



In vitro antioxidant and anti-inflammatory effects of *Plantago major*, *Cannabis sativa* and *Ranunculus multifidus* on LPS-stimulated RAW 264.7 macrophages

Zandile Nxumalo^{a,b}, Buntubonke Gom^c, Nelisiwe Masuku^a, Anjo Theron^b, Tsholofelo Mokoka^a, Sechaba Bareetseng^c, Lyndy McGaw^{a,*}

^a Phytomedicine Programme, Department of Paraclinical Sciences, University of Pretoria, Private Bag X04, Onderstepoort 110, Pretoria, South Africa

^b Synthetic Nanobiotechnology and Biomachine Group, Synthetic Biology and Precision Medicine Centre, CSIR, Pretoria, South Africa

^c Advanced Agriculture and Food Cluster, CSIR, Pretoria, South Africa

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ABSTRACT

Chronic inflammation is a major contributor to cancer progression, driven by persistent pro-inflammatory signalling and oxidative stress. Plant-derived bioactive compounds offer therapeutic potential by modulating these pathways. This study evaluated the antioxidant and anti-inflammatory effects of leaf extracts from *Cannabis sativa* L., *Plantago major* L., and *Ranunculus multifidus* Forssk. in LPS-stimulated RAW 264.7 macrophages. Extracts were tested for cytotoxicity (MTT assay), nitric oxide (NO) inhibition (Griess assay), cytokine modulation (ELISA), antioxidant activity (DPPH and ABTS assays), and 15-lipoxygenase (15-LOX) inhibition. UPLC-MS analysis was used to identify potential phytochemical constituents. All extracts were non-cytotoxic at 5 - 15 µg/mL concentrations. The extract from *R. multifidus* had the highest NO inhibition (20%), followed by *P. major* (17%), while *C. sativa* exhibited no significant effect. The ELISA assay revealed extract-specific cytokine modulation. *P. major* strongly suppressed IL-6 and IL-1β and enhanced IL-10 secretion, while *C. sativa* inhibited IL-6, PTGS2 and moderately reduced iNOS. In contrast, *R. multifidus* showed variable effects and did not inhibit iNOS and IL-1β. However, it induced variable effects on IL-6 inhibition and IL-10 secretion. The antioxidant assays revealed potent radical scavenging: *C. sativa* achieved 92.5% DPPH scavenging comparable to ascorbic acid, while ABTS scavenging exceeded 90% for *P. major* and *R. multifidus*, closely matching Trolox. All extracts inhibited 15-LOX, with *C. sativa* showing the highest inhibition (96.2%). UPLC-MS identified the presence of cannabigerolic acid (CBGA) and Δ⁹-THC in *C. sativa* and verbascode in *P. major*, while no major compounds were conclusively identified in *R. multifidus*. These findings provide the first evidence of cytokine modulation and anti-inflammatory activity by *R. multifidus*. Moreover, they highlight the complementary antioxidant and anti-inflammatory potential of *C. sativa* and *P. major*, supported by phytochemical evidence. These extracts represent promising natural immunomodulatory agents.

1. Introduction

Inflammation is a fundamental cellular defence mechanism that protects the body from harmful stimuli such as pathogens, toxins, and tissue injury. Primarily, it improves cellular growth by speeding up the removal of damaged cells as well as promoting tissue repair to restore homeostasis (del Carmen Pinto et al., 2007; Xie et al., 2025). However, when this tightly regulated process becomes chronic, it contributes to the initiation, promotion, and progression of cancer. Chronic

inflammation is driven by complex signalling networks that regulate the sustained production of inflammatory mediators such as cytokines, chemokines, lipid-derived molecules and reactive oxygen and nitrogen species (Nishida and Andoh, 2025). Persistent activation of these pathways leads to excessive generation of reactive oxygen and nitrogen producing species, as well as enzyme synthesis and denaturation varying from inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX) (Rahmawati et al., 2024). These can induce DNA damage, promote genomic instability, and dysregulate cellular signalling, ultimately

* Corresponding author.

E-mail address: lyndy.mcgaw@up.ac.za (L. McGaw).

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driving uncontrolled proliferation, angiogenesis, and metastasis (Kim and Lee, 2025). As such, this work targets inflammation that has emerged as a promising strategy for cancer prevention and immunotherapy.

According to Zhao et al. (2021), inflammation and oxidative stress are closely related biological processes that play host to the initiation, progression, and maintenance of carcinogenesis. Under these conditions, the key enzymes mediating these responses include cyclooxygenases (COX-1 and COX-2) and lipoxygenases (LOX), which metabolize arachidonic acid (AA) into bioactive lipid mediators, including prostaglandins and leukotrienes (Adebayo et al., 2015). These lipid mediators orchestrate a variety of cellular responses, including immune cell recruitment, angiogenesis, and tissue remodelling (Mbhele et al., 2022). Through LOX-mediated pathways, particularly 15-LOX (Saqib et al., 2025), AA is converted to leukotrienes and lipoxins, which serve as critical modulators of immune cell trafficking and the inflammatory environment. By maintaining chronic inflammation, these mediators can influence tumour cell survival, proliferation, and metastatic potential. In view of the key enzymes, COX enzymes generate prostaglandins that actively promote inflammation, cell proliferation, and neovascularisation, which are hallmarks of tumour progression (Adebayo et al., 2015; Zhao et al., 2021). In fact, both COX and LOX pathways contribute to oxidative stress through lipid peroxidation, producing reactive oxygen species (ROS) that can directly damage DNA, promote mutagenesis, and alter cellular signalling pathways. This is important because the lipid-derived mediators engage and amplify pro-inflammatory signalling cascades, including the NF- κ B and MAPK pathways (Wang et al., 2025). Activation of these pathways leads to transcriptional upregulation of cytokines and growth factors, which can be used to remodel the microenvironments around a tumour to favour proliferation, survival, and metastasis (Issa et al., 2025). For example, NF- κ B will function as a central transcriptional regulator of iNOS, COX-2, and multiple pro-inflammatory cytokines, including IL-6 (Cheng et al., 2022). It was also noted by (Thuya et al., 2025) that excessive NO production further enhances NF- κ B activation which establishes a positive feedback loop, a key regulator of chronic inflammation. Moreover, IL-6 activates the JAK/STAT3 signalling cascade, promoting oncogenic transformation and acting synergistically with NF- κ B to maintain a tumor-supportive inflammatory microenvironment (Thuya et al., 2025). This leads to the stimulation of COX-2/5-LOX-derived lipid mediators, which further stimulate IL-6 release and iNOS expression, underscoring the intricate crosstalk between lipid mediators, cytokines, and reactive nitrogen species. These interconnected pathways demonstrate that chronic inflammation is driven by an integrated signalling network rather than a single mediator. Therefore, simultaneous evaluation of multiple biomarkers such as COX-2, 5-LOX, iNOS/NO, and IL-6 are essential for assessing anti-inflammatory and anticancer interventions.

It is well known that medicinal plants are a rich source of bioactive compounds, including flavonoids, polyphenols, and terpenoids, that exhibit antioxidant as well as anti-inflammatory properties. These phytochemicals act on multiple molecular targets to suppress pro-inflammatory mediators to restore redox balance, and to regulate cytokine signalling networks (Thuya et al., 2025; Adebayo et al., 2015; Mbhele et al., 2022; Sadiq et al., 2024; Tajdari et al., 2024). It does so by disrupting the integrated pathways that sustain chronic inflammation. Plant-derived metabolites offer promising therapeutic potential for inflammation-associated diseases, including cancer (Mbhele et al., 2022; Tajdari et al., 2024; Zhao et al., 2021). As such, this study investigates the anti-inflammatory and antioxidant effects of three medicinal plants, namely *Plantago major* L., *Cannabis sativa* L., and *Ranunculus multifidus* Forssk. traditionally used for inflammatory and wound healing conditions. Recent studies by Triastuti et al. (2022) and Zaiachuk et al. (2023) have highlighted the comparative efficacy of the plants in modulating inflammation and oxidative stress. These studies draw from previous research on *P. major* extracts which have been shown to reduce pro-inflammatory cytokines, including IL-6 and TNF- α , as well as

oxidative stress in rodent colitis models (Farid et al., 2022). Additionally, in vitro studies using human dermal fibroblasts reported enhanced antioxidant activity and wound-healing potential (Bâldea et al., 2023). The cannabinoids (CBD) and Δ 9-tetrahydrocannabinol (Δ 9-THC) found in *Cannabis sativa* have been shown to significantly suppress TNF- α , IL-1 β , and IL-6 in LPS-stimulated murine RAW 264.7 and human THP-1 macrophages (Suryavanshi et al., 2022). Although previous research has demonstrated the anti-inflammatory activity of *P. major* species and cannabinoids in *C. sativa* using various models, data on *Ranunculus multifidus* remains limited.

Existing studies by Marrelli et al. (2022) on related *Ranunculus* species indicate that plants of this genus can exert significant anti-inflammatory effects. In particular, Hong et al. (2020) have shown that methanolic extracts of *Ranunculus bulbosus* can reduce nitric oxide production and suppress the expression of key pro-inflammatory mediators, including iNOS, COX-2, IL-1 β , and IL-6, in LPS-stimulated RAW 264.7 macrophages. This inhibition is associated with downregulation of NF- κ B activation via interference with upstream signalling, including I κ B α phosphorylation (Hong et al., 2020; Marrelli et al., 2022). These findings suggest that modulation of NF- κ B and its downstream targets may represent a common mechanism through which *Ranunculus* species mediate their anti-inflammatory effects. Therefore, in this study, we used LPS-stimulated RAW 264.7 murine macrophages, a well-established in vitro model of inflammation, to evaluate the ability of these extracts to modulate key markers of inflammatory and oxidative stress. Specifically, we assessed inhibition of nitric oxide (NO), expression of inflammatory mediators including PTGS2 (COX-2), IL-6, IL-10, IL-1 β , and iNOS activity using ELISA, 15-lipoxygenase (15-LOX) activity, and antioxidant capacity through DPPH and ABTS assays. For the purpose of this study, these analyses provide biological function indicators of how these plant extracts regulate inflammation and oxidative stress processes intimately linked to tumorigenesis.

2. Methods

2.1. Protocols for plant material preparation

Plant material (leaves) were collected from the Silverton area in Pretoria, Gauteng Province, South Africa. Voucher specimens of each plant species were deposited at the HGWJ Schweickerdt Herbarium, University of Pretoria, for taxonomic identification, as summarised in Table 1. The collected plant leaves were dried in an oven at 40–50 °C. The oven-dried plant material of each plant species was ground to a powder and mixed with 500 mL mixture of dichloromethane (DCM) and methanol (MeOH) in a 1:1 ratio as the extraction solvent system and magnetically stirred for 24 h. The DCM:MeOH (1:1) solvent system was selected following preliminary extraction comparisons with ethyl acetate, methanol, and dichloromethane, as it produced the highest crude extract yield and has been shown that the combination of dichloromethane and methanol provides a broader polarity range than either solvent alone, facilitating the recovery of diverse phytochemical constituents (Abubakar and Haque, 2020). After extraction the solvent was removed using a rotary evaporator yielding dried plant extracts, which were stored at 4 °C until further scientific investigations.

2.1.1. Cell culture and chemical reagents

All cell culture reagents and consumables were obtained from Whitehead Scientific (Pty) Ltd, South Africa. Dimethyl sulfoxide (DMSO; $\geq 99.9\%$ v/v, AR grade, purchased from Minema Chemicals (Pty) Ltd, South Africa) was used as the vehicle control for dilution of plant extracts. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) for antioxidant assays was supplied by Labchem (Pty) Ltd (South Africa). Ascorbic acid and Griess reagent were obtained from Merck Life Science (Pty) Ltd. Lipopolysaccharide (LPS) from *Escherichia coli* O111:B4 was procured from Merck Life Science (Pty) Ltd. NF- κ B p65 rabbit polyclonal IgG antibody was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Alexa

Table 1Information on *P. major*, *C. sativa* and *R. multifidus* traditionally used for inflammatory related conditions.

Scientific name (Family)	Common name	Voucher specimen number	Biological activity	Phytochemistry	References
<i>Plantago major</i> L. (Plantaginaceae)	English: Broadleaf plantain isiZulu: Izindlebe zathekwane	PRU0133599	Used in wound healing and has anti-inflammatory, immunomodulatory, antioxidant properties. Has been shown to inhibit NF-κB and PTGS2; downregulates IL-6, IL-2β, iNOS, prostaglandins; upregulates IL-10.	Iridoids, flavonoids, phenolic acids, terpenoids, and alkaloids.	(Bacanek et al., 2026; Farid et al., 2022; Triastuti et al., 2022)
<i>Cannabis sativa</i> L. (Cannabaceae)	English: Cannabis / Marijuana Hemp isiZulu: Insangu	PRU0131045	Immunomodulatory; balances pro- and anti-inflammatory responses; modulates IL-6, IL-2β, iNOS, prostaglandins; upregulates IL-10 via CB2 receptor.	Cannabinoids (THC, CBD), terpenes	(Henshaw et al., 2021; Marrelli et al., 2022; Mujahid et al., 2025)
<i>Ranunculus multifidus</i> Forssk. (Ranunculaceae)	English: Buttercup isiZulu: Uxhaphozi	PRU0133600	Traditional use for wound healing, malaria, and infections; general anti-inflammatory activity inferred at genus level. No direct evidence for cytokine modulation (IL-6, iNOS, COX-2/PTGS2, IL-10).	General phytochemistry is inferred at genus level to contain ranunculin, protoanemonin, flavonoids reported in genus.	(Dai et al., 2024; Hong et al., 2020; Marrelli et al., 2022)

Fluor 488-conjugated goat anti-rabbit IgG secondary antibody was obtained from Thermo Fisher Scientific. All organic solvents used during plant extraction and phytochemical analysis, including dichloromethane (DCM) and methanol (MeOH) were high-performance liquid chromatography (HPLC) grade and supplied by Minema Chemicals (Pty) Ltd, South Africa. For the 15-lipoxygenase (15-LOX) inhibitory assay, linoleic acid ($\geq 99\%$ purity), ferrous sulfate heptahydrate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$; $\geq 99\%$), sulfuric acid (H_2SO_4 ; $\geq 95\%$), xylitol orange ($\geq 95\%$ purity), and quercetin ($\geq 95\%$) were acquired from Merck Life Science (Pty) Ltd, South Africa.

2.2. RAW 264.7 cell culture

The RAW 264.7 murine macrophage cells (ATCC® TIB-71™, part of American Type Culture Collection, Manassas, VA, USA) used in this study were provided by the Phytomedicine Laboratory, Department of Paraclinical Sciences, University of Pretoria, South Africa. Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 100 U/mL penicillin-streptomycin. Throughout the experiment, the cells were incubated at a constant condition of 37 °C with 5% CO₂ and routinely sub-cultured every 2–3 days to monitor exponential growth.

2.3. Assessment of the effects of plant extracts on cell viability using MTT assay

In order to confirm that the observed effects were not due to cytotoxicity by plant extracts, cell viability was evaluated in RAW 264.7 macrophages using the tetrazolium-based MTT assay, originally described by Mosmann (1983). Once cells had reached confluency, they were counted, and RAW 264.7 cells were seeded in 96-well plates at a density of 1×10^5 cells/well and incubated for 24 h to allow attachment. After 24 h, the old medium was replaced with fresh DMEM, and cells were pre-treated with *P. major*, *C. sativa*, and *R. multifidus* extracts at varying concentrations (5–40 µg/mL) and stimulated with LPS (1 µg/mL) for an additional 24 h. Doxorubicin and untreated cells served as positive and negative controls respectively. Following treatment, the supernatant from treated and untreated cell wells was removed and transferred to a new plate for nitric oxide (NO) analysis. Subsequently, cells were washed twice, and this was followed by the addition of 10 µL of MTT solution (5 mg/mL) per well and incubated for 1–2 h at 37 °C. Formazan crystals were solubilised with 100 µL of DMSO and absorbance was measured at 570 nm using a BioTech Gen-5 microplate reader. All experiments were conducted in three independent biological repetitions or replicates. The IC₅₀ value, representing the concentration that reduces cell viability by 50%, was determined using the following

formula.

$$\text{Cell viability (\%)} = \left\{ \frac{(\text{OD sample} - \text{OD untreated control})}{(\text{OD untreated control})} \right\} \times 100$$

2.4. Assessment of the effects of plant extracts on nitric oxide (NO) release

The concentration of nitrite released from macrophages was quantified using the Griess reagent assay protocol adapted from Ondua et al. (2019) and Lebeloane et al. (2024). Briefly, RAW 264.7 cells were seeded in 96-well microplates at a density of 1×10^5 cells/well and incubated for 24 h to allow adherence. These cells were then treated with 100 µL of plant extracts at concentrations ranging from 5 to 40 µg/mL and incubated for 1 h at 37 °C with 5% CO₂. Following 1 h incubation, 50 µL of lipopolysaccharide (LPS, 1 µg/mL in Dulbecco's Modified Eagle Medium [DMEM]) were added in each well except for the untreated control and quercetin-treated positive control, and plates were incubated for an additional 24 h. Collected supernatants (50 µL) were mixed with an equal volume of Griess reagent (1% sulphanilamide and 0.1% N-(1-naphthyl) ethylenediamine in 2.5% H₃PO₄) in 96-well plates and incubated at room temperature for 10 min. This was followed by absorbance reading at 550 nm using a microplate reader (Biotek, USA). The percentage inhibition of nitric oxide production was calculated relative to the lipopolysaccharide (LPS)-stimulated control.

2.5. UPLC-QTOF-MS/MS analysis of *P. major*, *C. sativa* and *R. multifidus* extracts

Prior to the ultra-performance liquid chromatography analysis, *P. major*, *C. sativa* and *R. multifidus* extracts were reconstituted in high-performance liquid chromatography (HPLC) grade methanol to a final concentration of 1 mg/mL for each extract, then centrifuged at 10 000 rpm for 2 min. Phytochemical profiling was carried out using ultra-performance liquid chromatography coupled to a Waters® Synapt G2 quadrupole time-of-flight mass spectrometer (UPLC-QTOF-MS/MS). The compounds were separated on a Waters BEH C18 column with dimensions 2.1 mm x 100 mm x 1.7 µm, particle size) which was thermostatically controlled at 40 °C. The mobile phase consisted of two chemical components: (1): Solvent A (0.1% formic acid in ultra-pure grade water) and (2): Solvent B (acetonitrile with 0.1% formic acid). A gradient elution was employed to effectively separate the diverse polarities of metabolites of the two solvents, beginning at 3% B for 0.1 min, linearly increasing to 100% B over 13.9 min (beginning at 0.1–14.0 min), the system was then held at 100% B until 16 min before returning to the initial condition of 3% B over 0.5 min and re-equilibrating until 20

min. A constant flow rate of 0.400 mL/min was maintained throughout the experiment cycle or run, and a sample injection volume of 5 µL was used for all the analyses. Following this, a Waters Synapt G2 high definition QTOF mass spectrometer equipped with an Electrospray Ionization (ESI) source enabled data acquisition in both positive and negative ion modes. All the instrument operations, including data acquisition, were managed using the MassLynx V 4.1 software (Waters Inc., manufactured in Milford, Massachusetts, USA). The chemical profiling was achieved by the tentative identification of compounds based on high-resolution for accurate mass data. Molecular formulas were initially generated using the MassLynx software that quantifies the precursor ion mass and isotopic distribution patterns (known as iFit value). Following this, compound identity was then tentatively assigned by comparing the experimentally obtained MS/MS fragmentation patterns with literature data and known spectral libraries. Comprehensive identification, for known compounds, involved cross-referencing against multiple online databases such as PubChem, Chempid, The Dictionary of Natural Products, MassBank, and Meffusion, which provided the basis for correlating the chemical profile of each plant extract with the observed in vitro biological activities.

2.6. Assessment of the effects of plant extracts on 15-lipoxygenase (15-LOX)

The lipoxygenase (LOX) inhibitory assay was used to measure enzyme conversion from linoleic acid to linoleic hydroperoxide in the presence of LOX (Gala Marti et al., 2023). The resulting hydroperoxide oxidises Fe²⁺ ions in the FOX reagent (Ferrous Oxidation Xylenol Orange) to a trication Fe³⁺, which forms a coloured complex with acidified xylenol orange, exhibiting maximal absorbance at 560 nm (Adebayo et al., 2015). The assay principle is that plant extracts containing LOX inhibitors will reduce the formation of Fe³⁺ dye complex, providing a quantitative measure of enzymatic inhibition. The assay was carried out as described previously by del Carmen Pinto et al. (2007), with modifications adapted from later studies (Adebayo et al., 2015; Lebeloane et al., 2024). Essentially, in a 96 well plate, 40 µL of the 15-LOX enzyme was pre-incubated with 20 µL of plant extract (final concentration of 1 mg/mL) at room temperature (37 °C) for 5 min, followed by the addition of 40 µL of linoleic acid (at a final concentration of 140 µM) in Tris-HCL buffer (50 mM, pH 7.4) to all wells, excluding blanks. The reaction mixtures of LOX inhibition and linoleic hydroperoxide precipitation were incubated at 25 °C in the dark for 20 min. Subsequently, 40 µL of FOX reagent comprising 30 mM sulfuric acid, 100 mM xylenol orange, and 100 mM ferrous sulphate in a solution of methanol:water (9:1) was added to the end of each reaction. The negative control wells excluding blanks contained only enzyme and buffer, while quercetin (final concentration 1000 µg/mL) served as a positive control. After 30 min incubation, linoleic acid was added to the blanks, and the Fe³⁺ xylenol orange complex absorbance was measured at 560 nm using a microplate reader. The LOX inhibitory activity was quantified as the percentage inhibition of hydroperoxide formation according to the following formula:

$$\text{LOX - 15 Inhibition (\%)} = \left\{ \frac{(AC - AB) - (AS - AB)}{(AC - AB)} \right\} \times 100$$

Where: AC = Control only absorbance; AS = Sample treated with plant extract absorbance; AB = Blank

2.7. The ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) radical scavenging assay

The antioxidant capacity of each plant extract was evaluated using the ABTS [2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)] assay, as described by (Re et al., 1999) and modified by (Lebeloane et al., 2024). Briefly, in a 96-well microplate, 40 µL of methanol was added to

each well, followed by 40 µL of plant extract (at a concentration of 1000 µg/mL) in the first three wells, then serially diluted across the 96-well microplate. A 160 µL aliquot of ABTS solution was added to all wells and plates were incubated in the dark at room temperature (37 °C) for 30 min. Methanol served as a negative control, wells containing extracts without ABTS acted as blanks and Trolox (1000 µg/mL) was used as positive control. Absorbance was measured at 734 nm, and all assays were performed in triplicate. Percentage radical scavenging activity (%) was calculated based on the reduction in absorbance relative to the control of Trolox. The percentage of ABTS radical scavenging was calculated using the formula below:

% ABTS Radical Scavenger Activity

$$= \left\{ \frac{(\text{Absorbance Control} - \text{Absorbance Sample})}{\text{Absorbance Control}} \right\} \times 100$$

The 50% inhibitory concentration (IC₅₀) of each extract was calculated by fitting a non-linear regression curve of percentage radical scavenging activity versus the logarithm of sample concentration. All the assays were performed in triplicate and results were expressed as mean ± standard error of the mean (SEM).

2.8. The DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging assay

The antioxidant activity of *P. major*, *C. sativa* and *R. multifidus* extracts was evaluated using the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay adapted from Brand-Williams et al. (1995). This method uses 40 µL methanol that is added to each well of a 96-well microplate, followed by 40 µL of each plant extract (at a final concentration of 1000 µg/mL) diluted serially across the extract wells. Followed by adding 160 µL aliquot of DPPH solution to all column wells, except for the methanol blanks. Ascorbic acid (1000 µg/mL) served as positive control, and methanol as the negative control. The plates were incubated for 30 min in the dark at room temperature, and absorbance was read at 517 nm, with a calibrated optical density (OD) 0.91 - 0.99 reading of the DPPH solution using a Biotek microplate reader, USA. All measurements were performed in triplicate (n = 3) and results were presented as mean ± standard error of the mean (SEM). Accordingly, percentage scavenger activity was determined using the following equation:

% DPPH Radical Scavenging Activity

$$= \left\{ \frac{(\text{OD DPPH} - \text{OD Sample})}{\text{OD DPPH}} \right\} \times 100$$

2.9. Quantification of cytokine (PTGS2, IL-6, IL-10, IL-1β, iNOS) expression in LPS-stimulated RAW 264.7 macrophages using enzyme-linked immunosorbent assay (ELISA)

This assay aimed to assess the ability of *P. major*, *C. sativa*, and *R. multifidus* extracts to modulate LPS-induced inflammatory mediator production in RAW 264.7 macrophages. As indicated in Section 2.4, the RAW 264.7 murine macrophages were seeded at a density of 1 × 10⁵ cells/well and pre-treated with *P. major*, *C. sativa*, or *R. multifidus* extracts (15 µg/mL) for 1 h. The concentration of 15 µg/mL was determined to be non-cytotoxic to RAW 264.7 macrophages. Quercetin served as a positive control. Following the 1 h incubation, cells were then stimulated with lipopolysaccharide (LPS) (1 µg/mL) and incubated for a further 24 h at 37 °C in a humidified incubator with 5% CO₂. Following 24 h incubation, culture supernatants were harvested for each plant treatment and clarified by centrifugation at 3000 × g for 10 min. This was followed by the quantification of each plant extract's effect on Prostaglandin - Endoperoxide Synthase 2 (PTGS2), Interleukin-6 (IL-6), Interleukin-10 (IL-10), Interleukin-1 beta (IL-1β), and inducible nitric oxide synthase (iNOS) using the sandwich ELISA kits (Elabscience®) as directed by the manufacturer's instructions. Standards and sample

preparations were run in duplicate, and absorbance values were recorded at 450 nm. In turn, standard curves were generated from serial dilutions of the supplied standards for each cytokine, and sample concentrations were interpolated using curve-fitting. Lastly, percent inhibition for each mediator was calculated relative to the LPS-only control using the following equation:

$$\% \text{ Inhibition} = \left\{ \frac{(\text{LPS control} - \text{Treatment})}{\text{LPS control}} \right\} \times 100$$

2.10. Immunofluorescence visualisation of NF-κB nuclear localisation in LPS-stimulated macrophages

Immunofluorescence staining was performed to evaluate the sub-cellular localisation of NF-κB in LPS-stimulated RAW 264.7 macrophages as described by [Hankittichai et al. \(2020\)](#). Cells were cultured in 24-well plates and pretreated for 3 h with *C. sativa*, *P. major*, or *R. multifidus* extracts. Following pretreatment, cells were stimulated with lipopolysaccharide (LPS, 1 µg/mL) for 24 h to induce NF-κB activation. Untreated cells served as the negative control, while LPS-stimulated cells without extract treatment served as the inflammatory activation control. After stimulation, cells were fixed with 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) in phosphate-buffered saline (PBS) for 15 min at 37 °C, followed by three Phosphate Buffered Saline (PBS) washes (5 min each). Permeabilization was achieved using 0.3% Triton X-100 in PBS for 5 min, after which the cells were rinsed and blocked with 1% bovine serum albumin (BSA) in PBS for 1 h at room temperature to prevent nonspecific binding. Cells were then incubated overnight at 4 °C with a rabbit anti-NF-κB p65 primary antibody (1:400). After incubation, cells were washed three times with phosphate buffer saline (PBS) and thereafter, samples were incubated for 2 h in the dark at room temperature with Alexa Fluor 488-conjugated anti-rabbit IgG (1:500) and counterstained with Hoechst 33,342 (10 µg/mL) for nuclear visualization. Subsequently, the 24-well plates were washed three times with PBS and fluorescence images were captured using a BioTek Cytation 5 Cell Imaging MultiMode Microplate Reader.

3. Results

3.1. Lipoxigenase (15-LOX) inhibition by plant extracts

The anti-inflammatory potential of *Cannabis sativa*, *Plantago major*, and *Ranunculus multifidus* leaf extracts was evaluated by assessing their ability to inhibit 15-lipoxygenase (15-LOX) enzymatic activity using the xylenol oxidation assay. All dichloromethane/methanol (DCM/MeOH) extracts exhibited a concentration-dependent inhibition of 15-LOX activity. However, these inhibitory effects did not reach statistical significance when compared with the quercetin positive control across all treatments (one-way ANOVA, $p > 0.05$), indicating limited inhibitory potency under the experimental conditions. Among the extracts tested, *C. sativa* demonstrated the strongest inhibitory activity against 15-LOX, with an IC_{50} value of 4.7 µg/mL, followed by *P. major* ($IC_{50} = 15.2$ µg/mL). In contrast, *R. multifidus* exhibited weaker inhibition, with an IC_{50} value of 63 µg/mL (Table 2). Although the differences were not statistically significant, *C. sativa* and *P. major* consistently showed greater inhibitory effects on 15-LOX activity compared to *R. multifidus* across the tested concentration range.

3.2. ABTS and DPPH radical scavenging antioxidant activity

The antioxidant potential of the extracts was assessed using ABTS and DPPH radical scavenging assays because these assays are well-established, reproducible, and allow comparison of hydrogen-donating and electron-transfer-based antioxidant mechanisms. Among the three extracts tested, *C. sativa* exhibited the strongest radical scavenging

Table 2

Anti-inflammatory, antioxidant, and cytotoxic activities of *C. sativa*, *P. major*, and *R. multifidus* leaf extracts. DPPH and ABTS results are expressed as IC_{50} values (µg/mL). IC_{50} values were calculated independently for each biological replicate using linear regression ($y = mx + c$) and are presented as mean ± SD ($n = 3$). Statistical analysis was performed using two-way ANOVA followed by Dunnett's test post hoc multiple-comparison tests, with significance accepted at $p < 0.05$. Ascorbic acid and Trolox served as positive controls for the DPPH and ABTS assays, respectively. Quercetin was used as the positive control for nitric oxide and 15-LOX inhibition assays, while doxorubicin served as the positive control for cytotoxicity. Nitric oxide inhibition was calculated relative to the LPS-treated control, and cytotoxicity relative to untreated cells.

Extract (DCM: MeOH)	%NO inhibition IC_{50} (µg/mL)	% Cell viability IC_{50} (µg/mL)	DPPH IC_{50} (µg/mL)	ABTS IC_{50} (µg/mL)	15-LOX IC_{50} (µg/mL)
<i>C. sativa</i>	27.33 ± 1.27 ($P = 0.09$)	39.02 ± 3.4 ns	147 ± 0.001 0227 ($P = 0.001$)	13 ± 0.001 0,03 ($P = 0.001$)	0004 ± 0.0064 ns
<i>P. major</i>	25.51 ± 5.17 ($P = 0.02$)	38.94 ± 3.86 ns	87 ± 0.001 0151 ($P = 0.001$)	8 ± 0.001 0016 ($P = 0.001$)	0040 ± 0.0135 ns
<i>R. multifidus</i>	31.97 ± 4.4 ($P = 0.01$)	39.02 ± 3.40 ns	265 ± 0.001 0386 ($P = 0.001$)	147 ± 0.001 0240 ($P = 0.001$)	0062 ± 0.0247 ($P = 0.02$)
Quercetin	31.92 ± 8.28 ($P = 0.08$)		ND		0003 ± 0.0060
Doxorubicin	ND	33.78 ± 3.86 ns	ND	ND	ND
Ascorbic acid	ND	ND	1 ± 0.001	ND	ND
Trolox	ND	ND	ND	2 ± 0.003	ND

ND: Not Determined, ns- $(P > 0.05)$.

activity, with IC_{50} values of 8.1 µg/mL (ABTS) and 87 µg/mL (DPPH). This suggests that *C. sativa* contains a high proportion of active antioxidant constituents, likely linked to its cannabinoid and polyphenolic profile. However, *P. major* also demonstrated notable antioxidant capacity, with IC_{50} values of 13 µg/mL (ABTS) and 147.2 µg/mL (DPPH), which are only slightly higher than those of *C. sativa*. These results indicate that *P. major* possesses strong radical scavenging properties, possibly due to its rich phenolic and flavonoid content, including verbascoside, which is known for potent antioxidant activity. In contrast, *R. multifidus* showed comparatively weaker antioxidant activity, with IC_{50} values of 147 µg/mL (ABTS) and 265 µg/mL (DPPH). Although less active than the other two extracts, these values still reflect measurable antioxidant potential, which may be attributed to flavonoids and phenolic compounds reported at the genus level. The variability in IC_{50} values between ABTS and DPPH across all extracts highlights differences in compound polarity and antioxidant mechanisms, suggesting that each plant extract contains a unique profile of bioactive constituents contributing to its overall antioxidant effect.

3.3. In vitro cell viability of three plant extracts in RAW 264.7 murine macrophages

The in vitro cytotoxic effects of *C. sativa*, *P. major* and *R. multifidus* on RAW 264.7 macrophages were evaluated after 24-h incubation with increasing extract concentrations (5 - 40 µg/mL). Cell viability was assessed using the MTT assay and expressed as a percentage relative to the untreated control. Overall, all extracts were largely non-toxic at lower concentrations (5 - 15 µg/mL), maintaining cell viability between 78% and 100% (Fig. 1). This indicates that these concentrations are suitable for subsequent anti-inflammatory and antioxidant assays without compromising cell health. At higher concentrations (20 - 40 µg/

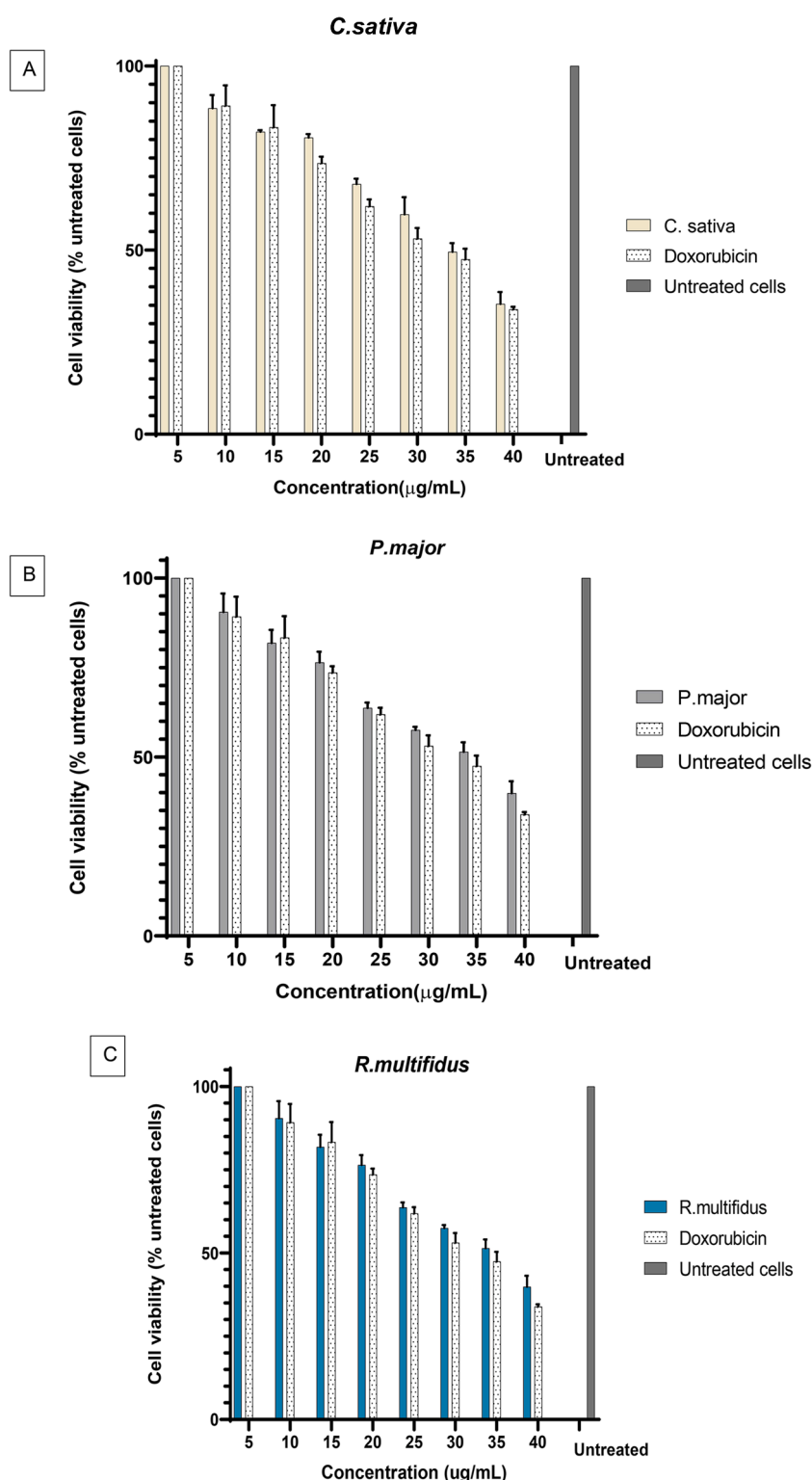


Fig. 1. Cell viability of RAW 264.7 macrophages treated with increasing concentrations (5–40 µg/mL) of (A) *Cannabis sativa*, (B) *Plantago major*, and (C) *Ranunculus multifidus* extracts or doxorubicin, and the MTT assay was used to assess cell viability relative to untreated control cells (100%). Doxorubicin was included as a positive control. Data represent mean $\pm$ SEM from three independent experiments, each performed in triplicate ($n = 3$). Statistical analysis was performed using one-way ANOVA followed by Dunnett's post hoc test, with comparisons made against untreated control cells. No statistically significant differences were observed (ns, $p > 0.05$).

mL), a concentration-dependent decrease in viability was observed, reflecting low to moderate cytotoxicity. Among the extracts, *R. multifidus* showed the lowest IC_{50} (1.34 µg/mL), followed by *P. major* (1.42 µg/mL) and *C. sativa* (2.25 µg/mL). Although these IC_{50} values provide insight into cytotoxic thresholds, they were not used for further

experiments because they fall within ranges that significantly reduce cell viability. Instead, non-cytotoxic concentrations (≤ 15 µg/mL) were selected for mechanistic studies to ensure observed effects were not confounded by cell death.

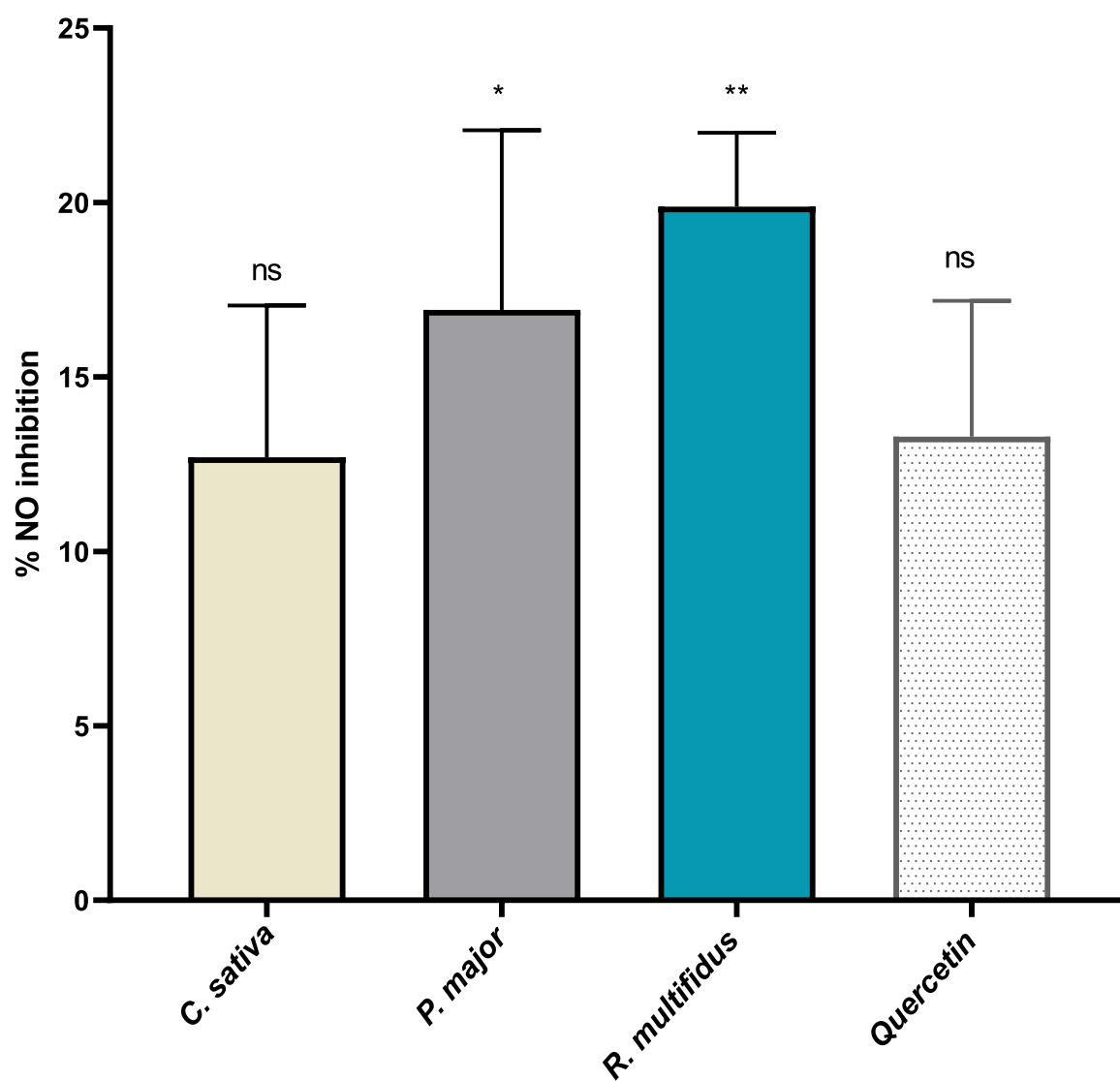


Fig. 2. Inhibitory effects of *C. sativa*, *P. major* and *R. multifidus* extracts on lipopolysaccharide (LPS)-induced nitric oxide (NO) production in RAW 264.7 macrophages. NO levels are expressed as percentages relative to LPS-treated cells, which served as the inflammatory baseline control. Data represent mean $\pm$ SEM from three independent experiments, each performed in triplicate ($n = 3$). Statistical significance was determined using one-way ANOVA followed by Dunnett's post hoc test, * $p < 0.02$; ** $p < 0.001$; ns, not significant.

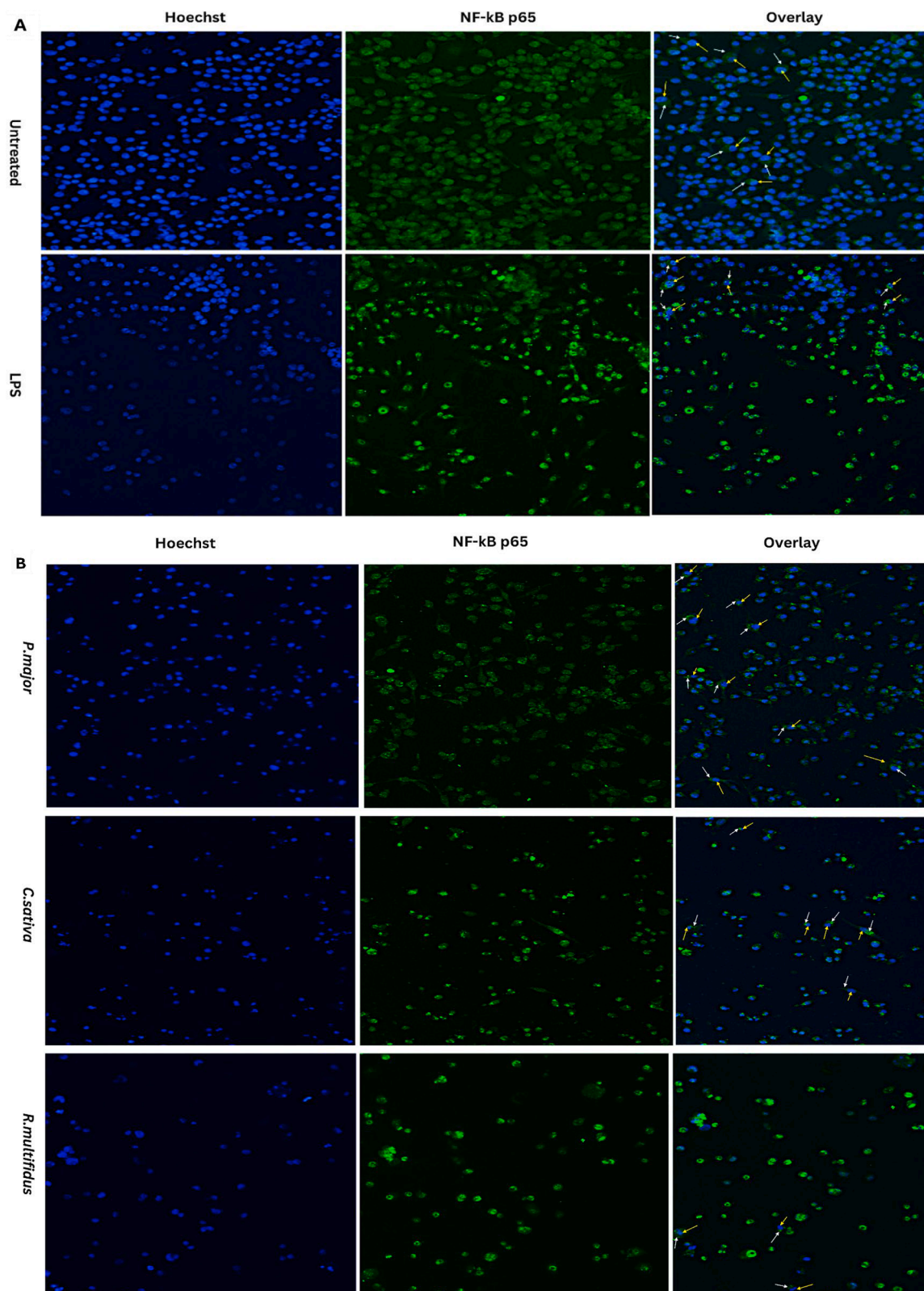


Fig. 3. Effects of *Plantago major*, *Cannabis sativa* and *Ranunculus multifidus* extracts on NF-κB p65 nuclear translocation in LPS-stimulated RAW 264.7 macrophages. Scale bar = 200 μm. Representative immunofluorescence images showing NF-κB p65 staining (green), nuclei stained with Hoechst 33,342 (blue), and merged overlay images in RAW 264.7 macrophages. (A) Untreated control cells and LPS-stimulated cells. (B) LPS-stimulated cells pretreated with *Plantago major*, *Cannabis sativa*, or *Ranunculus multifidus* extracts. Images were acquired at 10 × magnification and are representative of three independent experiments. Yellow arrows indicate nuclei, while white arrows indicate cytoplasmic localisation of NF-κB p65.

Table 3

ELISA quantification of iNOS, IL-6, IL-1 β , PTGS2, and IL-10 modulation by plant extracts in LPS-stimulated RAW 264.7. Data (n = 2) and are expressed as percentage inhibition relative to LPS control. Values are presented as mean $\pm$ SD (n = 3). Statistical analysis was performed using ordinary one-way ANOVA followed by Dunnett's multiple comparison test. Differences were considered statistically significant at $p < 0.05$.

Extract	iNOS (%)	IL-6 (%)	IL-1 β (%)	PTGS2 (%)	IL-10 (%)
<i>P. major</i>	49.02	68.10	82.58	6.99	−62.67
	ns	*	ns	**	ns
<i>C. sativa</i>	−13.73	71.33	68.58	6.84	−7.52
	ns	*	ns	**	ns
<i>R. multifidus</i>	15.69	−12.90	62.78	−1.19	−20.06
	ns	ns	ns	ns	ns
Quercetin	100.00	100.00	86.93	14.17	−127.86
	ns	**	ns	***	ns

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and ns- not significant.

3.4. Inhibitory effects of nitric oxide production of the three plant extracts

The anti-inflammatory potential of *C. sativa*, *P. major* and *R. multifidus* was assessed by measuring nitric oxide (NO) inhibition and quantified using the Griess assay in LPS-stimulated RAW 264.7 macrophages. In order to avoid cytotoxicity-related bias, NO inhibition was evaluated at concentrations of 15 $\mu\text{g/mL}$, where cell viability remained at an average of about 80%. In turn, quercetin, a well-characterised flavonoid, served as a positive control. All three dichloromethane: methanol (DCM:MeOH) extracts moderately suppressed NO production in a dose-dependent manner. At 15 $\mu\text{g/mL}$, *C. sativa*, *P. major* and

R. multifidus reduced NO production by 12.7%, 17%, and 20%. Although *R. multifidus* and *P. major* showed the strongest inhibition, these effects were not statistically significant across all extracts. When compared with the quercetin positive control, *P. major* ($25.51 \pm 5.17 \mu\text{g/mL}$) and *C. sativa* ($27.33 \pm 1.27 \mu\text{g/mL}$) showed slightly lower IC_{50} values for nitric oxide inhibition, suggesting inhibitory activity broadly comparable to quercetin (see Fig. 2). In contrast, *R. multifidus* exhibited an IC_{50} value ($31.97 \pm 4.40 \mu\text{g/mL}$) similar to that of quercetin ($31.92 \pm 8.28 \mu\text{g/mL}$), as shown in Table 2.

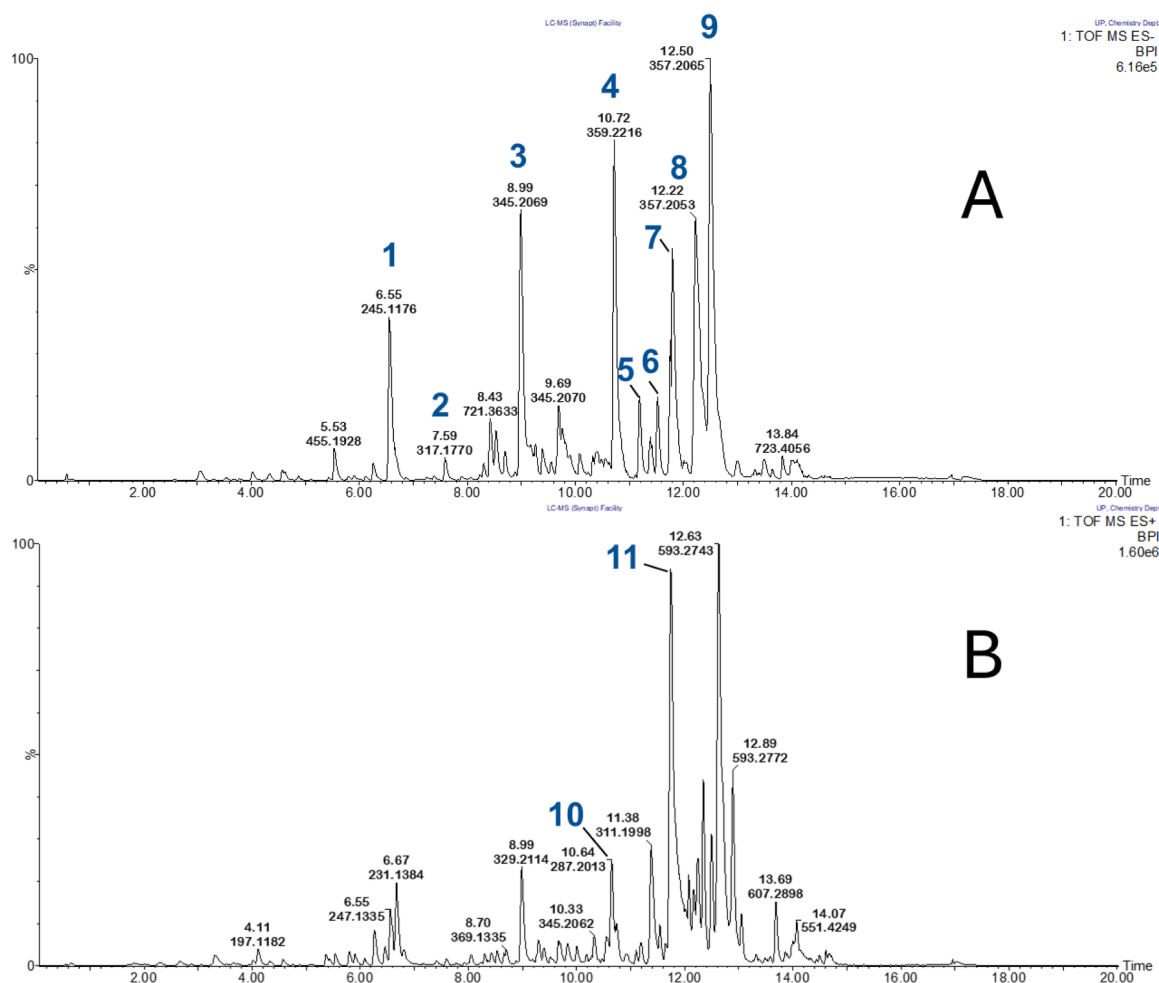


Fig. 4. UPLC-MS chromatogram of *Cannabis sativa* DCM: MeOH extract (1:1), (A) ESI negative mode and (B) ESI positive mode; compounds identified numbered 1–11.

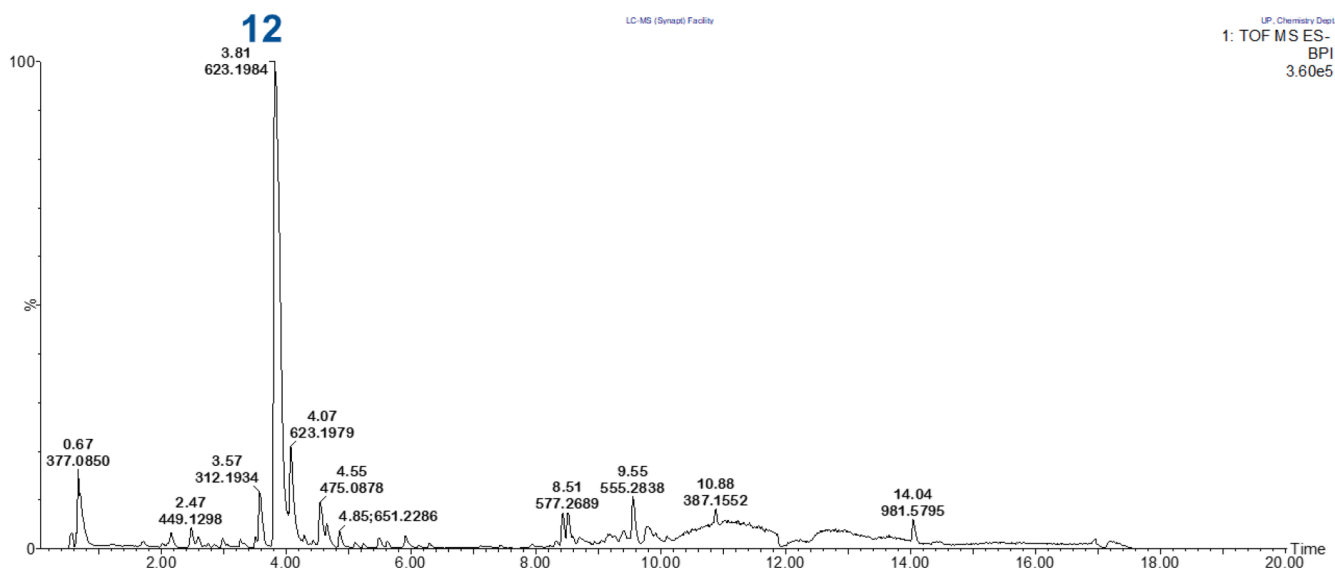


Fig. 5. UPLC-MS chromatogram of the *Plantago major* DCM: MeOH (1:1) extract in ESI negative mode.

3.5. Effects of plant extracts on NF- κ B localisation in LPS-stimulated RAW 264.7 cells

The ELISA analysis revealed that all three plant extracts moderately regulated the protein expression of key pro-inflammatory and anti-inflammatory mediators in LPS-stimulated RAW 264.7 macrophages. To further clarify these findings and understand the underlying mechanism of action by which the extracts regulate inflammatory responses, NF- κ B localisation was evaluated. This is important because NF- κ B is a central transcription factor that regulates the expression of many pro-inflammatory genes, including cytokines (IL-6, IL-1 β), enzymes (iNOS, COX-2), and other inflammatory mediators. Notably, its activity is controlled by its subcellular localisation. Therefore, immunofluorescence staining was performed to examine the subcellular localisation of the NF- κ B p65 subunit in activated RAW 264.7 macrophages. Specifically, NF- κ B p65 localisation was visualised by immunofluorescence staining (green) to detect its intracellular distribution, while nuclei were counterstained with Hoechst 33,342 (blue) to identify nuclear regions and facilitate assessment of NF- κ B nuclear translocation, as shown in Fig. 3. In LPS-only controls, NF- κ B p65 exhibited strong nuclear accumulation, confirming activation of inflammatory signalling pathways, whereas untreated cells exhibited predominantly cytoplasmic retention, indicative of basal NF- κ B activity. Pretreatment with *C. sativa* and *P. major* extracts markedly reduced LPS-induced nuclear translocation of NF- κ B p65, with *C. sativa* showing pronounced cytoplasmic retention and *P. major* demonstrating moderate inhibition. These findings suggest that the extracts suppress NF- κ B-mediated inflammatory signalling, thereby limiting downstream expression of pro-inflammatory mediators. In contrast, treatment with *R. multifidus* did not substantially inhibit p65 nuclear localisation, with localisation patterns comparable to LPS-only controls.

3.6. IL-6, IL-10, IL-1 β and iNOS in LPS-stimulated RAW 264.7 macrophages

The anti-inflammatory potential of *C. sativa*, *P. major* and *R. multifidus* was quantified using ELISA analysis to evaluate their effects on inflammatory mediator release in LPS-stimulated RAW 264.7 macrophages. The inflammatory mediators IL-6, IL-1 β , IL-10, inducible nitric oxide synthase (iNOS), and prostaglandin-endoperoxide synthase 2 (PTGS2) were quantified using the sandwich ELISA method and expressed as percentage inhibition relative to the LPS control. Positive values indicate inhibition of cytokine or mediator expression relative to

the LPS control, whereas negative values indicate that the extract did not inhibit, but instead permitted or enhanced mediator secretion (see Table 3). The results presented in Table 3 show that *P. major* inhibited iNOS (49%), IL-6 (68%), and IL-1 β (82%), while enhancing secretion of the anti-inflammatory cytokine IL-10 (–62.7%) and exerting minimal effect on PTGS2. *C. sativa* inhibited IL-6 (71.33%) and IL-1 β (68%), with comparatively weak inhibition of iNOS (13.7%) and little effect on PTGS2, while also modestly enhancing IL-10 secretion (–7.5%). In contrast, *R. multifidus* moderately inhibited IL-1 β (62%) and iNOS (15%), but increased IL-6 (–12.90%) and PTGS2 (–1.19%) levels, while also enhancing IL-10 secretion (–20.1%). Quercetin, used as a positive control, markedly suppressed all measured pro-inflammatory mediators and strongly enhanced IL-10 secretion (–127.9%).

3.7. UPLC-MS analysis of *C. sativa* and *P. major*

The UPLC chromatograms of the DCM: MeOH (1:1) extracts from the leaves of *C. sativa* (Fig. 4) and *P. major* (Fig. 5) show the major compounds depicted as peaks (1–12), generated from the extracts of these plant species. For example, in Fig. 4A and 4B, for *Cannabis sativa*, the tentatively identified cannabinoids (Tables 4 and 5) include 2,3-didehydro- β -cannabispnanol (peak 1, at 6.55 min [min]), cannabimovone (peak 3, at 8.99 min), cannabigerolic acid (CBGA, peak 4, at 10.72 min), cannabivarinic acid and cannabidivarinic acid [peaks 5 (at 11.16 min) and 6, (at 11.50 min)], tetrahydrocannabivarin (peak 10, at 10.64 min), and Δ^9 -tetrahydrocannabinol (Δ^9 -THC, peak 11, at 11.70 min). These compounds are likely the principal contributors to the observed anti-inflammatory and antioxidant properties of the plant extract, based on their known pharmacological activities reported in literature (Blebea et al., 2024). In *Plantago major*, the principal identified compound was verbascoside (Peak 12), a well-known phenylethanoid glycoside, which was the highest peak observed at a retention time of 3.77 min (see Table 6). In this study, the UPLC-MS identification yielded no conclusive identification of phytochemical compounds in *R. multifidus* extract (Fig. 6). This outcome is likely due to the lack of prior comprehensive phytochemical profiling for this species, and as such, limits the availability of validated reference spectra and structural annotations needed to progress from tentative to confirmed identifications. As a result, we were unable to make robust chemical inferences from the chromatographic features observed.

Table 4Tentative identification of phytochemicals in DCM:MeOH crude extract of *Cannabis sativa* in ESI negative mode.

Peak #	Retention time (min)	Acquired [M - X] ⁻ m/z	Adduct	Mass error (ppm)	Formula of possible structure	Theoretical [M - X] ⁻ m/z	Calculated accurate mass (Da)	Possible Compounds	Possible Structure
1	6.55	245.1177	[M - H] ⁻	-0.4	C ₁₅ H ₁₈ O ₃	245.1178	246.125,595	2,3-Didehydro beta-Cannabispiranol	
2	7.59	317.1763	[M - H] ⁻	3.2	C ₁₉ H ₂₆ O ₄	317.1753	318.183,110	(3aR,4R,5aR,6R,9aS,9bR)-6-hydroxy-5a-methyl-3,9-dimethylidene-4-(2-methylpropoxy)-3a,4,5,6,9a,9b-hexahydrobenzo[g][1]benzofuran-2-one	
3	8.99	345.2069	[M - H] ⁻	0.5	C ₂₁ H ₃₀ O ₄	345.2066	346.214,410	Cannabimovone	
4	10.72	359.2223	[M - H] ⁻	0.3	C ₂₂ H ₃₂ O ₄	359.2222	360.230,060	Cannabigerolic acid	
5	11.16	329.1759	[M - H] ⁻	1.8	C ₂₀ H ₂₆ O ₄	329.1753	330.183,110	Cannabivarinic acid	
6	11.50	329.1750	[M - H] ⁻	-0.9	C ₂₀ H ₂₆ O ₄	329.1753	330.183,110	Cannabidivarinic acid	

(continued on next page)

Table 4 (continued)

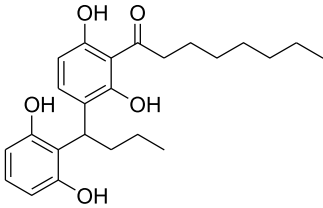
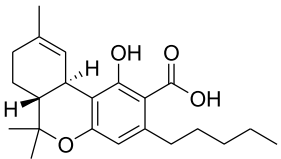
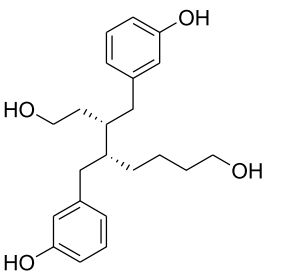
Peak #	Retention time (min)	Acquired [M - X] ⁺ m/z	Adduct	Mass error (ppm)	Formula of possible structure	Theoretical [M - X] ⁺ m/z	Calculated accurate mass (Da)	Possible Compounds	Possible Structure
7	11.73	399.2164	[M - H] ⁺	−1.8	C ₂₄ H ₃₂ O ₅	399.2171	400.224,975	1-[3-[1-(2,6-Dihydroxyphenyl)butyl]-2,6-dihydroxyphenyl]-1-octanone	
8	12.22	357.2062	[M - H] ⁺	−1.1	C ₂₂ H ₃₀ O ₄	357.2066	358.214,410	(8R,9S,10R,13S,14S,17S)-17-(2-hydroxyethyl)-10,13-dimethyl-3-oxo-2,8,9,11,12,14,15,16-octahydro-1H-cyclopenta[a]phenanthrene-17-carboxylic acid	
9	12.50	357.2065	[M - H] ⁺	−0.3	C ₂₂ H ₃₀ O ₄	357.2066	358.214,410	(3S,4R)-3,4-bis[(3-hydroxyphenyl)methyl]octane-1,8-diol	

Table 5Tentative identification of phytochemicals in DCM: MeOH crude extract of *Cannabis sativa* in ESI positive mode.

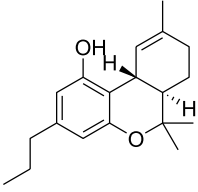
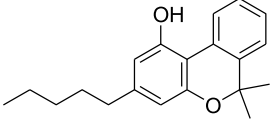
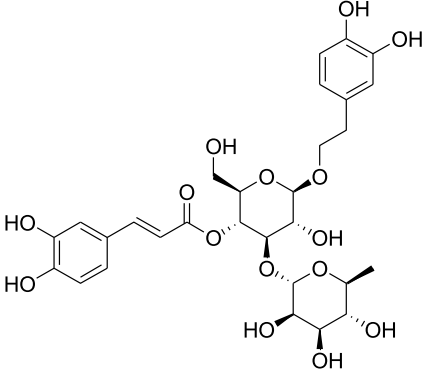
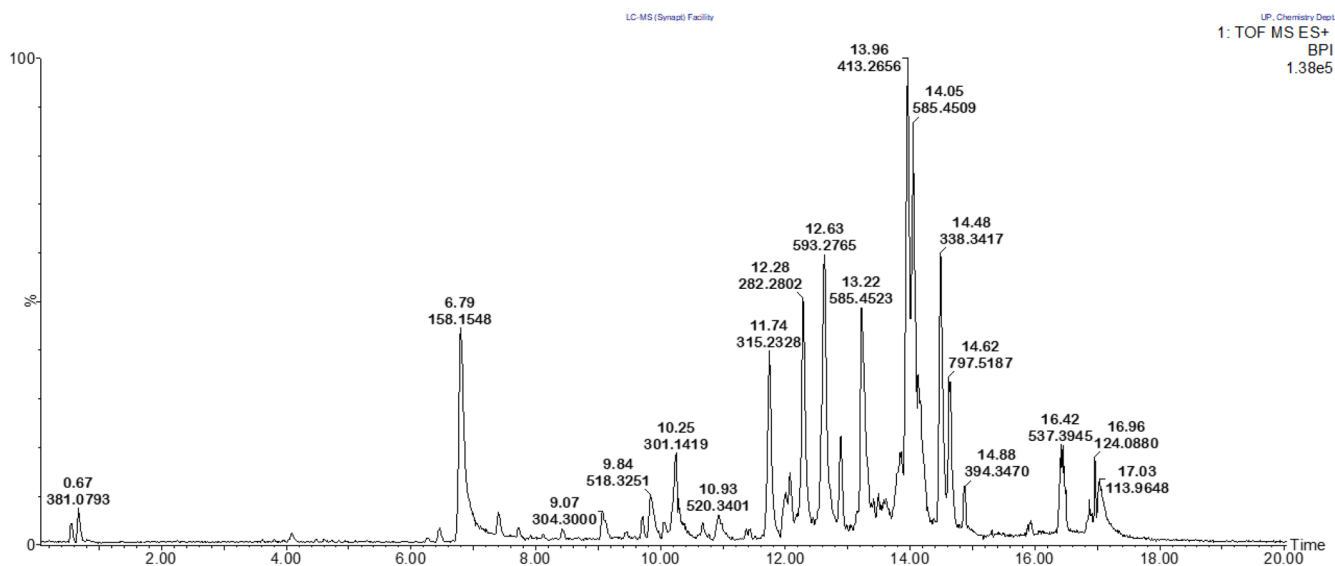
Peak #	Retention time (min)	Acquired $[M + X]^+$ m/z	Adduct	Mass error (ppm)	Formula of a possible compound	Theoretical $[M + X]^+$ m/z	Calculated accurate mass (Da)	Possible Compound	Possible Structure
10	10.64	287.2015	$[M + H]^+$	1.4	$C_{19}H_{26}O_2$	287.2011	286.193,280	Tetrahydrocannabivarin	
11	11.70	315.2318	$[M + H]^+$	-1.9	$C_{21}H_{30}O_2$	315.2324	314.224,580	Δ^9 -Tetrahydrocannabinol	

Table 6Tentative identification of phytochemicals in the DCM:MeOH (1:1) extract of *Plantago major* analysed in the negative ESI mode.

Peak #	Retention time (min)	Acquired $[M - X]^-$ m/z	Adduct	Mass error (ppm)	Formula of possible structure	Theoretical $[M - X]^-$ m/z	Calculated accurate mass (Da)	Possible compound	Possible structure
12	3.77	623.1980	$[M - H]^-$	1.0	$C_{29}H_{36}O_{15}$	623.1976	624.205,425	Verbascoside	

**Fig. 6.** UPLC-MS chromatogram of *Ranunculus multifidus* DCM: MeOH (1:1) extract in ESI positive mode.

4. Discussion

The present study investigated the antioxidant and anti-inflammatory activities of *Plantago major*, *Cannabis sativa* and *Ranunculus multifidus* crude extracts in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages. Overall, all three extracts exhibited concentration-dependent antioxidant activity together with variable anti-inflammatory effects, as demonstrated by their free radical-scavenging capacity in DPPH and ABTS assays and their modulation of inflammatory responses. Among the extracts, *C. sativa* exhibited the strongest overall antioxidant activity, followed closely by *P. major*, whereas *R. multifidus* showed comparatively weaker radical-scavenging capacity.

Cannabis sativa demonstrated marked radical-scavenging capacity. UPLC-MS analysis revealed the presence of cannabinoids, including cannabigerolic acid (CBGA; Peak 4) (Table 4) and Δ^9 -tetrahydrocannabinol (Δ^9 -THC; Peak 11) (Table 5), both of which possess phenolic hydroxyl moieties capable of donating electrons or hydrogen atoms to quench reactive oxygen species (Ceresa et al., 2025). However, because the individual constituents were not isolated, their discrete contributions to the overall antioxidant profile could not be determined. Nevertheless, the antioxidant activity of the extract is likely attributable to the combined effects of multiple phytochemical classes found in cannabis crude extract (Zaiachuk et al., 2023). Prior investigations have demonstrated that whole cannabis extract can elicit greater biological activity than single isolated constituents (Zaiachuk et al., 2023), suggesting that interactions between phytochemicals within crude extracts may influence bioactivity.

Similarly, *P. major* species have also been shown to possess high phenolic and flavonoid contents, which are considered major contributors to their antioxidant capacity (Gözcü and Atmaca, 2025; Sanna et al., 2022). In particular, the markedly elevated antioxidant capacity of *P. major* observed in this study is likely due to the presence of verbascoside, identified as Peak 12 in the UPLC-MS chromatogram and is summarised in Table 6. This caffeoyl phenylethanoid glycoside is recognised as a potent free radical scavenger, owing to the presence of multiple ortho-dihydroxy groups within its catechol moieties, which readily donate hydrogen atoms and stabilise reactive radical species (Alipieva et al., 2014). This might explain the pronounced DPPH and ABTS radical-scavenging activities observed. Consistent with the current findings, high verbascoside content has repeatedly been associated with enhanced antioxidant activity in *Plantago* species (Budzianowska et al., 2024; Kirkan et al., 2025; Sanna et al., 2022).

Although *R. multifidus* exhibited measurable antioxidant activity in both DPPH and ABTS assays, no major compounds were conclusively identified by UPLC-MS. This limitation constrains direct elucidation of the phytochemical determinants of its activity, particularly as this species remains comparatively underexplored. As a result, any proposed contribution of phenolic or flavonoid constituents remains inferential and is based on evidence from related *Ranunculus* species. In the present study, *R. multifidus* displayed a quantifiable, moderate level of antioxidant activity, aligning with previous reports that describe antioxidant flavonoids and phenolic compounds in members of the Ranunculaceae (Bhatti et al., 2015). These observations are consistent with data from related taxa within the Ranunculaceae family, including *R. arvensis*, which has been reported to contain flavonoids and phenolic compounds with documented antioxidant properties (Bhatti et al., 2015; Neag et al., 2017). More recently, caffeic acid and ferulic acid were identified among the principal phenolic contributors to antioxidant activity in *R. arvensis* pollen (Huseynova et al., 2025). Broader surveys of medicinal Ranunculaceae species have similarly documented substantial in vitro antioxidant potential (Heller et al., 2024). These comparisons remain indirect, and they provide a plausible phytochemical basis for the antioxidant activity observed in *R. multifidus*.

In addition to their radical-scavenging properties, all three extracts demonstrated a concentration-dependent inhibition of 15-lipoxygenase

(15-LOX), indicating a potential capacity to modulate lipid peroxidation and associated inflammatory signalling pathways (Singh and Rao, 2019). These findings are biologically significant because 15-LOX catalyses the oxidation of polyunsaturated fatty acids, resulting in the generation of hydroperoxides and other bioactive lipid mediators that are critically involved in the regulation of oxidative stress and inflammatory responses (Singh and Rao, 2019). Although the inhibitory effects of the extracts did not differ significantly from those of the quercetin positive control (Table 2), *C. sativa* exhibited the highest inhibitory activity, followed by *P. major*, whereas *R. multifidus* displayed comparatively lower activity.

The comparatively strong 15-LOX inhibition observed for *C. sativa* is consistent with reports showing that cannabis-derived extracts can modulate lipid mediator networks and inflammatory signalling in macrophages (Blebea et al., 2024; Zaiachuk et al., 2023). Similarly, the inhibitory activity of *P. major* aligns with previous studies describing anti-inflammatory effects of *Plantago* extracts and may, at least in part, reflect the activity of phenolic constituents such as verbascoside (Farid et al., 2022; Triastuti et al., 2022). In contrast, the weaker activity observed for *R. multifidus* suggests a more limited influence on lipoxygenase-associated pathways under the present experimental conditions. While anti-inflammatory effects have been reported for related *Ranunculus* species, the absence of definitive phytochemical characterisation prevents direct association of the observed activity with specific bioactive compounds or molecular targets (Hong et al., 2020; Marrelli et al., 2022).

All three extracts further reduced nitric oxide (NO) production in LPS-stimulated RAW 264.7 macrophages, indicating measurable anti-inflammatory activity under the present experimental conditions. These effects were evaluated at 15 $\mu\text{g}/\text{mL}$, within the non-cytotoxic concentration range, where cell viability remained above 80%. In activated macrophages, excessive NO production is primarily driven by inducible nitric oxide synthase (iNOS) and contributes to inflammatory tissue damage, oxidative stress, and amplification of pro-inflammatory signalling (Jimenez-Trinidad et al., 2026; Kim and Lee, 2025). The observed suppression of NO therefore suggests that these extracts can modulate inflammatory macrophage activation. Within the tested concentration at 15 $\mu\text{g}/\text{mL}$, *R. multifidus* showed more pronounced percentage inhibition of NO production, followed by *P. major* and *C. sativa*. However, the IC_{50} values were relatively close, ranging from $25.51 \pm 5.17 \mu\text{g}/\text{mL}$ for *P. major* to $31.97 \pm 4.40 \mu\text{g}/\text{mL}$ for *R. multifidus*, indicating broadly comparable NO-suppressive effects among the extracts. While NO inhibition was evident across all three species, the broader anti-inflammatory profiles differed substantially, suggesting that suppression of NO alone may not fully reflect the extent of immunomodulatory activity.

Consistent with their effects on NO production, the extracts showed distinct patterns of cytokine modulation in LPS-activated macrophages. *P. major* displayed the clearest anti-inflammatory profile, significantly suppressing IL-6, IL-1 β , and iNOS while simultaneously increasing secretion of the anti-inflammatory cytokine IL-10. Because IL-10 suppresses LPS-induced inflammatory responses through inhibition of NF- κ B-dependent signalling and reduction of pro-inflammatory mediator expression, this cytokine profile is indicative of robust anti-inflammatory activity (Liu et al., 2025; Zubair et al., 2019). These findings are consistent with previous reports showing that *Plantago*-derived phenolics and flavonoids enhance IL-10 production while suppressing inflammatory cytokine expression.

In contrast, *C. sativa* demonstrated a more selective immunomodulatory profile. The extract suppressed IL-6 and IL-1 β but exerted comparatively weaker effects on iNOS and PTGS2 expression. The modest increase in IL-10 observed is consistent with the complex immunopharmacology of phytocannabinoids, which have been reported to regulate cytokine production through cannabinoid receptor-dependent and receptor-independent mechanisms (Nagarkatti et al., 2009; Russo, 2011). *R. multifidus* exhibited weaker and more variable

cytokine responses, with modest inhibition of IL-1 β and iNOS but little effect on IL-6 or PTGS2. Such variability may reflect the presence of bioactive metabolites characteristic of the Ranunculaceae family, including ranunculin-derived compounds, which have been associated with context-dependent inflammatory responses. Overall, these findings demonstrate clear extract-specific differences in cytokine regulation, with *P. major* exhibiting the most consistent anti-inflammatory cytokine profile.

The anti-inflammatory effects observed in the present study appear to involve modulation of multiple interconnected inflammatory pathways, with NF- κ B emerging as a central regulatory target. NF- κ B is a key transcription factor controlling the expression of numerous pro-inflammatory mediators, including IL-6, IL-1 β , iNOS and PTGS2, and plays a critical role in macrophage activation following LPS stimulation (Mao et al., 2025). Therefore, the inhibition of NF- κ B activation can suppress multiple downstream inflammatory responses simultaneously. Consistent with this mechanism, immunofluorescence analysis demonstrated that *P. major* and *C. sativa* reduced LPS-induced nuclear translocation of the NF- κ B p65 subunit, indicating suppression of NF- κ B activation. The marked cytoplasmic retention of p65 observed in both extracts corresponded closely with their cytokine and nitric oxide profiles, including reduced IL-6, IL-1 β and NO production. These findings suggest that inhibition of NF- κ B signalling occurs upstream of inflammatory mediator release and may contribute substantially to the anti-inflammatory effects observed. Although the precise molecular events were not investigated, this effect may involve inhibition of I κ B kinase (IKK) activation or prevention of I κ B α degradation, thereby limiting nuclear translocation of NF- κ B (Jiang et al., 2018; Jiang et al., 2022; Kozela et al., 2010).

The phytochemical composition of the extracts provides further support for this interpretation. In *P. major*, verbascoside has been widely reported to suppress NF- κ B activation and downstream expression of pro-inflammatory mediators, providing a plausible explanation for the observed inhibition of IL-6, IL-1 β , iNOS and NO production, together with the increase in IL-10 secretion (Ma et al., 2020; Marčetić et al., 2025; Zubair et al., 2019). Likewise, in *C. sativa*, cannabinoids including CBGA and Δ^9 -THC have been shown to modulate inflammatory responses through cannabinoid receptor-associated signalling pathways and suppression of NF- κ B-dependent cytokine production (Suryavanshi et al., 2022; Zaiachuk et al., 2023). The selective inhibition of IL-6 and IL-1 β observed in the present study is therefore consistent with established cannabinoid-mediated immunoregulatory mechanisms.

In contrast, *R. multifidus* showed comparatively limited effects on NF- κ B activation, with p65 localisation resembling that of the LPS-treated control. This finding is consistent with its weaker and more variable cytokine profile, including limited suppression of IL-6 and PTGS2. Although *R. multifidus* reduced NO production and modestly affected certain inflammatory markers, the absence of significant NF- κ B inhibition suggests that its anti-inflammatory effects may involve alternative pathways not examined in the present study. Interpretation is further limited by the lack of definitive phytochemical characterisation. Members of the Ranunculaceae family contain bioactive compounds such as ranunculin, which is converted to the reactive metabolite protoanemonin following tissue damage and may produce context-dependent inflammatory responses (Sirak et al., 2025). This may partly explain the variable biological activity observed for *R. multifidus*.

In addition to direct effects on inflammatory signalling, the antioxidant activity demonstrated by *P. major* and *C. sativa* may have contributed indirectly to suppression of NF- κ B activation. Reactive oxygen species are recognised activators of redox-sensitive inflammatory pathways, including NF- κ B, and therefore the strong radical-scavenging activity observed in these extracts may reduce inflammatory signalling by limiting oxidative stress. Together with their inhibitory effects on 15-LOX, these findings suggest that the anti-inflammatory activity of *P. major* and *C. sativa* is mediated through a combination of antioxidant

activity, modulation of lipid mediator pathways, and suppression of NF- κ B-dependent inflammatory signalling.

Although the present study demonstrates antioxidant and anti-inflammatory activity in vitro, several limitations should be acknowledged. The extracts were phytochemically profiled using UPLC-QTOF-MS/MS but were not quantitatively standardised to defined marker compounds. In addition, the study was limited to biochemical and cell-based assays, and therefore the bioavailability, absorption, distribution, metabolism, systemic exposure, and potential drug interactions of the identified constituents were not assessed. Toxicity evaluation was restricted to in vitro cytotoxicity in RAW 264.7 macrophages, and no in vivo toxicity or LD₅₀ studies were performed. Furthermore, no direct assessment of synergistic interactions among phytochemical constituents was conducted. Therefore, extrapolation of these findings to human use will require further pharmacological, toxicological, and mechanistic investigation.

When the three extracts were compared in terms of antioxidant capacity, inflammatory mediator modulation, and pathway-related outcomes, a clear extract-specific differences emerged. *Plantago major* displayed the most coherent overall anti-inflammatory profile under the present experimental conditions. It suppressed IL-6, IL-1 β , iNOS, and nitric oxide production, enhanced IL-10 secretion, inhibited NF- κ B activation, and exhibited strong antioxidant activity, while remaining active within the selected non-cytotoxic concentration range. Overall, *P. major* exhibited the most favourable balance between anti-inflammatory activity and cellular safety within the tested concentration range. *Cannabis sativa* also demonstrated a favourable but more selective anti-inflammatory profile. The extract exhibited strong antioxidant and 15-LOX inhibitory activities, suppressed IL-6 and IL-1 β , and reduced NF- κ B nuclear localisation, supporting a mechanism involving both cannabinoid-mediated immunoregulation and suppression of inflammatory signalling pathways. In contrast, *Ranunculus multifidus* exhibited measurable but less consistent biological activity. Although it reduced nitric oxide production and modulated selected inflammatory markers, its overall anti-inflammatory profile was weaker, with limited effects on IL-6, PTGS2, and NF- κ B activation, together with less definitive phytochemical support. Taken together, these findings suggest that *P. major* provided the most balanced combination of antioxidant and anti-inflammatory activities, whereas *C. sativa* demonstrated potent but more selective immunomodulatory effects. *Ranunculus multifidus* remains a promising species; however, further phytochemical characterisation and mechanistic investigation are required to clarify its bioactive constituents and mode of action.

5. Conclusion

This study demonstrated that leaf extracts of *Cannabis sativa*, *Plantago major* and *Ranunculus multifidus* possess antioxidant activity and modulate inflammatory responses in LPS-stimulated RAW 264.7 macrophages. The extracts reduced nitric oxide production, altered the expression of selected inflammatory mediators, and exhibited differential effects on NF- κ B p65 nuclear localisation. Among the extracts evaluated, *P. major* produced the broadest anti-inflammatory response, characterised by suppression of pro-inflammatory mediators together with increased IL-10 production, whereas *C. sativa* exhibited strong antioxidant activity and selective modulation of inflammatory pathways. UPLC-QTOF-MS/MS profiling tentatively identified verbascoside in *P. major* and cannabinoids, including CBGA and Δ^9 -THC, in *C. sativa*, providing a plausible phytochemical basis for the biological activities observed. The extracts were non-cytotoxic at the working concentration used for mechanistic evaluation (15 μ g/mL), supporting their suitability for in vitro anti-inflammatory assessment. In contrast, the phytochemical composition of *R. multifidus* could not be conclusively characterised, limiting mechanistic interpretation of its comparatively variable biological effects. Overall, these findings support the ethnopharmacological use of these medicinal plants as potential sources of bioactive

compounds capable of modulating oxidative stress and inflammation. However, the extracts were not chemically standardised, and the study was limited to in vitro assays. Further work involving quantitative phytochemical standardisation, bioactivity-guided compound isolation, mechanistic validation, and in vivo evaluation will be necessary to confirm their therapeutic potential, establish safety profiles, and determine their translational relevance.

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Any opinion, findings and conclusions or recommendations expressed in this material are those of the author(s) and therefore the NRF do not accept any liability in regard thereto.

CRediT authorship contribution statement

Zandile Nxumalo: Writing – original draft, Visualization, Validation, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Buntubonke Gom:** Writing – review & editing, Formal analysis, Data curation. **Nelisiwe Masuku:** Data curation. **Anjo Theron:** Writing – review & editing, Resources. **Tsholofelo Mokoka:** Writing – review & editing, Supervision. **Sechaba Bareetseng:** Writing – review & editing, Supervision. **Lyndy McGaw:** Writing – review & editing, Supervision.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Given her role as an Editorial Board Member for this journal, Prof Lyndy McGaw had no involvement in the peer review of this article and had no access to information regarding its peer review. Full responsibility for the editorial process for this article was delegated to another journal editor. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available upon request.

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