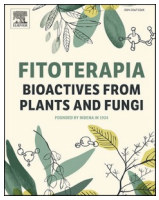
Fitoterapia 184 (2025) 106651

Contents lists available at ScienceDirect

Fitoterapia

journal homepage: www.elsevier.com/locate/fitote



Research Paper

In vitro nitric oxide inhibition of selected south African medicinal plants: A bio-guided purification of anti-inflammatory compounds from *Conyza scabrida*

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ARTICLE INFO

Keywords:
Anti-inflammatory
Nitric oxide (NO)
Conyza scabrida
Positive pressure solid phase extraction
5,3′,4′-trihydroxy-3,6,7,8-tetramethoxyflavone
Hautriwaic acid

ABSTRACT

This study aimed to investigate the anti-inflammatory activity of plant species by in vitro screening of extracts, fractions, and pure compounds on LPS-activated RAW 246.7 mouse macrophages. Plants were sourced from the plant repository in the Biodiscovery Centre, at the University of Pretoria. The plant materials were ground into fine powdered and extracted with dichloromethane: methanol (DCM:MeOH) (1:1) followed by 100 % MeOH, filtered, concentrated, and dried to generate extracts. Twelve extracts were fractionated using positive pressure solid phase extraction (ppSPE) Gilson liquid hander workstation, resulting in seven fractions per plant extract. The observed anti-inflammatory activity was mainly attributed to specific ppSPE fractions of the plant extracts rather than the entire crude extract.The most active plants species identified with 90 % inhibition at 25 μg/mL, were *Dodonaea. viscosa*, *Buxus natalensis*, *Flacourtia. indica*, and *Conyza scabrida*. The crude extract and ppSPE fractions (6 and 7) of *C. scabrida* demonstrated strong anti-inflammatory activity at 50 μg/mL with 97.8 % and 97.0 % inhibition, respectively. The Ultra-Performance Liquid Chromatography coupled with Quadrupole Time-of-Flight Mass Spectrometry Mass Spectrometry (UPLC-QTOF-MS) analysis of the active ppSPE fractions was used to tentatively identify two major compounds 5,3′,4′-trihydroxy-3,6,7,8-tetramethoxyflavone (C1) and hautriwaic acid (C2) and were subsequently purified using preparative high-performance liquid chromatography (prep-HPLC-MS) and confirmed by Nuclear Magnetic Resonance (NMR). At a concentration of 100 μM, they inhibited NO production by 96.6 % and 59.2 %, respectively. We have provided scientific preliminary evidence supporting ethnopharmacological claims of twelve South African medicinal plant species traditionally used to treat inflammatory diseases.

List of abbreviations

LPS	Lipopolysaccharide
ppSPE	Positive Pressure Solid Phase Extraction
UPLC-QTOF-MS	Ultra-high Performance Liquid Chromatography Quadrupole Time-of-Flight Mass Spectrometry
prep-HPLC-MS	Preparative High-Performance Liquid Chromatography Mass Spectrometry

1. Introduction

Inflammation is a crucial biological response that the body activates as a defence mechanism to protect itself from injury, infection, and harmful stimuli [1]. Tissue injury or the presence of pathogens prompts body cells and macrophages to release pro-inflammatory substances, which include microbial components, cytokines, and certain enzymes to facilitate inflammation by interacting with specialised receptors leading to initiation of critical intracellular signalling pathways [2]. This include the activation of protein kinase G (PKG), mitogen-activated protein kinase (MAPK) pathway, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), and the Janus kinase-signal transducer, to modulating inflammation, cell regeneration, and subsequent restoration of homeostasis [3–5]. However, dysregulated production of inflammatory mediators can become chronic and lead to various chronic diseases including autoimmune diseases, cardiovascular disease, diabetes and cancer [6,7].

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https://doi.org/10.1016/j.fitote.2025.106651
Received 8 January 2025; Received in revised form 11 May 2025; Accepted 29 May 2025
Available online 30 May 2025

Chronic inflammation is increasingly recognized as a significant public health problem, contributing to a wide range of non-communicable diseases [8]. Although chronic inflammation is a critical health issue, available treatment options remain limited and frequently raise concerns regarding their effectiveness and safety [9]. While corticosteroids and non-steroidal anti-inflammatory drugs (NSAIDs) are commonly used treatment options to alleviate pain and inflammation, they are linked with unbearable adverse effects [1]. Another limitation surrounding chronic inflammation is the development of effective and less toxic pharmaceutical agents, which remains complex due to intricate nature of inflammatory processes, translation gaps, and high drug development costs [10].

In low- and middle-income countries (LMICs), the burden of chronic inflammatory diseases is aggravated by various systematic issues including limited access to healthcare resources, inadequate infrastructure, and socioeconomic discrepancies [6]. However, the ongoing research in drug development holds promise for advancing treatment options in this area where the use of medicinal plants as an alternative treatment of inflammatory conditions has been widely investigated [11]. Recent reviews, demonstrate the anti-inflammatory potential of plant natural products, along with their unique mechanisms of action and historical significance, strongly supporting the pursuit of plants as a valuable source of new anti-inflammatory drug candidates [1,12]. demonstrated that plant-derived drugs offer safer and potentially more effective alternative treatments compared to traditional synthetic drugs [13,14].

Several plant-based natural products are commercially available as supplements and/or a stand-alone anti-inflammatory drug [11]. These include curcumin [15], *Boswellia serrata* [16], bromelain [17], flavonoids [18], parthenolide [19], cucurbitacin [20,21], 1,8-Cineole [22], and pseudopterosins [23]. The rich medicinal properties of plant-derived natural products great potential for discovering novel lead compounds, particularly in countries with rich biodiversity, such as South Africa, which is known for its exceptional variety of plant species [24]. The country boasts a remarkable variety of flora, featuring around 22,755 plant species, of which approximately 3000 are utilized in traditional medicine [25]. Among these, 495 species from 99 families are specifically used to treat pain and inflammatory diseases, highlighting a rich ethnobotanical knowledge that remains largely unexplored for their full pharmacological and biological potential [6,26]. Despite reasonable ethnobotanically use of medicinal plants, there remains a critical need to scientifically validate their medicinal claims, assess toxicity and identify their active compounds [27].

In this study, twelve South African medicinal plants were selected based on their reported traditional use in treating inflammation, pain and wound healing. The selected plants were prepared for preliminary in vitro anti-inflammatory evaluation as described by Mianda et al. (2022) [28], through measuring NO inhibition on LPS-activated RAW264.7 macrophages. The mouse macrophage cell line, RAW 264.7, is a well characterised and popular model to investigate the anti-inflammatory potential of test samples [29]. Cells are cultured in multi-well plates and activated by exposure to LPS which induces the expression of iNOS with concomitant nitric oxide formation. Changes in NO production are determined by measuring the levels of nitrite in the culture medium. Simultaneous evaluation of cell viability (MTT assay) is used to confirm the absence of cytotoxicity of the test sample.

Additionally, we report on the bio-guided purification and identification of active compounds from the most promising plant, *C. scabrida*, using HPLCMS based method. To the best of our knowledge, this is the first report on preliminary in vitro anti-inflammatory activity targeting NO suppression of *C. scabrida.* Additionally, we present the isolation of a polymethoxyflavone-type compound for the first time from *C. scabrida* exhibiting its potential anti-inflammatory properties. Given the extensive ethnobotanical use and the current preliminary data, this study represents a significant step toward scientifically validating the medicinal potential of South African plant.

2. Material and methodology

2.1. Reagents and chemicals

RAW 264.7 mouse macrophages were purchased from Cellonex (South Africa). Lipopolysaccharide (LPS), Griess reagent and aminoguanidine were purchased from Sigma-Aldrich (St. Louise, MO, USA). RPMI 1640 culture medium and foetal bovine serum (FBS) were purchased from Cytiva (Marlborough, MA, USA). Solvents for chromatographic purification were of HPLC grade purchased from MICROSEP (JHB, SA). All other chemicals were of analytical grade purchased from Sigma-Aldrich (St. Louise, MO, USA).

2.2. Selection and collection of plant species

The databases (Google, Google Scholar, ScienceDirect, PubMed and SciFinder) were used to search for publications on plant species using the keywords “South African medicinal plants traditionally used for the treatment of inflammation, pain and wound healing”. The selected plants were prioritized using a rating system that classified them as 1 - strong, 2 - medium, and 3 - weak. The evaluation criteria included:

a) Strength of traditional uses in inflammatory diseases: plants directly associated with inflammation and pain were rated 1, those traditionally used for conditions like fever received a rating of 2, while plants with other traditional applications, such as blood purification and treating diarrhoea, were rated 3.

b) strength of published use for inflammatory diseases (peer review): plants with substantial published research on inflammatory diseases were assigned a rating of 1, while those with limited or no published information received ratings of 2 and 3, respectively.

c) plant toxicity: plants with no or very low toxicity were rated 1, whereas those with medium and high toxicity received ratings of 2 and 3, respectively.

d) plant part used: leaves were rated 1, stems and bark received a rating of 2, and roots were assigned a rating of 3.

Plant samples, 10,000 dried, ground, and catalogued in a database that included their GPS coordinates, collection sites, and geographical information. Both the physical repository of samples and the associated database are made available at Biodiscovery Centre, Chemistry Department at the University of Pretoria [28]. Twelve plants with the highest scores were selected for further investigation (Table 1). Plant materials in the repository were already identified at the initial stage of the creation of the plant repository and a unique number was assigned for each plant material as illustrated in Table 1.

2.3. Extraction of plant material

Selected plant species and plant parts used are shown in Table 1. The samples were given a unique plant ID and the voucher specimen deposited at the National Herbarium of the South African Biodiversity Institute (Table 1). Approximately, 7.50 g of dried pulverized plant materials were extracted with 1:1 dichloromethane (DCM)-methanol (MeOH) in a sonicator at 35 °C for one hour, followed by 100 % MeOH, filtered and concentrated using BUCHI rotavapor R-300 and dried at 35 °C on HT-6 Genevac to generate a dry extract.

2.4. Prefractionation of extract

A modified method used by the National Cancer Institute (NCI) Programme for natural product discovery was adopted in this study [30]. About 250–300 mg plant extracts were weighed into barcoded tubes and dissolved in 4.5 mL of MeOH/EtOAc/MTBE (6:3:1). The solution was adsorbed into cotton rolls (1.27 cm × 3.81 cm) and dried in the HT-6 Genevac at 35 °C. Automated fractionation was performed using a customized positive pressure solid phase extraction (ppSPE) on a GX-214 Liquid handler workstation using a 2 g SPE cartridges (Thermo

Table 1
Selection of plant species from Biodiscovery repository.

Sample no	Plant species	Plant family	Plant part	Sample ID (Specimen no)	Extract yield (*w*/w)
1	*Agrimonia bracteata* E. Mey. ex C.A. Mey.	Rosaceae	Roots	P16477 (DS02604)	18.6
2	*Combretum zeyheri* Sond.	Combretaceae	Roots	P25314 (JM00273)	14.3
3	*Mimusops caffra* E.Mey. ex. A.DC	Sapotaceae	Leaves	P17927 (DS03029)	8.6
4	*Ziziphus mucronata* Willd.	Rhamnaceae	Roots	P25019 (DS04236)	10.0
5	*Calpurnia aurea* (Aiton) Benth. subsp. *aurea*	Fabaceae	Roots	P25694 (JM00345)	14.0
6	*Cyperus digitatus* subsp. *auricomus*	Cyperaceae	Roots	P13973 (HV00531)	14.0
7	*Dodonaea viscosa* subsp. *angustifolia*	Sapindaceae	Leaves	P24612 (FP01447)	8.3
8	*Buxus natalensis* (Oliv.) Hutch.	Buxaceae	Leaves	P16713 (DS02640)	16.7
9	*Agapanthus praecox* Willd	Amaryllidaceae	Stems	P12468 (DB00144)	5.0
10	*Flacourtia indica* (Burm. f.) Merr.	Salicaceae	Roots	P13195 (FP00317)	9.8
11	*Euclea natalensis* A. DC. *obovata*	Ebenaceae	Roots	P13307 (FP00550)	30.5
12	*Conyza scabrida* DC	Asteraceae	Leaves	P20296 (DS03082)	20.7

scientific hyperSpe C8 SPE non-end-capped). The cartridge was pre-conditioned and equilibrated using three-time column volumes each of MeOH/H_2O in a ratio of 100:0 and 1:19, respectively. The extracts were pre-fractionated to seven fractions using methanol (MeOH), water (H_2O) and acetonitrile (MeCN) in the following solvent systems. For fractions 1–6, MeOH/ H_2O in a ratio of 1:19, 1:4, 2:3, 3:2, 4:1, 100: 0, and 1:1 (MeCN/MeOH) for fraction 7, were used respectively. A controlled elution rate of <10 mL/min was maintained, and 8 mL of elution solvent was collected in pre-weighed 10 mL polypropylene barcoded tubes. Samples were dried at 35 °C on HT-6 Genevac high-performance centrifugal evaporation systems.

2.5. Standardization of plant extract and fractions

The mass of each fraction was weighed out using an automated weighing station. The formatting and dilution were done to generate standardised sample solutions of 5 mg/mL in DMSO using Hamilton Microlab STARlet automated liquid handle platform in 96-well plates. The standardised samples were transferred into polypropylene FluidX 2D barcoded 96 well microlitre plates for automated sample storage on the Hamilton Verso Q20 freezer, ready for ant-inflammatory screening and future reference. About 2 mg of samples (extracts and fractions) from 12 plants were tested for their anti-inflammatory effects on a mouse macrophage cell line, RAW 264.7. The most active plants were selected for further analysis and purification using the UPLCMS-QTOF-MS and preparative HPLC-QDa-MS, respectively.

2.6. UPLCMS-QTOF-MS analysis

The bio-active samples (extract, fractions, and compounds) prepared at 1 mg/mL in MeOH, were analysed on a Waters ACQUITY UPLC system connected to a quadrupole mass filter and a high-resolution time-of-flight mass analyzer. The Waters Xevo G2 high-definition mass spectrometer operating under MassLynx v. 4.1 software was used for compound separation and detection. Sodium iodide clusters were used to calibrate the mass spectrometer over a range of 50–1200 Da using the Intellistart software function. The instrument was optimized for ESI positive and negative mode with a source temperature of 120 °C, extraction cone voltage of 4.0 V, sampling cone of 30.0 V, and a capillary voltage of 2.0 kV. An internal lock mass control standard of 2 ng/μL leucine enkephalin (*m/z* 555.2693) was used to account for mass drift, which was monitored by infusing the lock mass solution at a rate of 3 μL/min every 10 s. Separation was achieved with a Waters BEH C_{18} (2.1 mm × 100 mm, 1.7 μm) column using a gradient elution method with solvents A (H_2O + 0.1 % formic acid) and B (MeCN +0.1 % formic acid), respectively. The flow rate was 0.4 mL/min, the injection volume of 5 μL, and the column temperature was 40 °C.

2.7. Isolation and purification of compounds from C. scabrida

Identified compounds from the active fraction of *C. scabrida* were isolated from DCM/MeOH extract using the following method: 4.34 g of DCM/MeOH extract was dissolved in 10 % H_2O in MeOH. Subsequently, the solution was partitioned three-times using 200 mL of n-Hex and DCM, successively. The resulting fractions were analysed by UPLCMS-QTOF-MS to confirm the presence of previously identified compounds in the active fraction. The DCM fraction showed the target compounds and was further fractionated on ppSPE following the method described in section 2.3. About 300 mg of ppSPE fraction was dissolved in MeCN/H_2O (2:1), filtered through a 0.22 μm filter, and purified on preparative high-performance liquid chromatography (prep- HPLC-QDa-MS) Waters chromatographic system. The prep-HPLC was fitted with a Waters PDA (Model 2998) and interfaced with an ACQUITY QDa detector (Waters Corporation, Milford, MA, USA). 500 μL was injected on an Xbridge® preparative C18 OBD™ (19 mm × 250 mm, 5 μm) column, where solvent A consisted of H_2O (0.1 % formic acid) and solvent B consisted of MeCN (0.1 % formic acid). The flow rate was kept constant at 5 mL/min. The following linear elution gradient was used: an initial hold at 40 % of solvent B (0–1 min), followed by a linear increase to 100 % of B (1–16 min), and kept at 100 % B (16–18 min), and then return to the starting conditions (20–24 min). Compounds C1 and C2 with *m/z* of 389.1176 and 331.2162 (negative ionization mode), respectively, were collected and dried on H6 Genevac. The compounds were identified as 5,3′,4′-trihydroxy-3,6,7,8-tetramethoxyflavone (C1) and hautriwaic acid (C2) using data from UPLC-QTOF-MS, 1D and 2D NMR spectroscopy, as well as SCXRD spectrometry.

2.8. NMR analysis

The ^{1}H, ^{13}C, and 2D NMR spectra were recorded using a Bruker Avance III HD 500 MHz NMR spectrophotometer at 18 °C with a cryoprobe. The samples were dissolved in deuterated methanol (CD_3OD) purchased from Aldrich Chemistry, Sigma-Aldrich, Milwaukee, WI, USA, and the chemical shifts were reported in parts per million (ppm), referenced to the residual solvent signals (CD_3OD δ_H 3.31, δ_C 49.15 ppm).

2.9. Crystal structure analysis

The crystalline structure of isolated compounds C1 and C2 were analysed by single crystal X-ray diffraction experiment. Intensity data was determined on a Bruker D8 Venture Microfocus with Photon III CCD area detector diffractometer with graphite-monochromated $MoK\alpha_1$ (l =

0.71073 Å) radiation at 173 K using an Oxford Cryostream 600 cooler. Data reduction was carried out using the program SAINT+, version 6.02 and empirical absorption corrections were made using SADABS [31]. Space group assignments were made using XPREP.[1] The structure was solved in the WinGX [32] Suite of programs, using intrinsic phasing through SHELXT-2018/2 [33] and refined using full-matrix least-squares/difference Fourier techniques on F [32] using SHELXL-2019/3 [33]. All C-bound hydrogen atoms were placed at idealized positions and refined as riding atoms with isotropic parameters 1.2 or 1.5 times those of their parent atoms. Diagrams and publication material were generated using ORTEP-3 [32] and PLATON [34].

2.10. In vitro anti-inflammatory activity of plant samples

The RAW264.7 cells were seeded in RPMI complete medium which consisted of RPMI1640 culture medium (with L-Glutamine and no antibiotics) supplemented with 10 % FBS in 96-well plates at a density of 1×10^5 cells per well and allowed to attach overnight at 37 °C in humidified environment with 5 % CO_2. The following day, the used culture medium was aspirated from the wells and replaced with 100 μL of complete RPMI medium. Plant samples were solubilized with DMSO to prepare a 100 mg/mL stock solution. To assess the anti-inflammatory activity of the plant samples (pure compounds, extracts, and fractions), 50 μL of medium containing LPS (final concentration 500 ng/mL) and 50 μL of sample diluted in RPMI complete medium was added to the corresponding wells. LPS and test samples were added at the same time. The final concentrations of extracts and fractions were 25 and 50 μg/mL, while compounds were tested at 0.1, 1.56, 3.13, 6.25, 12.5, 25, 50 and 100 μM. Aminoguanine (AG) at concentrations of 50, 100 and 200 μM was used as a positive control for anti-inflammatory effects. The cells were incubated for an additional 24 h at 37 °C in 5 % CO_2.

To quantify NO production, 50 μL of spent culture medium was transferred to a new 96-well plate after 24 h of incubation, and 50 μL of Griess reagent was added. The absorbances of four wells per concentration were measured at 540 nm using a Bio Tek Power Wave XS spectrophotometer. To determine the NO concentration in each sample, a standard curve of sodium nitrite dissolved in culture media was used. The percentage inhibition of NO production in response to LPS-activated macrophages of extract, fractions and compounds was calculated using the expression in eq. 1 below [35]. The data were reported as mean ± SD. $n = 1$, in technical quadruplicate.

$$\%NO\ inhibition = 100 - \frac{sample\ absorbance - blank\ absorbance}{control\ absobance - blank\ absorbance} x100 \quad (1)$$

2.11. Cytotoxicity screening of plant samples

To ensure that toxicity was not a factor in any of the samples, cell viability was assessed using the tetrazolium salt assay (MTT) on RAW 264.7 macrophages as described by Wu (2025) guidelines for anti-inflammatory assays [36]. This involved removing the medium and treatments from each well after incubation, washing once with 100 μL of PBS before adding 100 μL of medium containing 0.5 mg/mL MTT. The samples were then incubated at 37 °C in 5 % CO_2 for a further 30 min as longer incubation times can lead to over-reduction causing inaccurate quantification. The MTT was then discarded and 100 μL of DMSO was added to each well to dissolve the formazan crystals. Absorbance measurements were performed using a BioTek Power Wave XS spectrophotometer (Winooski, VT, USA) at a wavelength of 540 nm. Each sample was tested in quadruplicate for both the inflammatory response and the MTT test. The percentage cell viability was calculated using the expression in eq. 2 below [37]. Data were reported as mean ± SD and $n = 1$, in technical quadruplicate.

$$\%Cell\ viability = \frac{sample\ absorbance - blank\ absorbance}{control\ absobance - blank\ absorbance} x100 \quad (2)$$

2.12. Statistical analysis

The samples were subjected to in vitro screening against LPS-activated RAW macrophages at a concentration range of 25–50 μg/mL for extract and fractions, and 0.1–100 μM for isolated compounds over 8 concentration serial dilution points. Each sample was evaluated in single biological experiment in quadruplicate. The data was expressed as mean ± SD. A statistical comparison of control and treated group was conducted using Two-tailed Student *t*-test in GraphPad Prism (v8) to evaluate statistical significance. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Selection and extraction of plant material

Twelve plants from different families were selected for this study based on their traditional uses in the treatment of pain and inflammatory diseases (Tables 1 and 2). Generally, the roots and leaves were the most commonly used plant parts, with the stems being used for only one plant. The resulting extraction yields ranged between 5.0 % and 30.5 % depending on the plant species and plant part extracted (Table 1).

3.2. Anti-inflammatory screening of plant sample (extracts and fractions)

Plant extracts underwent a pre-fractionation through positive pressure solid phase extraction (ppSPE) yielding seven fractions for each plant before being screened for anti-inflammatory activity. In total, 84 ppSPE fractions were generated from twelve plants extract. This resulted in 96 samples of extracts and fractions, which were evaluated for their anti-inflammatory properties at two test concentrations of 25 and 50 μg/mL on LPS-activated RAW 264.7 mouse macrophages. Plant species that showed significant to moderate activity are shown in Fig. 1 and 2, were

Table 2
Reported traditional uses of 12 selected South African medicinal plants.

Plant species	Traditional medicinal uses
A. bracteata	Used to treat inflammatory and oxidative-related diseases [40]. Infusions and decoctions are used to treat the respiratory system and urinary tract, gastrointestinal disorders, and persistent wounds or ulcers [41].
C. zeyheri	Infused leaves are drunk for genitourinary system inflammation, pain, poisonings, diarrhoea, cancer and cold [42].
M. caffra	The leaves and bark are used to treat inflammatory wounds and malaria, and the root is used to treat sexually transmitted infections, tuberculosis, and womb problems [43,44].
Z. mucronata	The whole plant is used to treat various inflammatory conditions [45].
C. aurea	Used to treat various inflammatory conditions, such as snake bites, amoebic dysentery, diarrhoea, stomach pain, and bowel problems [46,47].
C. digitatus	The powdered tuber is inhaled to treat cold and fever [48], gastrointestinal and respiratory issues, menstrual irregularities, and inflammatory conditions [49].
D. viscosa	Used to treat rheumatism, skin infections, [50,51] pain, oral thrush, measles, antipruritic in skin rashes, and toothaches [52,53].
B. natalensis	The bark is traditionally used by elderly people to treat memory loss [54]. Buxus species are also reported to treat inflammatory conditions like wounds, gout, skin problems rheumatism and malaria [55].
A. praecox	The plant is used to treat pregnancy complications, augmented labour, heart disease, paralysis, coughs, colds, and chest discomforts [56].
F. indica	Used to treat inflammatory conditions, indigestion, and stomach pains [57,58].
E. natalensis	The roots and bark are used for swelling, worms, stomach disorders, toothache, headache, chest complaints, pleurisy, and urinary tract infections. [59,60].
C. scabrida	The leaves decoction is used to alleviate backache and inflammation, headaches, general pain, and toothache [6,61].

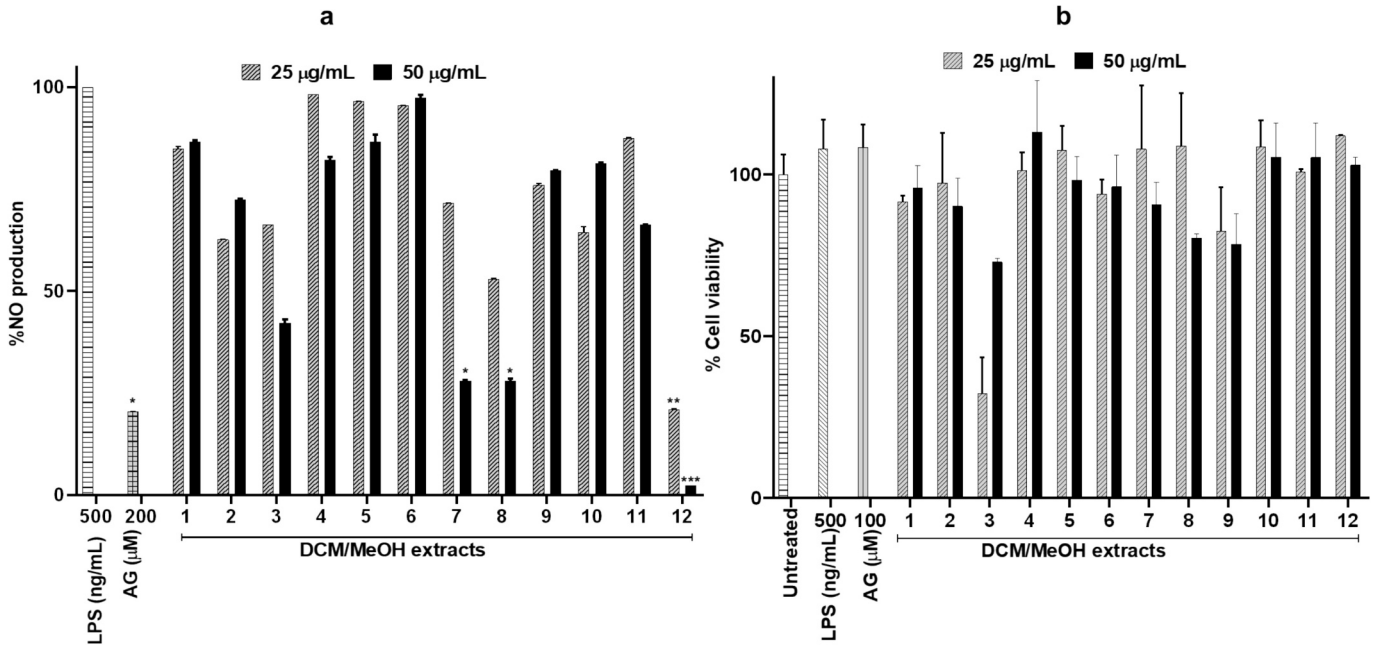

Fig. 1. a, Anti-inflammatory activity of 12 plant extracts at 25 and 50 µg/mL measured in vitro using Griess assay. The anti-inflammatory activity was indicated by the decrease in NO concentration in response to the LPS-activated RAW 264.7 mouse macrophages. b, Cell viability (%) of LPS-activated macrophages after 24 h of exposure to treatments. Bar graph represents quadruplicate values of one experiment. Error bars represent the standard deviation of the mean. *, $P < 0.05$; **, $P < 0.01$; ***$P < 0.001$.

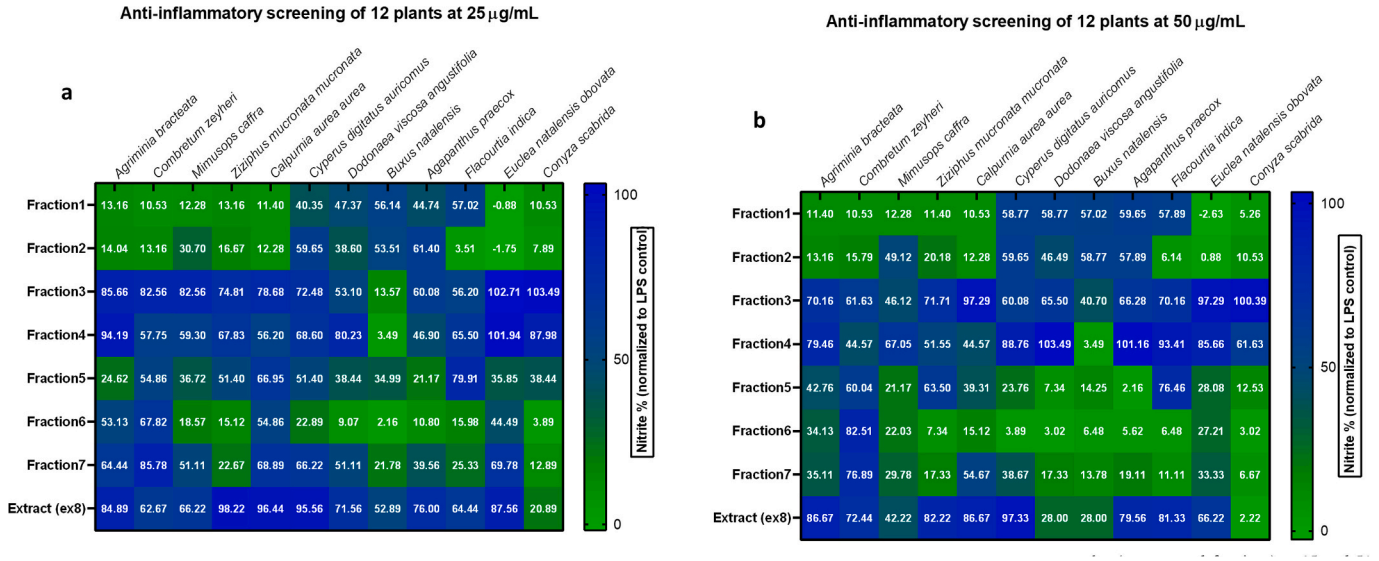

Fig. 2. Anti-inflammatory activity of 12 plant samples (extracts and fractions) at 25 and 50 µg/mL measured in vitro using the Griess assay. The mice macrophage RAW 264.7 cell line was activated with Lipopolysaccharide (LPS) and the anti-inflammatory activity of 96 samples was indicated by the decrease in nitrite concentration in response to LPS-activated RAW macrophages with no effect on cell viability. The colour coding in the heat map indicates biological activity, with blue representing lower activity levels (<50) and green signifying higher activity levels (≥50) with $P < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

discussed to identify the hit for further investigation.

The following classification criteria described by Mutombo (2023) [38] was used to categorise plant samples as active or inactive:

a) Good activity:

> 70 % inhibition at 50 µg/mL and > 50 % inhibition at 25 µg/mL

b) Moderate activity:

> 70 % inhibition at 50 µg/mL and < 50 % inhibition at 25 µg/mL.
< 70 % inhibition at 50 µg/mL and > 50 % inhibition at 25 µg/mL.
50 to 70 % inhibition at 50 µg/mL and < 50 % inhibition at 25 µg/mL

c) No or minimal activity:

< 50 % inhibition at 50 µg/mL and < 50 % inhibition at 25 µg/mL.

The aminoguanidine (AG) was used as a positive control on this screening experiment to validate the anti-inflammatory potential of tested samples targeting NO pathways by demonstrating the expected

reduction in NO-mediated inflammation through selective NO inhibition [39]. Four DCM/MeOH plant extracts demonstrate potential anti-inflammatory activity by suppressing NO production on LPS-activated macrophages (Fig. 1a). Specifically, moderate NO suppression in response to LPS-activated macrophages was observed on the leave extract of *M. caffra* (no.3) showing 33.4 % suppression at 25 μg/mL and 57.8 % at 50 μg/mL. The leave extract of *D. viscosa* (no.7) exhibited moderate NO inhibition of 28.3 % at 25 μg/mL and 72 % at 50 μg/mL (Fig. 1a). The leave extracts of *B. natalensis* (no. 8 in Fig. 1a) showed moderate activity by exhibit 47.1 % at 25 μg/mL and 72 % NO inhibition at 50 μg/mL. Lastly, the leave extract of *C. scabrida* (no.12) displayed significant NO inhibition of 79.1 at 25 μg/mL and 97.8 % at 50 μg/mL (Fig. 1a). At 50 μg/mL the extract of *C. scabrida* significantly inhibition NO production more effectively than the positive control (AG) at 200 μg/mL. The extracts from *M. caffra*, *D. viscosa*, and *B. natalensis* at 50 μg/mL showed NO inhibition comparable to AG (Fig. 1a). These findings indicate that these DCM/MeOH leaf extracts have great potential as inhibitors of NO production under inflammatory conditions, suggesting promise anti-inflammatory agents by modulating macrophage-mediated inflammatory responses.

The anti-inflammatory activity shown in Fig. 2 demonstrates that each plant species contains one or more fractions that significantly suppress NO production in LPS-activated RAW macrophages. Eleven of twelve plant species demonstrated more than 80 % inhibition of NO production, while the least effective species showed an inhibition of 77.1 % NO production. These results underscore the importance of pre-fractionation of extract before screening and provide a better understanding of poor NO inhibition observed in the plant extracts which may be due to a low concentration of an active compound or an antagonistic effect in the complex extract matrix. The most active fractions exhibited above 90 % NO inhibition at 25 μg/mL and were obtained from four plants species. Specifically, Fraction 6 from the leaves extract of *D. viscosa* showed 90.9 % NO inhibition. Faction 4 and 6 from the leaves extract of *B. natalensis* exhibited 96.5 % and 97.8 % NO inhibition respectively. Additionally, Fraction 2 of the leaves extract of *F. indica* showed 96.4 % NO inhibition, while Fraction 6 of the leaves extract of *C. scabrida* exhibited 96.1 % NO inhibition. The plants were further prioritized based on the anti-inflammatory activity, toxicity observed, and the availability of plant material for large-scale extraction. This resulted to this study focusing on further investigation of the chemical composition and potential anti-inflammatory compounds in *C. scabrida* rather than *B. natelensis*, despite exhibiting 97.8 % NO inhibition.

3.3. Cell viability

The cell viability experiment demonstrated that, out of twelve tested plant extracts, nine were found significantly non-toxic to the RAW 264.7 mouse macrophages (Fig. 1b). Most of these extracts promoted cell proliferation compared to the untreated control, indicating that the observed anti-inflammatory activity of the extract was not a result of cell damage. At 25 μg/mL, the ppSPE fractions from twelve selected plants were found to be significantly cytotoxic, specifically the region from polar fractions (1–4), as shown in Fig. 3. The leaves extract of *C. scabrida* demonstrated the highest anti-inflammatory activity and was found to be substantially non-toxic and promoted cell proliferation (Figs. 1 and 3). The MTT assay has provided adequate evidence for the best plant selection for further purification of the compounds responsible for the observed anti-inflammatory effects (Figs. 1 and 3).

3.4. UPLC-QTOF-MS analysis of C. scabrida

The crude extract and the active ppSPE fractions (6 and 7) were analysed using UPLC-QTOF-MS to tentatively identify the active compounds. Two major compounds were selected for identification as shown in the chromatograms in positive mode ESI+ ion, BPI, illustrated in Fig. 4. The first compound, 5,3′,4′-trihydroxy-3,6,7,8-tetramethoxyflavone (C1), was detected at *m/z* 391.1176 $[M + H]^+$ at a retention time (RT) of 9.60 min, the elementary composition determined through MassLynx software algorithm was $C_{19}H_{18}O_9$ (calculated 391.1029). The molecular formula matches that of gardenin E, a polymethoxyflanone previously isolated from plants [62].

The second compound, hautriwaic acid (C2), appeared at m/z 333.2162 $[M + H]^+$ with a retention time of 11.52 min and a corresponding molecular formula of $C_{20}H_{28}O_4$ (calculated 333.2056) matches that of hautriwaic acid, a clerodane diterpenes previously reported in literature [63]. The compound was previously identified in *C. scabrida* and its anti-inflammatory properties is well-known. The observed anti-inflammatory activity of the leaves extract from *C. scabrida* may be attributed to the presence of hautriwaic acid. However, further purification and screening of this compound in its pure form is necessary to confirm its anti-inflammatory effects and potential applications.

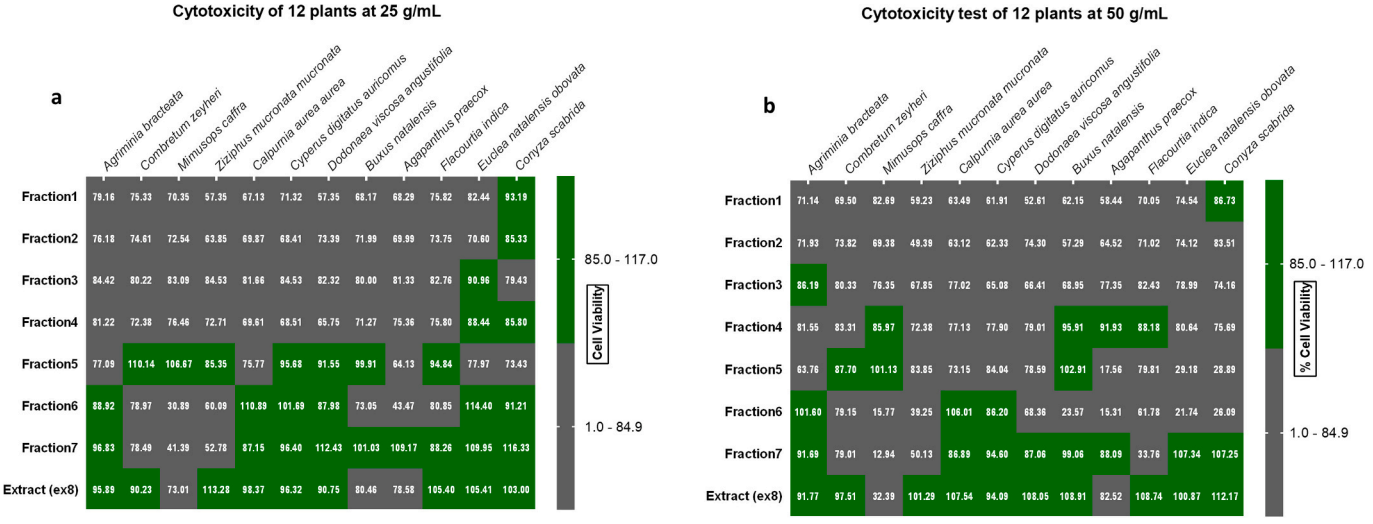

Fig. 3. Cell viability (%) of LPS-activated macrophages after 24 h of exposure to treatments as indicated in the Figures. The heatmap graph per cell represents quadruplicate values of one experiment. The colour coding indicates cytotoxicity activity, with grey representing cytotoxic sample levels (<85.0) and green showing samples with no significant cytotoxicity activity levels (≥85). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

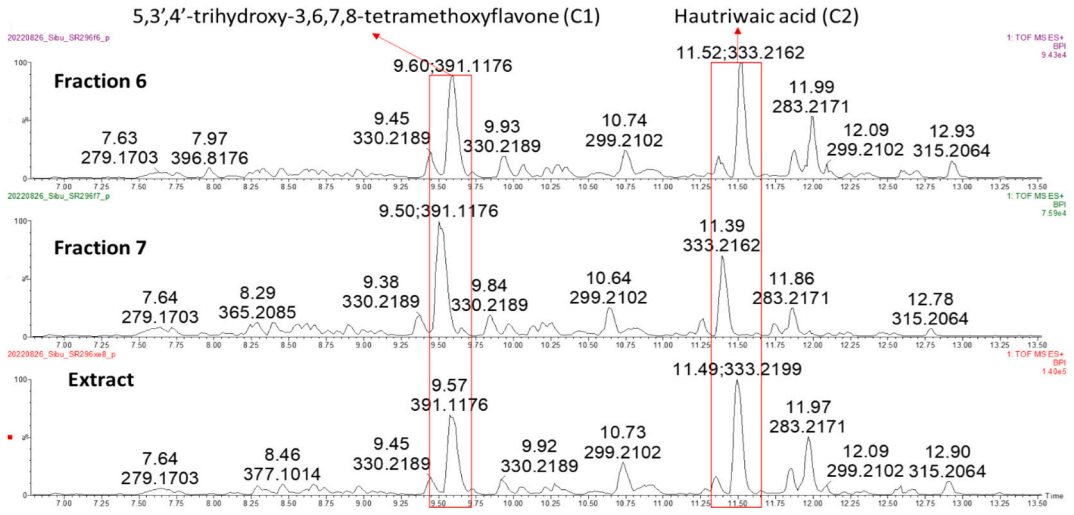

Fig. 4. UPLC-QTOF-MS chromatograms of *C. scabrida* active extract and fractions obtained in BPI positive ESI ionization mode. Peak 1 corresponds to 5,3′,4′-trihydroxy-3,6,7,8-tetramethoxyflavone (C1) and peak 2 to hautriwaic acid (C2).

3.5. HPLC-based purification and structure elucidation of active compounds from C. scabrida

Both compounds (C1 and C2) were isolated as pure compounds from a 300 mg ppSPE fraction of *C. scabrida* using preparative HPLC-QDa-MS (as detailed in section 2.6). About 4.7 mg of C1 was collected at a retention time of 9.50 min, while 3.2 mg of C2 was obtained at 12.50 min. The HPLC fractions were subsequently analysed with UPLC-QTOF-MS to verify their purity, and their structures were elucidated through 1D and 2D NMR, as well as single crystal X-ray diffraction (SCXRD).

Compound C1. was obtained as a yellow amorphous powder. UPLC-QTOF-MS analysis in ESI positive ionization mode revealed that 5,3′,4′-trihydroxy-3,6,7,8-tetramethoxyflavone (C1) exhibits an *m/z* 391.1176 $[M + H]^+$, corresponding to the molecular formula of $C_{19}H_{18}O_9$ and showing 11 degrees of unsaturation (Supplementary Fig. S1). The ^1H NMR spectrum showed the presence of four methoxy proton signals at δ_H 3.89 (3H, s, 3-OCH3), δ_H 3.82 (3H, s, 6-OCH_3), δ_H 4.08 (3H, s, 7-OCH_3), and δ_H 3.94 (3H, s, 8-OCH_3). The 1D and 2D NMR experiments established that compound C1 had the same planar structure as the previously reported compound gardenin E [62]. Subsequently, the compound was recrystallized in MeOH at room temperature, resulting in the formation of yellow crystals. Single-crystal X-ray diffraction analysis reveal that the compound crystallizes in monoclinic space group P21/c, unit cell parameters a = 19.581(3) Å, b = 6.5811(11) Å, and c = 15.248 (2) Å and molecular formula of $C_{19}H_{18}O_9$ corresponding with UPLC-QTOF-MS data (Fig. 5, supplementary data Table S2). The crystallographic data confirm that the compound is achiral, and display the positions of the four methoxy groups, which were consistent with the NMR assignments for the compound.

Compound C2. The compound was identified as hautriwaic acid and obtained as a colourless amorphous powder. In the UPLC-QTOF-MS analysis, under ESI positive ionization mode, hautriwaic acid appeared at *m/z* 333.2162 $[M + H]^+$ which corresponded with a molecular formula of $C_{20}H_{28}O_4$ with 7 degrees of unsaturation (Supplementary Fig. S2). The structure of C2 was elucidated through both 1D and 2D NMR analyses, which provided distinct signals consistent with classification of a clerodane diterpene drawn in the published literature data [63] (Fig. 6, compound C2, supplementary Table S3). Hautriwaic acid was recrystallized in MeCN/H_2O (2:1) in a cold room for about one week. Well-shaped single crystals were analysed using X-ray diffraction and the experiment confirms the molecular formula of $C_{20}H_{28}O_4$, which is consistent with the data obtained from the UPLC-QTOF-MS. The compound crystallizes in orthorhombic space group P 2_1 2_1 2_1, unit cell parameters a = 10.2101(3)Å, b = 10.5191(4) Å, c = 16.3089(6) Å. The absolute structure and anomeric configuration were determined (supplementary data Table S4), proving that compound C2 has a chiral centre at (5*S*, 8*R*, 9*S* 10*R*)-(−)-hautriwaic acid configuration. The UPLC-

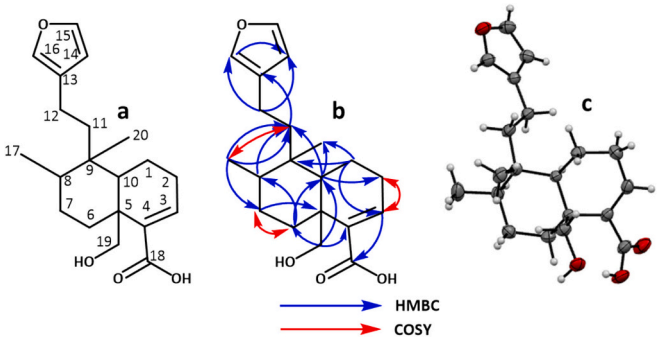

Fig. 6. Chemical structure a, compound C2 (Hautriwaic acid), b selected HMBC and COSY correlation and c crystal structure of compound C2.

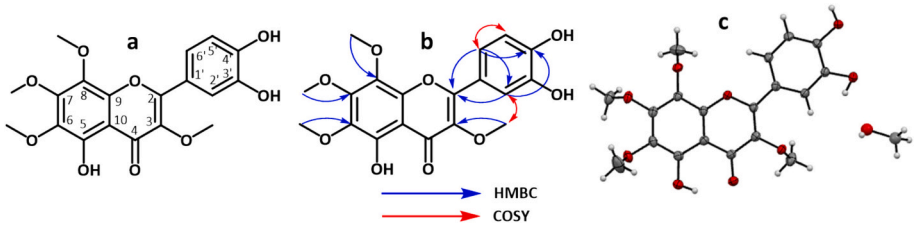

Fig. 5. Chemical structure a, compound C1 (5,3′,4′-trihydroxy-3,6,7,8-tetramethoxyflavone), b selected HMBC and COSY correlation and c crystal structure of compound C1.

HRMS, ^{1}H and ^{13}C NMR, and SCXRD data are provided in the supplementary file.

3.6. Anti-inflammatory activity of isolated compounds from C. scabrida

The isolated compounds 5,3′,4′-trihydroxy-3,6,7,8-tetramethoxyflavone (C1) and hautriwaic acid (C2) were screened for their anti-inflammatory effect using LPS-activated RAW 264.7 mouse macrophages as described in section 3.2. Both compounds showed strong anti-inflammatory activity. Compound C1 at the highest concentration of 100 μM inhibited 96.6 % NO production in response to LPS-activated macrophages without affecting cell viability (Fig. 7a, supplementary data Table S6 and S7). The activity of compound C1 at 100 μM showed better activity than the positive control (Aminoguanidine) tested at a higher concentration of 200 μM. Compound C2, also showed potent anti-inflammatory activity at higher concentration (100 μM), almost equivalent to that of a positive control (Fig. 7a, supplementary data Table S6 and S7). Additionally, the cytotoxicity of the compounds was evaluated on the same cell line using the MTT assay, and no evidence of cytotoxicity was observed at the tested concentrations after 24 h (Fig. 7b, supplementary data Table S7).

4. Discussion

In this study, we provide scientific validation of twelve (12) South African medicinal plants traditionally used to treat inflammatory diseases and highlight their potential as effective natural remedies. The selected plant species demonstrated strong anti-inflammatory activity, attributed to their ppSPE fractions. Each species displayed one or more fractions that significantly inhibited NO production by more than 70 % in LPS-activated macrophages. In this present study, *D. viscosa*, *B. natalensis*, *F. indica* and *C. scabrida* fractions exhibited more than 90 % NO inhibition. The *D. viscosa* is a well-known plant species in traditional medicine for its potent anti-inflammatory effects [64]. Previous pharmacological studies demonstrated that the extracts of *D. viscosa* shows potential antidiabetic, antioxidant, antimicrobial and anti-inflammatory properties [65]. Compounds have been identified from this plant;, studies suggest that its anti-inflammatory activity might be attributed to the presence of hautriwaic acid [66]. Hautriwaic acid is one of the abundant compounds found from *D. viscosa* with significant inhibition of edema in mice, with approximately 87.1 % reduction at a dose of 0.25 mg/ear [67]. According to Martínez-Casares et al. (2023) [68], this compound inhibits the production of prostaglandins and leukotrienes by selectively binding to cyclooxygenases (COX-1 and 2) and 5-lipoxygenase (5-LOX). Furthermore, the DCM extract of *D. viscosa*, exhibited a remarkable 97.8 % anti-inflammatory effect at a dose of 3 mg/kg [50]. Another review highlighted the exceptional anti-inflammatory activity of hautriwaic acid, noting its ability to inhibit TNF- α, IL-6 and IL-1β and increase IL-10 [68].*D. viscosa* have a great potential in the discovery of new anti-inflammatory drug, however, further pharmacological studies are needed to elucidate its mode of action.

Limited studies have been identified on *B. natalensis,* despite that the species plays a vital role in South African traditional medicine in the treatment of a wide range of inflammatory diseases [69]. The species is very rich in alkaloid-type compounds including cyclovirobuxine D, known for its strong anti-inflammatory activity, aligning with traditional uses of this plant [54]. The species has not been extensively studied, however, the literature reports and the findings from this study suggest that *B. natalensis* has potential in the treatment of various inflammation diseases drawn from its unique alkaloid profile.

F. indica, is known for its potent traditional uses in the treatment of inflammatory diseases [70]. The methanol extract of *F. indica* shows strong analgesic, anti-inflammatory, and diuretic effects in in vivo studies [71]. Another study demonstrated that the ethyl acetate fraction of *F. indica* shows good antimalarial activity ($IC_{50} = 3$ μg/mL) and three compounds were isolated from this fraction, namely Pyrocetechol, Homaloside D and Poliothrysoside [57]. Additionally, the fruit extract of this species has also been reported to exhibit potential anti-inflammatory and antioxidant properties [70]. The literature supports the anti-inflammatory activity demonstrated in the current study. The findings suggest that *F. indica* have a potential in anti-inflammatory drug candidate, and further studies are needed.

Despite limited pharmacological studies, *C. scabrida* has been traditionally used for the treatment of pain and inflammation [6]. According to a review report by Maroyi (2019) [72], the acetone and methanol leaves extracts of *N. ivifolia*, also known as *C. scabrida*, showed potent antibacterial and antifungal activity. Other studies reported the phytochemicals present in *C. scabrida* which comprises diterpene acids like hautriwaic acid, conyscabraic acid, 5α-hydroxy, 10β-hydroxyprintzianic acid, nidoresedaic acid, and printziaic acid. [72,73]. The anti-

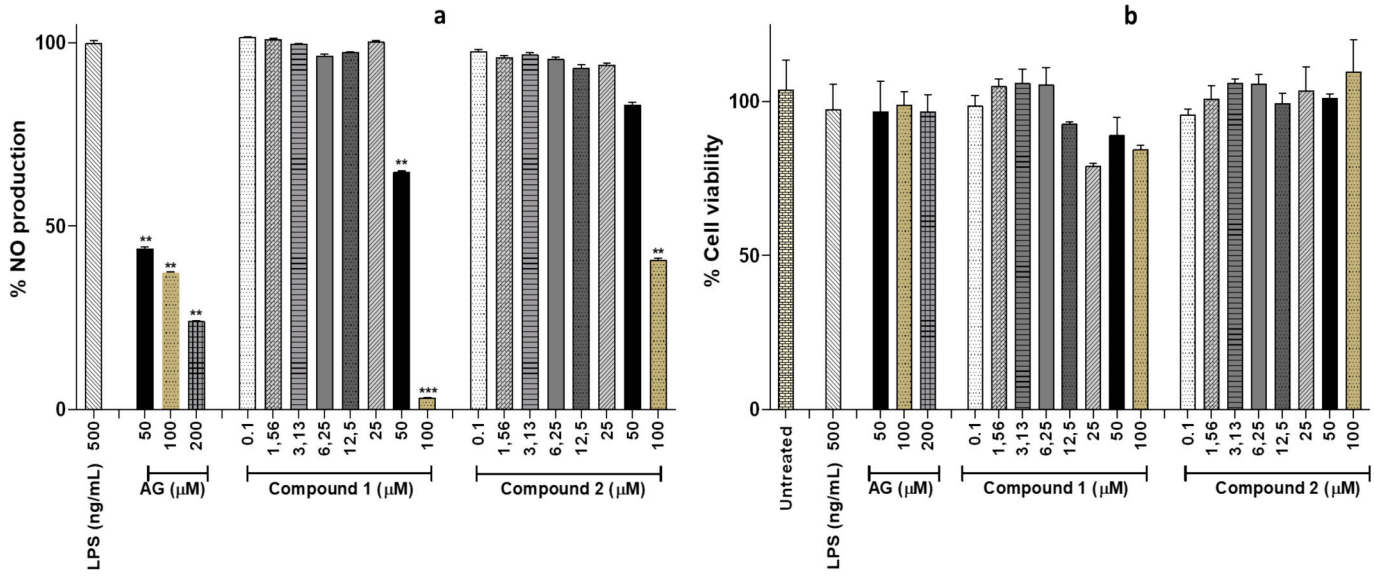

Fig. 7. a, Illustration of nitric oxide production in LPS-activated RAW 264.7 mouse macrophages cell line treated with compound C1 (5,3′,4′-trihydroxy-3,6,7,8-tetramethoxyflavone) and compound C2 (Hautriwaic acid) and b, cell viability (%) of LPS-activated macrophages after 24 h of exposure to treatments. Each bar graph represents the percentage cell viability mean ± SD., $n = 4$. Error bars are the standard deviation of the mean. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

inflammatory properties of hautriwaic acid are well established, as it effectively inhibits the production of pro-inflammatory cytokines, including interleukin-1 beta (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α), which are crucial in mediating inflammatory processes in various cell lines [74]. The present study, DCM/MeOH extract of *C. scabrida* showed potent anti-inflammatory effects by reducing 97.8 % of NO produced in response to LPS at 50 μg/mL without toxicity observed. Similar activity was observed from fractions 6 and 7, and two major compounds, namely 5,3′,4′-trihydroxy-3,6,7,8-tetramethoxyflavone and hautriwaic acid were identified as possible components responsible for the efficacy. Hautriwaic acid's anti-inflammatory activity is well reported, however, to the best of our knowledge, there are no literature reports on the anti-inflammatory activity of 5,3′,4′-trihydroxy-3,6,7,8-tetramethoxyflavone and it was isolated for the first time from *C. scabrida*, but similar polymethoxyflavones (PMFs) have been reported to show anti-inflammatory effects [75]. In the current study, 5,3′,4′-trihydroxy-3,6,7,8-tetramethoxyflavone showed strong anti-inflammatory properties and this compound may be used as a drug candidate in the development of a novel anti-inflammatory drug. The results are promising, however further studies are needed to fully elucidate the underlying mechanisms and explore the potential therapeutic applications of *C. scabrida* extract, its fractions, and the isolated compounds which may lead to the discovery of novel anti-inflammatory drug formulation. Future studies should specifically investigate the mechanism, including the effect on secretion of pro-inflammatory cytokines.

5. Conclusion

This study highlights the promising anti-inflammatory potential of twelve South African medicinal plants traditionally used for inflammatory conditions. Prefractionation of extracts prior to biological screening proved effective for early bio-guided identification of active compounds. Notably, 5,3′,4′-trihydroxy-3,6,7,8-tetramethoxyflavone (C1) was isolated for the first time from *C. scabrida,* alongside the known anti-inflammatory compound hautriwaic acid. Both compounds inhibited NO production in LPS-activated RAW 264.7 macrophages without cytotoxicity, indicating their therapeutic potential. Further studies are needed to fully validate the anti-inflammatory activity of these plants, elucidate their mechanisms of action, and identify additional bioactive compounds.

CRediT authorship contribution statement

Sibusiso Rali: Methodology, Investigation, Formal analysis, Data curation. **Bongiwe Mshengu:** Supervision, Methodology, Investigation. **Maryna Van De Venter:** Methodology, Formal analysis, Data curation. **Vinesh J. Maharaj:** Writing – review & editing, Validation, Supervision, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors confirm that we have all contributed in preparation of this manuscript and declare that there are no conflicts of interest related to this submission.

Acknowledgements

The authors express their gratitude to the Biodiscovery Centre, Chemistry Department at the University of Pretoria for the collection of plant material in their repository. We would like to thank the BioAssaix Screening Facility, Department of Biochemistry and Microbiology, Nelson Mandela University for in vitro bioassays. Dr. Luke Invernizzi for running of samples on UPLC-QTOF-MS instrument. Dr. Andreas Lemmerer from the University of the Witwatersrand for running single-crystal diffractometer analysis and the National Research Foundation National Equipment Programme (NEP grant 129920) for financing the purchase of the single-crystal diffractometer. This research study received support from a grant provided by the Department of Science and Innovation (DSI) of South Africa, awarded to Vinesh Maharaj. Sibusiso Rali was supported by funds from the University of Pretoria Postgraduate Research Support Bursary,

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fitote.2025.106651.

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