62.50	125	4.24 ± 0.01
14. *Helichrysum splendidum* (Thunb.) Less	500	500	8.99 ± 0.11
15. *Heteromorpha arborescens* (Spreng.) Cham. & Schltdl.	500	500	69.24 ± 1.27
16. *Leucas martinicensis* (Jacq.) R. Br.	500	500	43.90 ± 1.60
17. *Nigella sativa L.*	> 500	> 500	b
18. *Parthenium hysterphorus* L.	500	500	b
19. *Scadoxus puniceus* (L.) Friis & Nordal	500	500	37.65 ± 0.67
20. *Tabarnaemontana elegans* Stapf.	500	500	102.5 ± 4.51
21. *Urtica dioica* (L.)	500	500	109.10 ± 2.26
Fraction 5–7 from *C. anisata*	500	500	b
Fractions 210–213 from *C. anisata*	31.25	31.25	b
trans-4-hydroxy-N-methylproline isolated from *C. anisata*	> 500	> 500	b
Tetracycline[c]	a	3.13	b
Vitamin C[d]	b	b	1.98 ± 0.01

[a] Not clear.

[b] Not tested.

[c] Positive control for antibacterial assay.

[d] Positive control for anti-oxidant assay.

3.2. Antioxidant activity

The hydrolytic activity of *P. acnes* on increased sebum levels is known to cause the release of free fatty acids (FFA) within the pilosebaceous unit. These FFA act as triggers for cells of the immune response causing disruption of the follicular walls of the pilosebaceous unit,

culminating in oxidative damage through the release of free radicals (Portugal et al., 2007; Sharma et al., 2013). In the presence of antioxidants, which can successfully donate an electron, the DPPH radical is neutralised to a stable compound and characterised by a colour change from purple to yellow (Du Toit et al., 2001; Mishra et al., 2012). The DPPH free radical scavenging activity was determined for the selected plant extracts (Table 2). *Gunnera perpensa* exhibited the highest radical scavenging activity with an IC_{50} of 1.10 ± 0.01µg/ml. Similar results were observed in a study by Muleya et al (2014) who determined the IC_{50} of the acetone extract against DPPH to be 1.07 µg/ml. Of the species which showed good antibacterial activity *H. kraussii* showed better scavenging activity with an IC_{50} of 4.24 ± 0.01µg/ml when compared to that of *C. anisata* which exhibited an IC_{50} of 34.46 ± 0.12µg/ml. The flowers of this species have been identified to contain antioxidant flavonoid compounds with high specificity for DPPH radical scavenging and could explain the difference in activity (Legoalea et al., 2013). *Clausena anisata* DPPH scavenging activity was performed on the 50% methanol extract of both stored and fresh leaves and twigs in a study by Amoo et al (2012). The extracts made from stored and fresh plant material exhibited an IC_{50} of 33.20 ± 3.89 and 26.80 ± 2.06 µg/ml, respectively. The DPPH scavenging activity of *C. anisata* is similar to that of the extract tested in the present study. The extract of *R. officinalis* had an IC_{50} of 109.30 µg/ml indicating that the extract of *C. anisata* compared well with popular herbs that are used as alternative acne treatments (Vora et al., 2017). The positive control used was ascorbic acid which exhibited an IC_{50} of 1.98 ± 0.01µg/ml. The IC_{50} value obtained for ascorbic acid compares well with other published data which reported an IC_{50} of 2.1 ± 0.05 µg/ml (Amoo et al., 2012).

3.3. Cytotoxicity

Based on the significant antibacterial activity and anti-oxidant activities of *H. kraussii* and *C. anisata* these two extracts were tested for their potential cytotoxic effect on human leukemic monocyte lymphoma (U937) cells to determine the non-lethal concentrations which could be used to treat cells for collection of supernatant and analysis of anti-inflammatory potential. *C. anisata* was found to be moderately toxic to U937 cells with IC_{50} at 74.46 ± 0.42 µg/ml (Fig. 2A). Moderate toxicity is described for extracts showing an IC_{50} between 50 and 100 µg/ml. Two compounds that have been isolated from *Clausena anisata* have shown similar cytotoxicity results on human leukaemia cells (HL-60) with Clausamine B and C exhibiting IC_{50} at 80.3 ± 1.5 and 79.7 ± 0.9 µM, respectively (Ito et al., 2009). Pentoxyfilline (PF) and phorbol-12-myristate-13-acetate (PMA) were found to be non-toxic to U937 cells with IC_{50} at

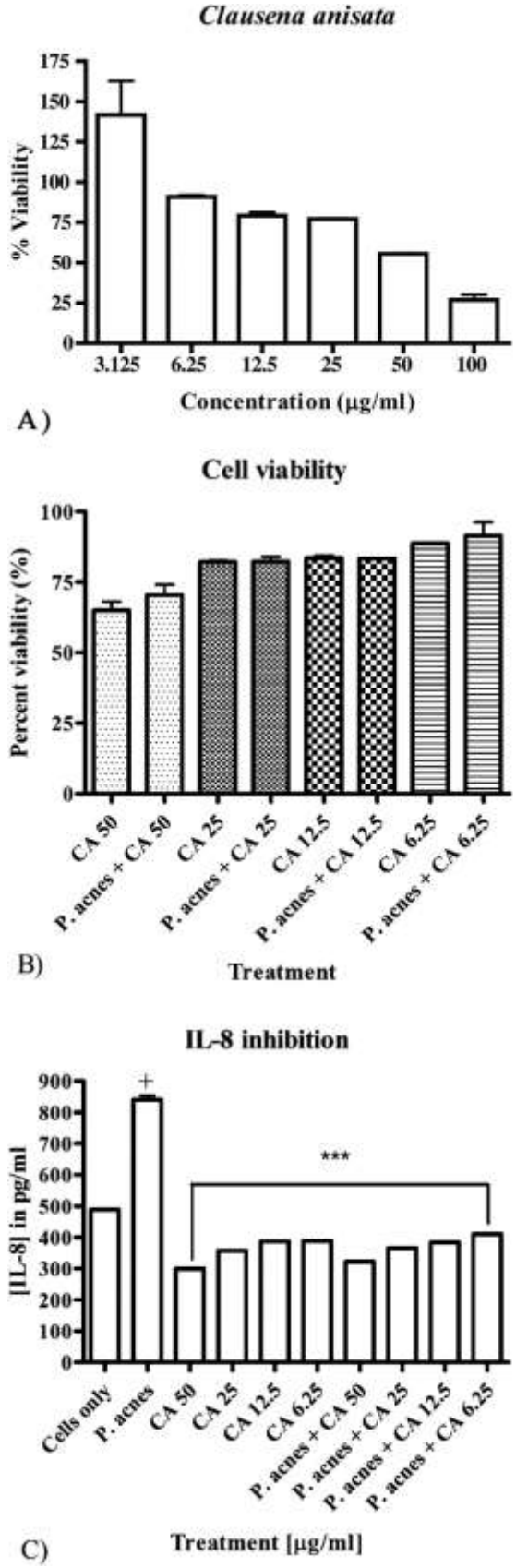

Fig. 2. (A) Percentage viability for cells treated with *C. anisata*, (B) the cell viability after 24 h of treatment with non-lethal concentrations of *C. anisata* extract and heat-killed *P. acnes* and (C) the inhibition of IL-8 in cells treated with extract only and also in cells treated with both *P. acnes* and extract (***$p < .001$).

169.50 ± 7.80 and 317.60 ± 6.40 µg/ml, respectively. These were used as cell stimulants for the production of IL-8 and used as controls. PF and PMA were non-toxic which was described for test samples showing IC_{50} values > 100 µg/ml. The selectivity index (SI) was also determined for *H. kraussi* and *C. anisata* comparing the antibacterial activity in relation to the cytotoxicity (IC_{50}/MIC). The SI for *H. kraussii* was 0.41 and for *C. anisata* this was much higher at 2.38 which indicates that this species was more antibacterial than it is toxic towards the U937 cells.

After having obtained significant antibacterial and antioxidant activity as well as determining the effects of the extracts on U937 cells, the extract of *C. anisata* was then selected for further investigation as a potential anti-inflammatory activity through the effects of pro-inflammatory cytokine IL-8 and lipase inhibition. It was also investigated for its inhibitory effects against hyaluronidase activity. These are some of the major targets associated with the progression of acne.

3.4. IL-8 inhibition

The cell viability after 24 h of treatment exhibited a dose-response (Fig. 2B). Cell viability at the lowest concentration (6.25 µg/ml) was above 80% for both the extract treatment alone and the bacteria-extract combination treatment. The lowest viability observed was for the bacteria-extract treatment at 50 µg/ml which was 65%. The viability levels therefore suggest that any IL-8 inhibition was due to treatment effects and not due to decreased cell viability. The results obtained in this study have shown that U937 cells treated with 100 µg/ml heat-killed *P. acnes* produced increased amounts of IL-8 when compared to that of the untreated cell control.

The IL-8 concentration for the *P. acnes* treatment was quantified as 840.52 pg/ml (Fig. 2C). It is important, however, to note that the IL-8 protein recorded for the untreated cell control was relatively high at 488.76 pg/ml (Fig. 2C) when compared to that of a similar study by Sharma et al. (2013). This phenomenon was previously observed in peripheral blood monocyte cells where they spontaneously produced IL-8 making it difficult to differentiate between the effects of various cell stimuli (Kasahara et al., 1991; Vowels et al., 1995). IL-8 is known for its ability to attract neutrophil cells to the pilosebaceous unit (Kim et al., 2002). *Propionibacterium acnes* ingestion by activated neutrophil cells results in the release of hydrolase enzymes. These are known to disrupt the follicular wall of the pilosebaceous unit inducing inflammation. This explains the involvement of IL-8 in the pathogenic process of *P.*

acnes and also the increased amounts of IL-8 protein secreted in U937 cells treated with the bacterium in this study.

Protein levels of IL-8 detected in U937 cells treated with various (non-lethal) concentrations of *C. anisata* extract without *P. acnes* stimulation were compared with the untreated cell control. Low levels of IL-8 inhibition were observed. Inhibition was dose-dependent with 50, 25, 12.5 and 6.25 µg/ml showing IL-8 levels at 299.24 ± 0.13, 357.82 ± 0.07, 387.14 ± 0.12 and 388.74 ± 0.19 pg/ml, respectively. This means that *C. anisata* was able to reduce the levels of IL-8 from 488.76 ± 0.06 pg/ml (Fig. 2C).

Protein levels of IL-8 detected in U937 cells treated with various (non-lethal) concentrations of *C. anisata* extract with *P. acnes* stimulation were compared with U937 cells stimulated with *P. acnes* only. The inhibition levels were lower than that of cells treated without *P. acnes* stimulation. Treatment of cells with 50, 25, 12.5 and 6.25 µg/ml of *C. anisata* extract in the presence of heat-killed *P. acnes* showed IL-8 levels at 322.48 ± 0.07, 365.98 ± 0.24, 383.62 ± 0.08 and 409.52 ± 0.13 pg/ml respectively (Fig. 2C). This means that the *C. anisata* extract was able to decrease the secretion of IL-8 from 840.52 ± 0.06 pg/ml. After showing significant biological activity, step gradient isolation was performed in order to isolate potentially active compounds.

3.5. GC-MS of oil fraction from *C. anisata*

The essential oil constituents from the hexane fraction of *C. anisata* were analysed using GC-MS. The compounds reported in Table 3 were obtained from comparisons with the NIST05 GC-MS database. Only compounds with a percentage similarity above 90% were reported. The major constituent in the oil fraction was methyl eugenol (69.97%). The major constituents reported for *C. anisata* in other studies are largely monoterpenes such as α-pinene, β-pinene and Sabinene (Govindarajan, 2010; Senthilkumar & Venkatesalu, 2009) and could be due to the extraction method used. Hydrodistillation will most likely be the reason for the difference in compound composition when compared with fraction collection with hexane from a crude extract. *Clausena pentaphylla*, a species from the same genus and family, however, also contained methyl eugenol as a major constituent making up 38.10% of the composition (Pandey et al., 2012). The presence of fatty acids and their esters were identified as methyl palmitate, ethyl palmitate, methyl 14-methylhexadecanoate and phytol, these have been previously reported in other studies (Ngassoum, 1999; Yakoob et al., 2016). The compounds α-selinene and β-selinene have also been reported in *Clausena dentata*. Spathulenol was

observed in *C. excavata* making up 11.9 and 1.9% in *C. engleri*. This confirms that the ethanolic extract of *C. anisata* contains some volatile components within the hexane fractions that could contribute to the activity. The components have also been identified in other *Clausena* species (Trung et al, 2014).

Table 3. Major constituents found in the oil fraction (5–7) of the chromatographed extract of *Clausena anisata.*

Retention time	Library/ID	Formula	Percentage of composition	Quality compared to NIST05 database
14.9083	Methyleugenol	$C_{11}H_{14}O_2$	69.97	96
15.0494	Caryophyllene	$C_{15}H_{24}$	0.20	94
15.9595	1,4,7,-Cycloundecatriene, 1,5,9,9-tetramethyl-, Z,Z,Z-	$C_{15}H_{24}$	0.68	99
16.665	Acenaphthene	$C_{12}H_{10}$	0.10	91
16.7709	β-Selinene	$C_{15}H_{24}$	0.14	99
16.9402	α-Selinene	$C_{15}H_{24}$	0.34	97
18.8804	Spatulenol	$C_{15}H_{24}O$	0.82	94
19.0003	Caryophyllene oxide	$C_{15}H_{24}O$	0.44	91
19.085	Fluorene	$C_{13}H_{10}$	0.34	90
20.6795	α-Cadinol	$C_{15}H_{26}O$	0.21	98
23.7062	Anthracene	$C_{14}H_{10}$	0.91	93
24.6587	Hexahydrofarnesyl acetone	$C_{18}H_{36}O$	0.38	96
25.7946	1-Methylphenanthrene	$C_{15}H_{12}$	0.20	93
25.9145	1-Methylphenanthrene	$C_{15}H_{12}$	0.23	96
26.0556	Farnesyl acetone	$C_{18}H_{30}O$	0.12	98
26.1756	4H-Cyclopenta[def]phenanthrene	$C_{15}H_{10}$	0.30	93
26.3661	Methyl palmitate	$C_{17}H_{34}O_2$	1.12	99
27.3327	2-Phenylnaphthalene	$C_{16}H_{12}$	0.26	93
27.6925	Ethyl palmitate	$C_{18}H_{36}O_2$	0.10	97

Retention time	Library/ID	Formula	Percentage of composition	Quality compared to NIST05 database
27.756	Methyl 14-methylhexadecanoate	$C_{18}H_{36}O_2$	0.09	94
28.4333	3,6-Dimethylphenanthrene	$C_{16}H_{14}$	0.14	94
28.9906	Fluoranthene	$C_{16}H_{10}$	2.30	96
29.541	Linoleic acid methyl ester	$C_{19}H_{34}O_2$	0.50	99
29.6538	Linolenic alcohol	$C_{18}H_{32}O$	1.31	94
29.9078	Phytol	$C_{20}H_{40}O$	7.62	90
29.9643	Pyrene	$C_{16}H_{10}$	2.55	93
30.7756	Benzonaphthofuran	$C_{16}H_{10}O$	0.16	93
31.6082	1-Methylpyrene	$C_{17}H_{12}$	0.16	91
31.714	2,3-Benzofluorene	$C_{17}H_{12}$	0.53	94
32.0456	2,3-Benzofluorene	$C_{17}H_{12}$	0.22	93
32.1302	4-Methylpyrene	$C_{17}H_{12}$	0.20	90
33.9717	4,8,12,16-Tetramethylheptadecan-4-olide	$C_{21}H_{40}O_2$	0.22	97

3.6. Molecular docking of (4R)-4-hydroxy-1-methyl-L-proline

The molecular docking study was performed to determine the binding mode of the compound trans-4-hydroxy-N-methylproline in the active sites of both lipase and hyaluronidase enzymes. For both enzymes, residues of approximately 6 Å of co-crystal ligand were selected as active site residues. For the lipase enzyme, Gly122, Gly123, Gly124, Val127, Gly208, Ser209, Ala210, Gly211, Phe296, Leu297, Phe415, His449, and Ser450 were considered as active site residues. The analysis of active site revealed it is rich with hydrophobic residues. The compound trans-4-hydroxy-N-methylproline was docked and exhibited a GOLD fitness score of 31.26. A single H-bond was observed with residue Glu208 (Fig. 3). Similarly, the active site of the hyaluronidase enzyme was analysed. Binding residues consisted of Ala221, Leu222, Gly223, Thr224, Leu225, Lys226, Ile227, Thr271, Gly273, Lys274 and Leu275. The compound trans-4-hydroxy-N-methylproline was docked within the active site of hyaluronidase and exhibited GOLD fitness score of 17.20 (Fig. 3). When comparing the two

enzymes, the docking fitness score was better for the lipase enzyme as compared to hyaluronidase. This may be due to the large difference in size of the active site and compound. The compound showed a better fit into the lipase active site with more proximity to the active site residues, therefore allowing for more interactions.

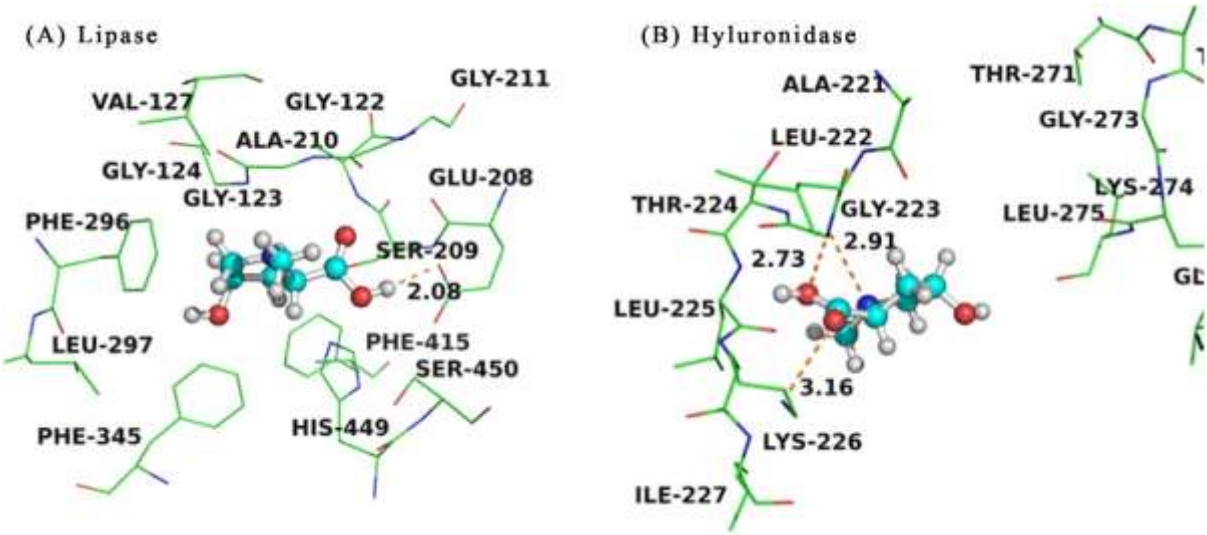

Fig. 3. Docked compound trans-4-hydroxy-N-methylproline in the active site of lipase (A) and Hyluronidase (B) prepared using PyMol molecular visual program.

3.7. Lipase inhibition

Propionibacterium acnes lipase enzymes degrade sebum triglycerides into FFA which are known to cause skin irritation by acting as inflammatory inducers within the pilosebaceous unit (Patil et al., 2012). *Clausena anisata* showed low levels of inhibitory activity even at the highest concentration tested. The percentage inhibition at 500 µg/ml was 21.93 ± 3.78% (Fig. 4A). The ethyl acetate fraction of three extracts of *Achillea santolina*, *Zizyphus lotus*, and *Inonotus hispidus* exhibited 50% inhibition of *Candida rugosa* lipase at higher concentrations of 2.32, 3.70 and 2.22 mg/ml, respectively. The butanol fractions of *A. santolina* and *Z. lotus* also exhibited high IC_{50} values of 4.83 and 8.27 mg/ml, respectively. This indicates that higher concentrations of extract are needed for the inhibition of lipase. The phenolic extraction of the ethyl acetate fractions, however, increased activity of *A. santolina*, *Z. lotus* and *I. hispidus* to an IC_{50} of 0.37, 0.45 and 0.56 mg/ml, respectively. The saponin extraction of the butanol extracts of *A. santolina* and *Z. lotus* showed an IC_{50} of 1.19 and 11.53 mg/ml, respectively against lipase activity. The inhibition of lipase activity by *C. anisata* exhibited a sigmoidal dose-response suggesting that higher concentrations of the extract or the phenolic fractions of

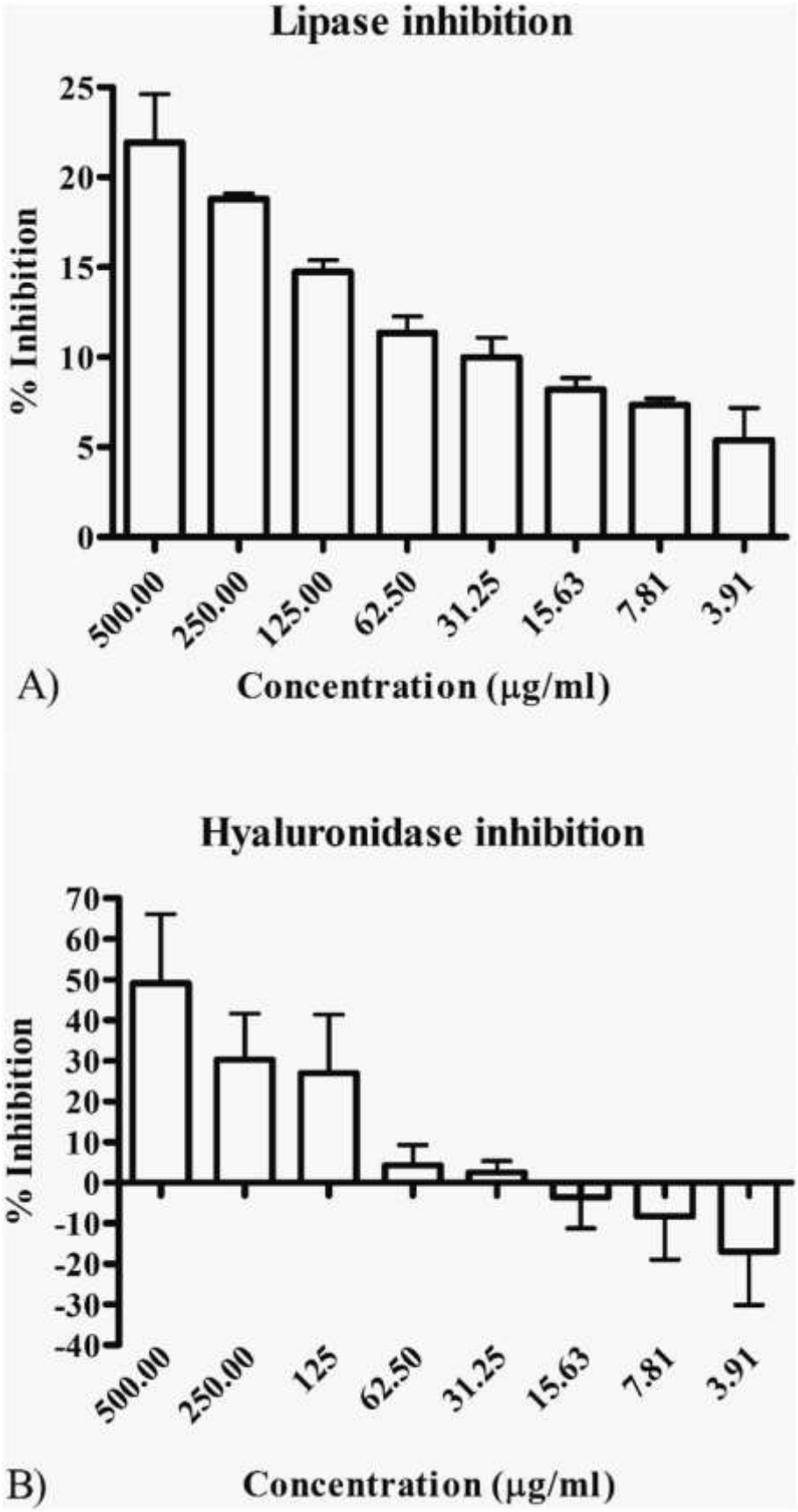

Fig. 4. **(**A) Percentage inhibition of *Candida rugosa* lipase activity and (B) *Streptococcus pyogenes* hyaluronidase activity of the *C. anisata* extract.

this extract could potentially show increased activity similar to that observed in the study by Benarous et al (2013). The methanol extract of *Terminalia chebula*, *Embelia ribes*, *Picrorhiza kurroa* and *Vitex negundo* however, showed excellent lipase activity with an IC_{50} of 1.02, 1.11, 47.89 and 19.25 µg/ml, respectively. This could indicate that the more polar fractions or extract of *C. anisata* could potentially inhibit lipase activity more effectively. The isolated compound chebulagic acid (from *T. chebula*) exhibited an IC_{50} of 57.4 µg/ml which was less than that of the total extract. This could indicate that lipase inhibition requires a mixture of compounds as opposed to single isolated constituents. This could explain why the isolated compound trans-4-hydroxy-N-methylproline showed no activity against the lipase enzyme (IC_{50} > 500 µg/ml) (Patil et al., 2012).

3.8. Hyaluronidase inhibition

Propionibacterium acnes excretes extracellular hyaluronidase which degrades the extracellular matrix of the skin connective tissues, which is made up of hyaluronic acid. It is estimated that 50% of this substrate, is found in the human skin. A number of microorganisms, including *P. acnes*, secrete this enzyme to allow for bacterial spread and tissue penetration (Tyner & Patel, 2015). The *C. anisata* extract showed 49.02% inhibition at 500 µg/ml (Fig. 4B). Common inhibitors of hyaluronidases include a number of bioactive secondary plant metabolites including those that show antioxidant and anti-inflammatory. Considering that the extract of *C. anisata* showed IL-8 inhibition and radical scavenging activity against DPPH, this could possibly explain the inhibition of the bacterial hyaluronidase activity (Girish et al., 2009). There have, however, been few studies that test plant extracts as bacterial hyaluronidase inhibitors, although there have been many reports on the inhibition of other types of hyaluronidases, particularly those isolated from snake and bee venoms and also from bovine testes (Girish & Kemparaju, 2005; Nema et al., 2011). The compound (trans-4-hydroxy-N-methylproline) showed no activity against the hyaluronidase enzyme (IC_{50} > 500 µg/ml).

4. Conclusion

Results obtained from the current study showed the potential of *C. anisata* for the treatment of AV based on its ability to act as an antimicrobial, antioxidant and an anti-inflammatory agent. The ethanolic extract showed an MIC at 31.25 µg/ml on *P. acnes* (ATCC 11827), the causative bacterial pathogen associated with AV. It only showed moderate toxicity on U937 cells with an IC_{50} at 74.46 µg/ml and an SI of 2.38. *Clausena anisata* showed significant inhibitory

activity on IL-8 secretion, however, it does not show activity on some other targets of acne pathogenesis. However, the use of polyherbal formulations and combinations of plant extracts is becoming popular practice. Therefore, *C. anisata* could be further investigated for its combined effect with other existing AV treatments.

Author contributions

M.D.C. (marcodecanhasa@gmail.com) designed, carried out experimentation, collected and analysed data for the study. N.K. (kishore.navneet6@gmail.com) aided in the isolation process. N.L. (namrita.lall@up.ac.za) and S.N. (snehar1805@yahoo.co.in) who edited the manuscript. V.K. (vivek494@gmail.com) carried out the molecular docking. D.M. (dmeyer@uj.ac.za) for providing expertise on cytokine investigation and analysis. B.S. (bikramsingh@ihbt.res.in) helped with the structure elucidation.

Conflicts of interest

The authors declare that they have no competing interests.

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