

Untargeted Metabolomics Profiling of Ethanolic Extract of *Garcinia microphylla* Merr. Stem Bark and Evaluation of Antioxidant and Anti-Inflammatory Activities

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Abstract

Garcinia microphylla Merr. is a species within the *Garcinia* genus that has not been studied yet. This study was conducted to determine the metabolite profile via LC-HRMS in the ethanolic extract of *G. microphylla*, as well as to analyse Total Phenolic Content (TPC), Total Flavonoid Content (TFC), and the in vitro antioxidant and anti-inflammatory activities both in vitro and in vivo. Antioxidant activity was assessed using the 1,1-diphenyl-2-picryl-hydrazyl (DPPH) and 2,2-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS) methods, while anti-inflammatory activity was evaluated in vitro through heat and hypotonic-induced haemolysis inhibition tests, antiproteinase tests, and protein denaturation inhibition tests. In vivo, tests were conducted on paw oedema and peritonitis inhibition in rats induced by carrageenan. Numerous active compounds were detected in the LC-HRMS results, including phenolic compounds, terpenoid groups, coumarins, and fatty acids; this is corroborated by the high TPC and TFC levels observed in ethanolic extracts. It is established that *G. microphylla* extract has potential as an anti-inflammatory agent, with the treatment group demonstrating significant differences compared to the control group, and no significant differences when compared to the standard sodium diclofenac. This is supported by its antioxidant activity and the abundance of compounds in the extract.

Introduction

Over 300 medicinal plants, primarily located in the rainforests of India, Indonesia, the Andaman and Nicobar Islands, Vietnam, Thailand, Malaysia, China, Africa, and Brazil, belong to the *Garcinia* species of the Clusiaceae (formerly Guttiferae) family. *Garcinia* sp. has been demonstrated in numerous research studies to possess both pharmacological and nutraceutical benefits¹. From the observations of herbarium specimens and literature studies, it turns out that 64 types of *Garcinia* (*Garcinia* spp.) are found in Indonesia. In Kalimantan, there are 25 varieties of *Garcinia*, while Sumatra and Sulawesi each have 22 types. Maluku and Irian Jaya (Papua) each possess 17 types, Java has 8, and Nusa Tenggara features just five types of *Garcinia*. One species of *Garcinia* that has not been studied, namely *Garcinia microphylla* Merr, is known to originate from the Maluku distribution area². *Garcinia* species contain high concentrations of xanthenes, benzophenones, phloroglucinols, and bioflavonoids. These compounds are linked to various pharmacological effects, including antibacterial, antifungal, anticancer, antioxidative, and anti-inflammatory properties³.

Inflammation is an organism's defence mechanism against internal and external stimuli caused by chemical, mechanical, or biological stressors. Both steroidal and non-steroidal anti-inflammatory medications (NSAIDs) reduce inflammation, but with unfavourable side effects. Research into bioactive compounds with anti-inflammatory properties isolated from natural sources is becoming increasingly intriguing, as they can offer certain advantages over synthetic medicines, such as a reduced risk of side effects in patients⁴. Inflammation arises from various stimuli, including pathogens and toxins, physical injury, chemical irritants, and cell damage. Macrophages and dendritic cells recognise these stimuli through pattern recognition receptors that detect pathogen-associated molecular patterns released by microbes or damage-associated molecular patterns released by injured cells. Upon activation, cells

secrete pro-inflammatory cytokines (such as TNF- α , IL-1 β , and IL-6), chemokines, and lipid mediators, including prostaglandins and leukotrienes, which collectively result in vasodilation and increased vascular permeability. The response facilitates the migration of leukocytes to the site of injury, where they eliminate pathogens and clear damaged tissues through enzymatic degradation and hydrolysis⁵. The proinflammatory cytokines interact with TLRs, IL-1 receptor (IL-1R), IL-6 receptor (IL-6R), and the TNF receptor (TNFR) which trigger crucial intracellular signalling pathways including the mitogen-activated protein kinase (MAPK), nuclear factor kappa-B (NF- κ B), and Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathways. Importantly, inflammation is a protective process, but persistent and uncontrolled activation can result in chronic inflammation and the progression of chronic diseases⁶. Activated neutrophils release lysosomal components, such as proteinases, during an inflammatory response, which leads to further tissue inflammation and a variety of diseases. By first stabilizing the lysosomal membranes and then preventing the release of their constituents, a number of anti-inflammatory medications reduce the inflammatory response. Hydrogen bonding between polyphenols at the bilayer surface may reduce access to dangerous chemicals (oxidants), maintaining the structure and functionality of the membrane. Phenolics may interact with the polar head of phospholipids at the water-lipid interface, increasing membrane rigidity, decreasing fluidity, and boosting the resilience of mechanical lipid bilayers. Numerous investigations have demonstrated that interactions with polyphenolic chemicals enhanced proteins heat stability. Therefore, compounds that may prevent the denaturation of proteins would be helpful in the development of novel anti-inflammatory medications⁷.

Compounds with pertinent medicinal qualities have been found in abundance in members of the genus *Garcinia*. The genus *Garcinia* comprises a wide variety of physiologically active metabolites, and in recent decades, they have drawn a lot of interest due to the chemical makeup of their extracts, which is rich in phenolic derivatives. Extracts from *Garcinia*'s epicarp, seeds, and pericarp have shown antiprotozoal, leishmanicidal, anti-inflammatory, and antioxidant properties. Previous study explained that the enzymatic hydrolysate of *G. kola* seeds has excellent in vitro anti-inflammatory properties. The hydrolysate produced from the enzyme:substrate ratio of 1:16 had the highest hemolysis inhibition percentage of $84.45 \pm 0.007\%$, protein denaturation inhibition at a concentration of 1 mg/ml of $53.36 \pm 0.01\%$ and showed the highest DPPH radical scavenging activity with a termination percentage of $87.24 \pm 0.10\%$. The enzymatic pepsin hydrolysate of *G. kola* further has the potential to be developed as a functional food and as a pharmaceutical industry therapy in the treatment of diseases associated with oxidative stress and inflammation⁸. *G. morella* fruit methanol extract dramatically reduced TNF- α levels and nitrite release in LPS-induced RAW 264.7 cells ($p < 0.05$). Additionally, pretreating mice with carrageenan-induced paw edema with 20 mg/kg bioactive fraction considerably ($p < 0.05$) reduced paw inflammation and regulated nitrite and cytokine levels³.

Meanwhile oxygenated free radicals serve as mediators of inflammation, perpetuating an inflammatory state. When the ROS production exceeds the neutralising capacity of endogenous antioxidants, the imbalance leads to oxidative stress. The intimate connection between oxidative stress and inflammation

may justify the employment of compounds with antioxidant properties in anti-inflammatory therapies. Flavonoids, polyphenols, and vitamin C are examples of antioxidants that can either interact with pro-inflammatory cytokines or block the active sites of cyclooxygenase (COX), lipoxygenase (LOX), arachidonic acid formation, prostaglandin, and leukotriene production, subsequently lowering reactive oxygen species levels and reducing oxidative stress⁹

Of all the types of garcinia found in Indonesia, *G. microphylla* is a species that has never been studied. To date, based on the researcher's knowledge, no study has elucidated the bioactivity of *G. microphylla*. By referring to the bioactivity exhibited by other species of the Genus Garcinia, the study aimed to determine the anti-inflammatory activity in vitro and in vivo activity. To support the anti-inflammatory mechanism, an in vitro antioxidant activity assay was conducted.

Methods

Materials

The reagents used included ethanol 96% highest grade that is accessible commercially, gallic acid (CAS 149-91-7), quercetin > 95% (CAS 117-39-5), 2,2-diphenyl-1-picrylhydrazyl (DPPH) (CAS 1898-66-4), 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) (CAS 30931-67-0), potassium persulfate from Sigma Aldrich CAS 30931-67-0, perchloric acid 70–72% p.a Merck 100519, casein (Sigma catalog C7078), trypsin (Sigma catalog T4799), tris(hydroxymethyl) aminomethane CAS 77-86-1 from Himedia, and L-Ascorbic acid from Sigma CAS-No 50-81-7, carrageenan from Sigma Aldrich CAS 9000-07-1, Turk solution from Sigma Aldrich 1.09277.0100, and sodium carboxymethylcellulose (CMC-Na) from Sigma Aldrich CAS 9004-32-4.

Plant collection

The stem bark of *G. microphylla* was collected from Mokwam Village, Warmare District, Manokwari, West Papua, Indonesia. This plant was identified by botanist Deni Sahroni, S.Si and verified by Dr. Atik Retnowati at the Herbarium Bogoriense, Botany Division, Research Center for Biology, Indonesian Institute of Sciences, Bogor, Indonesia, with a voucher specimen (YP 6). The authors obtained permission to collect plant samples in accordance with applicable guidelines and legislation. The use of plants in this study complies with international, national, and institutional guidelines. All plant collection procedures complied with the IUCN Policy Statement on Research Involving Species at Risk of Extinction and the Convention on the Trade in Endangered Species of Wild Fauna and Flora.

Preparation of extract samples

G. microphylla tree stem bark was taken as much as 1 kg, cut into small pieces, then dried and grounded into a fine powder. The refined sample was extracted using the maceration method with ethanol solvent,

with a yield of up to 250 g, at a ratio of 1:10 (w/v)¹⁰. The maceration process was carried out for 2 x 24 hours with stirring; then, the filtrate was filtered through a filtration process and concentrated using a rotary evaporator to obtain a thick extract¹¹.

Total Phenolic Content (TPC)

A stock solution of gallic acid was prepared by dissolving a known quantity of gallic acid (e.g., 1 mg/mL) in distilled water or methanol. From this stock, a series of standard solutions with varying concentrations (e.g., 10, 20, 40, 60, 80, and 100 µg/mL) was prepared by diluting the stock solution with distilled water. These standards were then used to create a calibration curve to determine the total phenolic content. Twenty microliters of plant extract (10 mg/ml) were combined with 100 µL of Folin-Ciocalteu reagent and vortexed. Then, 80 µL of 7.5% Na₂CO₃ was added, and the mixture was mixed and incubated at 45°C for 30 minutes. The absorbance was then measured at 750 nm using a UV-visible spectrophotometer. This experiment was carried out in triplicate. TPC was calculated as mg GAE per g dry weight of the material using the gallic acid calibration curve¹².

Total Flavonoid Content (TFC)

Twenty-four microlitres of plant extract (10 mg/mL) were mixed with 28 µL of NaNO₂ (50 g/L) and allowed to stand for 5 minutes. Subsequently, 28 µL of AlCl₃ (100 g/L) was added, and the mixture was allowed to sit for 6 minutes. Following this, 120 µL of 1 M NaOH was added to the mixture, and the absorbance at 510 nm was measured immediately. This experiment was carried out in duplicate. TFC was calculated as mg QE/g dry weight¹².

LC-HRMS Analysis

Metabolite profiling of the ethanolic extract of *G. microphylla* was conducted using a Liquid Chromatography system (Thermo Scientific™ Vanquish™ UHPLC Binary Pump) coupled with Orbitrap high-resolution mass spectrometry (Thermo Scientific™ Q Exactive™ Hybrid Quadrupole-Orbitrap™ High-Resolution Mass Spectrometer), employing ThermoScientific™ Accucore™ Phenyl-Hexyl 100 mm × 2.1 mm ID × 2.6 µm as the separation column. The MS ionisation source utilised electrospray ionisation (ESI), with a spray voltage of 3.30 kV, a capillary temperature of approximately 320°C, and sheath gas, auxiliary gas, and sweep gas flow rates set at 32, 8, and 4 Arbitrary Units (AU), respectively, alongside a spray voltage of 3.30 kV. The flow rate from the delivery system was calibrated to 0.3 mL/min, the column oven was kept at 40°C, and the sample injection volume was 3 µL. The mobile phase consisted of 0.1% formic acid in water (A) and 0.1% formic acid in methanol (B). A linear gradient elution programme was implemented as follows: 0.01 min (5% B), 16.00 min (90% B), 20 min (90% B), and 25 min (5% B). The MS scan range (66.7–1000 m/z) was acquired in both positive and negative modes, with the resolution 70000 for full MS and 17600 for dd-MS2. An open-source program called MZmine is used

to process raw spectral data produced by several MS platform¹³. The detected compounds were selected based on a matching accuracy of approximately 90%¹⁴.

Antioxidant Activity Test

DPPH Method

The antioxidant activity of the plant extract was measured using the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay. This procedure was according to Hussien, 2023. A solution of DPPH (0.1 mM) was prepared in methanol. Different concentrations of the plant extract (e.g., 10, 20, 30, 40 & 50 µg/mL) were mixed with 0.4 mL of the DPPH solution and adjusted to a final volume of 2 mL with methanol. The mixture was then incubated in the dark at room temperature for 30 minutes. The absorbance was measured at 517 nm using a UV-Vis spectrophotometer. Ascorbic acid was used as a positive control, and methanol served as the blank. Antioxidant activity is expressed as % inhibition of DPPH free radicals, which is used to calculate the IC₅₀ value determined by probit analysis. The percentage of DPPH radical scavenging activity was calculated using the formula¹⁵:

$$\text{Scavenging activity (\%)} = \frac{(\text{Abs control} - \text{Abs sample})}{\text{Abs control}} \times 100\%$$

Information:

Abs_{control} : the mixture of methanol and DPPH solution

Abs_{sample} : the mixture of the sample extract and the DPPH solution

ABTS Method

The ABTS [2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)] radical scavenging assay evaluates the antioxidant activity of plant extracts. The working solution was prepared by diluting the ABTS^{•+} solution with methanol to achieve an absorbance of 0.70 ± 0.02 at 734 nm. Five µL of extract at various concentrations (10, 20, 30, 40, and 50 µg/mL) were mixed with 4000 µL of ABTS^{•+} solution and left in the dark for 16 hours at room temperature. The absorbance was measured at 734 nm using a UV-Vis spectrophotometer with methanol as a blank. The reactivity of the various concentrations of each solvent extract was compared with that of ascorbic acid as a standard. All measurements were conducted in triplicate¹⁶. The equation below was employed to compute the percentage inhibition. The results were expressed as inhibitory concentration (IC₅₀).

$$\text{ABTS}^{\bullet+} \text{ scavenged (\%)} = ((\text{AB}-\text{AS})/\text{AB}) \times 100\%$$

Information:

AB : absorbance of the blank solution

AS : absorbance of ABTS radical + plant extract.

In Vitro Anti-Inflammatory Test

Collection and Preparation of Erythrocyte Suspension.

Prior to fresh whole blood being intravenously extracted from healthy volunteer donors at the hematology laboratory of Universitas Nahdlatul Ulama Surabaya, informed consent was obtained from the university's Health Research Ethics Committee. The volunteers who had not taken any NSAIDs for two weeks before the trial were selected. All venous blood collection procedures were performed in accordance with the World Health Organization (WHO) guidelines on drawing blood: best practices in phlebotomy, and were carried out by a phlebotomist. Blood samples were collected into heparinised vacutainers to prevent coagulation. Packed blood cells were obtained by mixing the collected blood samples with 0.9% saline and centrifuging for 10 minutes at 3000 rpm. The centrifugation was repeated three times. The blood volume was determined following centrifugation and reconstituted using an isotonic buffer solution (10 mM sodium phosphate buffer pH 7.4) in a 10% (v/v) suspension¹⁷.

Membrane Stabilisation Activity

The membrane-stabilising activity of the plant extracts was evaluated using the hypotonic-induced hemolysis assay described by Adekola et al, 2021 with slight modifications. For the assay, 0.5 mL of the erythrocyte suspension was mixed with 1 mL of hypotonic solution (distilled water) containing various concentrations of the plant extract (e.g., 50–500 µg/mL). The mixtures were incubated at 37°C for 30 minutes and then centrifuged at 2000 rpm for 10 minutes. The supernatant was collected, and the absorbance was measured at 540 nm using a UV-Vis spectrophotometer to quantify the extent of hemolysis. A solution containing erythrocyte suspension in a hypotonic solution without the extract served as the negative control, while erythrocyte suspension in isotonic PBS served as the baseline control. The percentage of hemolysis inhibition was calculated using the formula¹⁸:

$$\% \text{ Inhibition of hemolysis} = \frac{\text{Abs. control} - \text{Abs. sample}}{\text{Abs. control}} \times 100$$

Abs control

The membrane-stabilising activity of the plant extracts also evaluated using the heat-induced hemolysis assay described by Adekola et al, 2021 with slight modifications. The examination commenced by taking 1 mL of a 10% erythrocyte suspension in a test tube and adding 1 mL of 0.9% saline; then, the mixture was homogenised. This tube served as a negative control. For a positive control mixture, add 1 mL of sodium diclofenac (10, 20, 30, 40, and 50 µg/mL) to 1 mL of 10% erythrocyte suspension, and the sample was homogenised as previously described. The same treatment was performed on *G. microphylla* samples with varying concentrations of 10, 20, 30, 40, and 50 µg/mL. All prepared tubes were incubated for 30 minutes at 56°C. Cool with running water—centrifuge at 2500 rpm for 5 minutes. The absorbance of the mixture was measured using a UV-Vis spectrophotometer (Thermo Scientific GENESYS 10S) at a wavelength of 560 nm. The percentage of inhibition is calculated using the same formula as above¹⁸.

Antiproteinase test

The antiproteinase test was conducted as described by Mba et al. (2021). This procedure begins with the preparation of a reaction mixture (2 mL) containing 0.001% trypsin, approximately 0.06 mg, 1 mL of 1 mM Tris HCl buffer (pH 7.4), and 1 mL of an extract sample with varying concentrations (10, 20, 30, 40, and 50 µg/ml). Prepare a standard solution containing 0.06 mg of trypsin, 1 mL of 20 mM Tris-HCl buffer (pH 7.4), and 1 mL of Na-diclofenac. Subsequently, prepare a blank solution containing 1 ml of 20 mM Tris-HCl buffer (pH 7.4). Add the same treatment to the sample and standard reaction mixture with concentrations of 10, 20, 30, 40, and 50 µg/mL. Then, incubate the mixture at 37°C for 5 minutes before adding 1 mL of 0.02% (w/v) casein. Incubate the mix again for 20 minutes at 37°C. Then, add 1 ml of 2% perchloric acid to halt the reaction. The turbid suspension is then centrifuged, and the absorbance of the supernatant is measured at 210 nm using a UV-Vis spectrophotometer (Thermo Scientific GENESYS 10S), against the buffer as a blank. The percentage of proteinase activity inhibition is calculated using the same formula as above¹⁹.

Protein Denaturation Test

The protein denaturation test was conducted according to the procedure outlined by Derbel et al., 2023. A 0.2% BSA solution was prepared by dissolving 0.2 grams of BSA in a 100 mL measuring flask, then brought to the mark with TBS (Tris Buffer Saline) solution, which consisted of 1.51 grams of Tris (0.05 M) and 2.1 grams of NaCl (0.15 M) dissolved in 250 mL of distilled water. Subsequently, the mixture was homogenised, and the pH was adjusted to 6.6 using an HCl solution (0.2% BSA buffer solution). Test tubes were filled with 1 mL of 0.2% BSA and 1 mL of methanol as a positive control. In another tube, 1 mL of standard sodium diclofenac ($C_{14}H_{10}Cl_2NNaO_2$) with a concentration range of 10–50 µg/mL was added, along with 1 mL of *G. microphylla* extract sample, also at a concentration range of 10–50 µg/mL. Following this, all tubes were incubated at room temperature for 20 minutes before being heated in a water bath at 72°C for 5 minutes. They were then cooled to room temperature, and the absorbance was measured at a wavelength of 660 nm using a UV-Vis spectrophotometer (Thermo Scientific GENESYS 10S) with a methanol blank. The percentage of protein denaturation inhibition was calculated using the same formula as previously mentioned²⁰.

In vivo anti-inflammatory test

Ethics Statement

Every procedure involving the care and treatment of animals at the Animal Laboratory, Faculty of Medicine, Universitas Nahdlatul Ulama Surabaya, complies with the guidelines established by the university's Health Research Ethics Committee, which is directed by the ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments). The Health Research Ethics Committee of Universitas Nahdlatul Ulama Surabaya approved these studies, which were carried out in accordance with institutional rules (No.0357/EC/KEPK/UNUSA/2024).

Animals

Thirty male albino Wistar rats (aged 5–7 weeks) weighing 150–200 g were purchased from the Indonesian Veterinary Center (Balai Besar Veteriner Farma Pusvetma) or BBVF Pusvetma, Surabaya, Indonesia. The rats were housed in a controlled environment (30°C ± 2°C, 70°C ± 5% relative humidity with a 12-hour light/dark cycle) at the Animal Laboratory, Faculty of Medicine, Universitas Nahdlatul Ulama Surabaya. The rats were acclimatized for 7 days, fed standard feed and water as needed. All animal care procedures were based on the Guide for the Care and Use of Laboratory Animals (National Research Council, 2012). The experiment began when the rats reached a weight of 200–250 g.

Carrageenan-induced paw oedema

The rats were randomly assigned to five groups, each with six animals. The animals were fasted overnight but had free access to drinking water. The rats were then injected subcutaneously with 0.1 ml of 1% Carrageenan on the right hind paw of the animal to induce inflammation. One hour prior to the Carrageenan injection, the following treatments were administered to the animals³.

Table 1
Route of administration in vivo antiinflammatory assay

Group Label	Description	Route of administration
Control	Carboxymethylcellulose (CMC) 0.3%	Oral
Positive Control	20mg/kg Na-Diclofenac	
Treatment group GME 1	20 mg/kg	
Treatment group GME 2	40 mg/kg	
Treatment group GME 3	60 mg/kg	

Extract was dissolved in carboxymethylcellulose (CMC) 0.3%. Paw volume was measured before (0 hour) and after carrageenan injection at 1, 2, 3, 4, and 5 hours using a plethysmometer²². Calculation of the % inhibition of paw oedema is done using the following formula:

Percentage % inhibition of oedema = $\frac{(\text{At-Ao})_{\text{control}} - (\text{At-Ao})_{\text{treatment}}}{(\text{At-Ao})_{\text{control}}} \times 100\%$

Where

(At – Ao) = difference change in mouse paw oedema volume.

Ao = paw oedema volume at t = 0.

At = paw oedema volume at various times.

Carrageenan-induced peritonitis

The grouping of animals and route of administration are similar to those described above. After 30 minutes, carrageenan (0.1 mL at 1.0%) was administered into the peritoneal cavity. Then, after 4 hours, the animals were given injections of xylazine (10 mg/kg) and ketamine (80 mg/kg) to induce death while under profound anesthesia. The peritoneal cavity was washed with 5 mL of heparinised PBS solution (10 IU/mL). Turk's solution (1/20) was taken as much as 0.380 mL and then added to 0.02 mL of peritoneal exudate. The number of leukocytes was counted using a Neubauer chamber under an optical microscope. The total number of leukocytes was determined according to the following formula: $\text{cells/mm}^3 = N \times 20 \times 2.5$, where N is the number of cells, 20 is the dilution factor of the Turk's solution, and 2.5 is the total volume into the Neubauer chamber. The results are expressed as total leukocytes/mm³ ²³.

Results

TPC and TFC Tests

The results of the total phenolic and flavonoid content assay in ethanolic extract *G. microphylla* were obtained from the calibration curve of gallic acid and quercetin using the UV-Vis spectrophotometer method. The TPC value of ethanolic extract *G. microphylla* was $498.148 \pm 32.07 \mu\text{g/mL}$, while the the TFC value was $29.164 \pm 0.546 \mu\text{g/mL}$.

Result of LC-HRMS Analysis

The chemical components included in the ethanol extract of *G. microphylla* were identified using LC-HRMS. Phenolic groups predominated among the substances found in the samples and a small amount of terpenoid groups, coumarins, and fatty acids. Compared to the negative mode, more chemicals were found in the positive mode. The metabolites found in the samples at the MS1 and MS2 levels are listed in Table 3. Accurate mass, retention time, predicted molecular weight and fragmentation patterns from mass spectrometry (MS) were used to identify the substances.

Table 2
Chemical components from extract of *G. microphylla*

No.	Rt [minute]	Molecular Formula	Major ion [M + H] + 1	Calculate Molecular Weight	Name
1.	11.963	C ₁₆ H ₁₄ O ₄	271.0962	270.0889	(2 <i>R</i>)-5-Hydroxy-7-methoxy-2-phenyl-3,4-dihydro-2H-1-benzopyran-4-one
2.	12.806	C ₁₈ H ₃₀ O ₃	295.2262	294.2190	9-Oxo-10(<i>E</i>),12(<i>E</i>)-octadecadienoic acid
3.	8.134	C ₃₀ H ₂₂ O ₁₁	559.1225	558.1154	(2 <i>S</i> ,3 <i>S</i>)-8-[(2 <i>R</i> ,3 <i>S</i>)-5,7-Dihydroxy-2-(4-hydroxyphenyl)-4-oxo-3,4-dihydro-2H-1-benzopyran-3-yl]-3,5,7-trihydroxy-2-(4-hydroxyphenyl)-3,4-dihydro-2H-1-benzopyran-4-one
4.	12.248	C ₁₆ H ₁₄ O ₄	271.0963	270.0889	(2 <i>R</i>)-5-Hydroxy-7-methoxy-2-phenyl-3,4-dihydro-2H-1-benzopyran-4-one
5.	8.946	C ₁₆ H ₁₄ O ₄	271.0963	270.0888	7-Hydroxy-5-methoxy-2-phenyl-3,4-dihydro-2H-1-benzopyran-4-one
6.	8.345	C ₃₀ H ₂₂ O ₁₁	559.1223	558.1154	(2 <i>S</i> ,3 <i>S</i>)-8-[(2 <i>R</i> ,3 <i>S</i>)-5,7-dihydroxy-2-(4-hydroxyphenyl)-4-oxo-3,4-dihydro-2H-1-benzopyran-3-yl]-3,5,7-trihydroxy-2-(4-hydroxyphenyl)-3,4-dihydro-2H-1-benzopyran-4-one
7.	13.057	C ₁₈ H ₃₀ O ₃	295.2264	294.2190	9-Oxo-10(<i>E</i>),12(<i>E</i>)-octadecadienoic acid
8.	5.562	C ₂₁ H ₂₀ O ₁₁	449.1073	448.1000	(1 <i>ξ</i>)-1,5-Anhydro-1-[2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4-oxo-4H-chromen-8-yl]-D-galactitol
9.	8.889	C ₁₄ H ₁₀ O ₆	275.0546	274.0474	NP-006274
10.	9.245	C ₃₀ H ₂₂ O ₁₀	543.1280	542.1209	(2 <i>S</i> ,2' <i>S</i> ,3 <i>R</i>)-5,5',7,7'-Tetrahydroxy-2,2'-bis(4-hydroxyphenyl)-2,2',3,3'-tetrahydro-4H,4'H-3,8'-bichromene-4,4'-dione
11.	15.543	C ₁₈ H ₃₇ NO	284.2944	283.2870	Stearamide
12.	15.227	C ₂₁ H ₄₂ O ₄	359.3148	358.3074	1-Stearoylglycerol
13.	14.615	C ₁₈ H ₃₅ NO	282.2786	281.2713	Oleamide
14.	5.764	C ₂₁ H ₂₀ O ₁₁	449.1073	448.1000	(1 <i>ξ</i>)-1,5-Anhydro-1-[2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4-oxo-4H-chromen-8-yl]-D-galactitol
15.	9.466	C ₃₀ H ₂₂ O ₁₀	543.1282	542.1209	(2 <i>S</i> ,2' <i>S</i> ,3 <i>R</i>)-5,5',7,7'-Tetrahydroxy-2,2'-bis(4-hydroxyphenyl)-2,2',3,3'-

No.	Rt [minute]	Molecular Formula	Major ion [M + H] + 1	Calculate Molecular Weight	Name
					tetrahydro-4H,4'H-3,8'-bichromene-4,4'-dione
16.	10.216	C ₁₅ H ₁₂ O ₄	257.0808	256.0735	Pinocembrin
17.	14.905	C ₁₈ H ₃₅ NO	282.2787	281.2714	Oleamide
18.	15.847	C ₁₈ H ₃₇ NO	284.2944	283.2870	Stearamide
19.	15.518	C ₂₁ H ₄₂ O ₄	359.3150	358.3074	1-Stearoylglycerol
20.	9.085	C ₁₂ H ₁₇ NO	192.1381	191.1308	DEET
21.	5.532	C ₁₄ H ₁₅ N	198.1273	197.1200	Dibenzylamine
22.	8.337	C ₁₄ H ₈ O ₄	241.0492	240.0417	Alizarin
23.	14.549	C ₁₈ H ₃₀ O ₂	279.23166	278.2242	α-Eleostearic acid
24.	14.266	C ₁₈ H ₃₀ O ₂	279.23157	278.2242	α-Linolenic acid
25.	12.353	C ₁₈ H ₂₈ O ₃	293.21072	292.2034	12-oxo Phytodienoic Acid
26.	12.35	C ₁₈ H ₃₀ O ₂	279.23163	278.2242	α-Eleostearic acid
27.	13.394	C ₁₈ H ₃₂ O ₇	361.22171	360.2144	Citroflex 4
28.	7.538	C ₁₄ H ₈ O ₄	241.04906	240.0418	Alizarin
29.	9.047	C ₁₁ H ₁₀ O ₄	207.06488	206.0576	Scoparone
30.	8.126	C ₁₄ H ₈ O ₄	241.04922	240.0418	Alizarin
31.	2.591	C ₆ H ₁₁ NO	114.0914	113.0842	Caprolactam
32.	11.989	C ₁₅ H ₂₂ O	219.1740	218.1667	Nootkatone
33.	7.763	C ₁₄ H ₈ O ₄	241.0491	240.0418	Alizarin
34.	4.395	C ₉ H ₁₀ O ₅	199.0599	198.0526	Syringic acid
35.	4.941	C ₈ H ₈ O ₃	153.0546	152.0473	Vanillin
36.	7.833	C ₃₀ H ₂₂ O ₁₁	559.1231	558.1154	(2 <i>S</i> ,3 <i>S</i>)-8-[(2 <i>R</i> ,3 <i>S</i>)-5,7-dihydroxy-2-(4-hydroxyphenyl)-4-oxo-3,4-dihydro-2H-1-benzopyran-3-yl]-3,5,7-trihydroxy-2-(4-hydroxyphenyl)-3,4-dihydro-2H-1-benzopyran-4-one

No.	Rt [minute]	Molecular Formula	Major ion [M + H] + 1	Calculate Molecular Weight	Name
37.	5.275	C ₂₁ H ₂₂ O ₁₀	435.1282	434.1209	5,7-Dihydroxy-2-(4-hydroxyphenyl)-6-[3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]-3,4-dihydro-2H-1-benzopyran-4-one
38.	13.987	C ₂₁ H ₃₈ O ₄	355.2838	354.2765	1-Linoleoyl glycerol
39.	14.449	C ₂₀ H ₃₄ O ₈	425.2141	402.2249	Citroflex A-4
40.	12.604	C ₁₈ H ₃₀ O ₂	279.2318	278.2242	α-Eleostearic acid
41.	11.179	C ₁₆ H ₁₄ O ₄	271.0969	270.0889	7-Hydroxy-5-methoxy-2-phenyl-3,4-dihydro-2H-1-benzopyran-4-one
42.	0.779	C ₆ H ₇ NO ₂	126.0550	125.0477	NP-019811
43.	14.582	C ₂₁ H ₄₀ O ₄	357.2990	356.2917	Monoolein
44.	15.045	C ₁₈ H ₃₇ NO	284.2944	283.2870	Stearamide
45.	9.5	C ₁₄ H ₁₀ O ₅	259.0599	258.0526	Norlichexanthone
46.	0.979	C ₆ H ₅ NO ₂	124.0393	123.0320	Nicotinic acid
47.	6.863	C ₁₆ H ₁₄ O ₅	287.0914	286.0840	7-Hydroxy-2-(4-hydroxyphenyl)-5-methoxy-3,4-dihydro-2H-1-benzopyran-4-one
48.	13.919	C ₂₄ H ₂₆ O ₆	411.1800	410.1729	1,3,7-Trihydroxy-6-methoxy-4,5-diisoprenylxanthone
49.	0.979	C ₆ H ₆ N ₂ O	123.0553	122.0480	Nicotinamide
50.	15.939	C ₂₂ H ₃₄ O ₃	347.2574	346.2500	NP-014113
51.	8.212	C ₁₆ H ₁₇ NO ₂	256.1329	255.1256	NP-001798
52.	13.312	C ₁₈ H ₃₀ O ₃	295.2266	294.2194	9-Oxo-ODE
53.	17.53	C ₂₀ H ₃₈ O ₂	311.2939	310.2866	Ethyl oleate

Antioxidant Activity Assay

The concentration of plant extracts that could scavenge 50% of the free radicals was determined by calculating the IC₅₀ values. Lower IC₅₀ values indicate more potent antioxidant activity, while high IC₅₀ values indicate weak antioxidant activity. The antioxidant activities which is marked as IC₅₀ values of ethanolic extract *G. microphylla* was 1.20 µg/ml in DPPH radical scavenging assay and 2.67 µg/ml in

ABTS radical scavenging assay. The results were compared with ascorbic acid, a well-known antioxidant standard, with an IC₅₀ value of 1.88 µg/ml and 2.19 µg/ml in the DPPH assay and in the ABTS assay, respectively. This extracts had strong antioxidant activities in both antioxidant assays, in the DPPH assay and in the ABTS assay.

In Vitro Anti-inflammatory Test Results

Membrane Stabilization Test

Figure 3 illustrates how each extract of *G. microphylla* stabilised membranes. The outcome showed that, in a concentration-dependent way, the extract exhibited excellent erythrocyte membrane stabilising action against hypotonic and heat-induced hemolysis. Extract with the various concentrations (0.5–50 ppm) produced the maximum membrane-stabilising activities in ethanolic extract and standard Na diclofenac ($89.997 \pm 5.445\%$ and $76.766 \pm 0.3166\%$) on hypotonic induced hemolysis and maximum inhibition ($98.145 \pm 0.161\%$ and $99.161 \pm 0.171\%$) on heat-induced hemolysis, respectively. At a similar concentration (50 ppm), however, each extract of *G. microphylla* demonstrated stabilising potential. Based on the data in Table 3, the IC₅₀ value in vitro anti-inflammatory test is known to be the lowest IC₅₀ value, indicating the highest heat-induced hemolysis inhibition activity. A comparison of the graph of the relationship between concentration and the percentage of hemolysis inhibition can be seen in Fig. 2 (a) and (b).

Antiproteinase test

The results of the antiproteinase test on all *G. microphylla* etanolic extracts showed significant inhibition. This is shown in Fig. 2 (c) above that the percentage of proteinase inhibition of extracts is highest at a concentration of 50 ppm ($84.068 \pm 0.080\%$) and higher than the standard Na diclofenac ($66.643 \pm 0.040\%$). The IC₅₀ value in the antiproteinase test can be seen in Table 3. It is known that the IC₅₀ value in the antiproteinase test of ethanolic extract of *G. microphylla* was 2.76 µg/ml, lower than standard drug 3.96 µg/ml. But, both have IC₅₀ value < 50 µg/ml, which means that extracts have very strong proteinase inhibition and have the potential as candidates for anti-inflammatory drugs.

Table 3
Result of IC₅₀ value ethanolic extract and standard drug on in vitro anti-inflammatory assay

Extract	Hypotonic induced hemolysis	Heat induced hemolysis	Antiproteinase	BSA Denaturation
Ethanol	3.30	1.94	2.76	4.42
Standard	5.24	1.24	3.96	2.21

Protein Denaturation Inhibition Test

Based on the Fig. 2 (d), it shows that the percentage of protein denaturation inhibition using Bovine Serum Albumin (BSA) in the ethanolic chloride extract, which is $87.626 \pm 1.948\%$ higher than the Na diclofenac standard ($87.401 \pm 1.878\%$). The increasing trend in the percentage of protein denaturation inhibition against concentration between the extract and the standard is depicted in Fig. 2(d). When viewed from the IC₅₀ value, *G. microphylla* extracts have IC₅₀ values higher than standard, but both have IC₅₀ values <50 µg/ml, which suggests the ability of the extracts in inhibiting of protein denaturation, including the Na diclofenac standard.

In Vivo Anti-inflammatory Test Results

Carrageenan-induced paw oedema

Results of the effect of the treatment of ethanolic extract of *G.microphylla* on carageen-induced paw oedema rats can be shown below in Table 4. Carrageenan was injected into the abdominal cavity of the left hind leg of rats to cause the development of oedema observed in the volume of oedema at 0, 1, 2, 3, 4, and 5 hours with a plethysmometer. Rats treated with various doses of *G. microphylla* ethanol extract showed a significant decrease in paw oedema volume (P < 0.05 in data with superscript *) compared to the control group. The maximum inhibition percentage (79.31%) of *G. microphylla* ethanol extract on carrageenan-induced oedema occurred at 20 mg/kg at the 5th hour.

Table 4
Effect of Ethanolic extract on *G. microphylla* on carageen induced paw oedema rats

Groups	Paw oedema volume (mL) Mean ± Sd (% inhibition of paw volume)					
	0h	1h	2h	3h	4h	5h
Control	1.004 ± 0.051	1.584 ± 0.062	1.584 ± 0.089	1.562 ± 0.092	1.582 ± 0.046	1.526 ± 0.095
Standard Drug	1.006 ± 0.043	1.396 ± 0.038* (32.75%)	1.358 ± 0.085* (39.31%)	1.256 ± 0.105* (55.195%)	1.188 ± 0.091* (68.51%)	1.134 ± 0.063* (75.47%)
Treatment group GME 1	0.956 ± 0.045	1.34 ± 0.055* (33.79%)	1.274 ± 0.037* (45.17%)	1.232 ± 0.100* (50.53%)	1.176 ± 0.058* (61.93%)	1.064 ± 0.087* (79.31%)
Treatment group GME 2	0.996 ± 0.068	1.328 ± 0.133* (42.75%)	1.302 ± 0.161* (47.24%)	1.286 ± 0.122* (48.02%)	1.218 ± 0.099* (61.59%)	1.146 ± 0.059 (71.26%)
Treatment group GME 3	0.994 ± 0.056	1.354 ± 0.082* (37.93%)	1.284 ± 0.109* (50.00%)	1.272 ± 0.058* (50.18%)	1.214 ± 0.097 (61.93%)	1.128 ± 0.110 (74.33%)

The mean ± standard deviation (n = 6) is used to display the results. The asterisk indicates the significance levels concerning the control: *P < 0.05. A one-way ANOVA analysis was followed by Tukey's post hoc analysis.

Carrageenan-induced peritonitis

Administration of ethanol extract of *G. microphylla* to rat model of peritonitis induced by carrageenan significantly decreased the total number of leukocytes. Based on Fig. 3 shows that the total number of leukocytes was highest in the control group that received carrageenan induction without treatment. The total number of leukocytes was lowest in the treatment group of ethanol extract of *G. microphylla* with a dose of 60 mg/kg bw ($5.33 \pm 3.686 \times 10^3/\text{mm}^3$) with a value almost the same as the standard Na diclofenac ($6.4625 \pm 2.407 \times 10^3/\text{mm}^3$). The results of the One-way ANOVA test, followed by Tukey's post hoc analysis, showed that there was a significant difference ($P < 0.05$) in all treatment groups when compared to the control group.

Discussion

Based on previous research shows that the factors that affect the phytochemical content of medicinal plants include the stage of plant development, plant parts, and solvents used for the extraction and isolation of phytochemicals. The type and polarity of the solvent can affect the quality of the extract, quantity, extraction rate, inhibitory compounds, toxicity, and other biological activities, as well as biological safety. The total amount of secondary metabolites and their antioxidant capacity highly depend on the solvent and plant parts used for extraction²⁴. Based on research conducted by Wairata, 2022, the results showed that the n-Hexane extract of the *G. forbesii* King plant had the highest total phenolic content, namely 187.37 ± 0.06 mg GAE/g, while the highest total flavonoid content was found in the methanol extract with a value of 35.97 ± 0.02 mg EQ/g. Significant total phenolic content was also present in extracts with significant flavonoid concentration¹⁶. It is well known that flavonoids have varying solubilities in different solvents and that polarity often influences the extraction solvent selection. The variability inherent in plant materials may cause variations in flavonoid concentration. Because flavonoids are now an indispensable part of many pharmaceutical, medicinal, cosmetic, and nutraceutical uses, a high flavonoid content indicates the value of the plant material. Furthermore, most oxidising chemicals, including singlet oxygen and other free radicals, are effectively scavenged by flavonoids. Depending on the kind of functional group and how it is positioned around the nuclear structure, flavonoids have different antioxidant capacities²⁵.

Based on LC-HRMS data, it can be seen at several retention times of 11.963, the molecular formula $\text{C}_{16}\text{H}_{14}\text{O}_4$ with $[\text{M} + \text{H}] + 1$ at m/z 271.0962 was annotated as a compound with a benzopyran-4-one framework. In accordance with Gupta's research, 2023 stated that the benzopyran-4-one skeleton has been widely used as a model for the synthesis of novel compounds with a variety of therapeutic applications due to its wide range of pharmacological characteristics, which include antitubercular, anticancer, antiallergic, anti-inflammatory, antidiabetic, antimicrobial, antihypertensive, and anti-HIV effects²⁶. Similarly, the compound detected at a retention time of 9.425, the molecular formula $\text{C}_{30}\text{H}_{22}\text{O}_{10}$ with $[\text{M} + \text{H}] + 1$ at m/z 543.12805 was annotated as a compound with a bichromene-4,4'-dione framework. A chromenone with the keto group at the 2-position is called coumarin. Based on the

benzopyran substitutions, coumarins have a variety of biological actions, including growth control, antioxidant, antiviral, antibacterial, anti-inflammatory, and anticancer properties. Coumarin's anti-inflammatory properties are ascribed to its suppression of lipoxygenase, cyclooxygenase, prostaglandin production, and neutrophil-dependent superoxide anion generation²⁷. In addition, at a retention time of 8.337, the molecular formula $C_{14}H_8O_4$, with $[M + H]^+ 1$ at m/z 241.0492 was annotated as a compound named Alizarin. Xu's study, 2022 showed that pharmacological research has demonstrated the anti-inflammatory, anticancer, antioxidant, hemostatic, antibacterial, and other pharmacological properties of *Radix rubiae*'s active components, which include a range of hydroxyl anthraquinone derivatives, including alizarin²⁸. In addition, there are several conjugated fatty acids, such as compounds detected at retention times of 12.806 ($C_{18}H_{30}O_3$), 15.543 ($C_{18}H_{37}NO$), 15.227 ($C_{21}H_{42}O_4$), 14.615 ($C_{18}H_{35}NO$), 14.549 ($C_{18}H_{30}O_2$), 12.353 ($C_{18}H_{28}O_3$), 13.312 ($C_{18}H_{30}O_3$), and 17.53 ($C_{20}H_{38}O_2$) with $[M + H]^+ 1$ at m/z of 295.2262, 284.2944, 359.31485, 282.2786, 279.2316, 293.2107, 295.2266 and 311.2939. Based on a study conducted by Salsinha, 2023, it was suggested that conjugated fatty acids have positive effects by reducing inflammatory responses in the hypothalamus and aberrant behaviour, oxidative reactions, and neuronal death caused by Saturated Fatty Acids. Much focus has been placed on additional PUFAs that could have comparable effects. For example, the conjugated linoleic acid (CLA) and conjugated linolenic acid (CLNA) included in diets can reduce the adipose tissue's synthesis of TNF- α , PGE2, nitric oxide (NO), IL-1, and IL-6, among other pro-inflammatory chemicals²⁹.

DPPH free radical assay is one of the most common methods to evaluate the antioxidant activity of plant extracts. DPPH $\cdot$ is a stable radical which can efficiently scavenge free radicals but is not dimerised. DPPH radicals can obtain an electron or a hydrogen atom from an antioxidant molecule, and then the reduced form appears. At this time, the deep purple color of the DPPH stock solution turns to light yellow and the ability to absorb ultraviolet light decreases. Therefore, the scavenging activity of natural products and synthetic compounds can be determined. Previous research explains that the methanol extract sample of *G. nigrolineata* is able to ward off free radicals at an IC_{50} of 2.5 $\mu g/mL$. Several previous studies have stated that the high content of phenolic compounds in the extract sample can increase the activity of scavenging free radicals³⁰. The ABTS method is based on spectrophotometric monitoring of the decomposition of the ABTS radical cation, which is generated by the oxidation of ABTS. The ABTS radical cation reacts with most antioxidants, including phenolic compounds. ABTS $\cdot^+$ is reduced in the presence of antioxidants. This method converts the blue ABTS radical cation to a colourless neutral form. The removal of colour represents the rate of ABTS $\cdot^+$ inhibition³¹. In accordance with the research results of Rifaldi, 2023 which explains that the n-hexane extract of *G. bancana* has antioxidant activity with an IC_{50} value of $1.22 \pm 0.02 \mu g/ml$. Antioxidant capacity experiments that use the 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS $\cdot^+$) radical cation are among the most used. The coupling is a particular reaction for some antioxidants because some, at least phenolic ones, can form coupling adducts with ABTS $\cdot^+$, while others can undergo oxidation without coupling. Further oxidative degradation of these coupling adducts might result in hydrazindylidene-like and/or imine-like adducts, the marker chemicals for which are 3-ethyl-2-oxo-1,3-benzothiazoline-6-sulfonate and 3-ethyl-2-imino-1,3-

benzothiazoline-6-sulfonate, respectively¹¹. The results of LC-HRMS of the ethanol extract of *G. microphylla* showed that the phenolic compound group (norlichexanthone, syringic acid, alizarin, and vanilin) dominated the most considerable content. This group of phenolic compounds contributes significantly to antioxidant capacity³².

The results of this study align with the research of Fidele, 2022 which explains that *G. kola* seed extract can inhibit hemolysis caused by hypotonic solutions with an IC_{50} value of 1.02 ± 0.2 mg/mL. The protective effect of the extract on red blood cells in preventing hemolysis is supported by its antioxidant properties³³. The lysosomal membrane's vulnerability to harm from harmful substances is crucial in controlling the inflammatory response. Damage to the lysosome may cause an overabundance of proteolytic enzymes to be released, exacerbating the inflammatory processes since the lysosomal membrane can be well-modelled by the erythrocyte membrane. The extract's potential to stabilise lysosomal membranes is implied by its capacity to stabilise the membrane of red blood cells³⁴.

Localised at lysosomes, neutrophils are a substantial source of serine proteinase. Leukocyte proteinase is crucial in developing tissue damage during inflammatory reactions, and protease inhibitors, such as flavonoids, have been shown to offer a considerable degree of protection. Inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX-2) isoforms produce a substantial amount of these mediators, prostaglandins and nitric oxide generation, involved in inflammation. Numerous flavonoids have been proven in countless recent studies to have a significant role in the antioxidant and anti-inflammatory properties of various plants. Thus, the anti-inflammatory properties of the leaves might be attributed to their bioactive components³⁵. This follows LC-HRMS data, which shows that *G. microphylla* contains several flavonoid compounds (pinocembrin and benzopyran derivatives). Flavonoids have been shown to suppress adhesion molecules and reactive C protein, two additional mediators of the inflammatory process and enzymes. Data on antiprotease action, however, are scarce. In a similar vein, the findings of this work support the notion that trypsin functions as a mediator of inflammatory response during in vitro research. The plant extracts utilised in this investigation function as protease inhibitors and are anti-inflammatory drugs offering the highest protection level. It is thought that the plant extract attaches to the trypsin molecule during the proteinase inhibition experiment, signalling the end of the inflammatory process³⁶.

When proteins are subjected to outside stressors or substances, they lose their quaternary, tertiary, and secondary structures. This process is known as protein denaturation, typically resulting in losing the proteins' biological functions. One of the well-established causes of inflammatory and arthritic disorders is the denaturation of tissue proteins³⁷. The oedema inhibition did not differ substantially ($P > 0.05$) from the standard drug (sodium diclofenac), but it was statistically significant ($P < 0.05$) when compared to the control. In the carrageenan model, the fraction's capacity to reduce the early stage of inflammation (1–2 hours) raises the possibility that it inhibited the production of bradykinin, serotonin, and histamine. Excessive bradykinin protease activity, cyclooxygenase (COX) generation of prostaglandins, and releasing proteolytic enzymes from the lysosome mediate the late-phase events. According to the

results, the fraction might have prevented the late stage of inflammation in this model by reducing prostanoid secretion and activity³⁴. Higher TPC and TFC values of these extracts confirmed their anti-inflammatory potential and the abundance of polyphenols. Consequently, it was proposed that *G. microphylla*'s phenolic components were likely responsible for its anti-inflammatory properties. The new study's findings are similar to those of Nazar et al., who showed that the ethanol extract of *G. microphylla* considerably decreased the rat paw oedema caused by carrageenan injection³⁸.

The control group that received carrageenan as a phlogistic agent had a significant inflammatory response, as indicated by an increase in white blood cells compared to the standard and sample treatment groups (Fig. 3)³⁹. In preclinical and experimental settings, carrageenan is frequently employed as an inflammatory stimulus to assess the anti-inflammatory qualities of different substances. In addition to activating peritoneal macrophages to create proinflammatory cytokines, including TNF- α and IL-1 β , it also causes neutrophil migration and activation. These cytokines encourage the generation of reactive oxygen species (ROS) via activating tyrosine kinase signalling pathways. Through the generation of ROS, TNF- α mediates endothelial degradation, which may result in the presence of erythrocytes and inflammatory cells in the peritoneal cavity. Common positive control medications like diclofenac reduce inflammation by blocking the production of prostaglandins, inhibiting cyclooxygenase (COX), and modifying K⁺ channel opening, all of which are essential mediators of the inflammatory response. Additionally, these medications frequently block neutrophil aggregation and cause inflammatory cell death, mediated mainly by prostaglandin signalling. As a result, mice given carrageenan either by itself or in conjunction with diclofenac showed a reduction in the number of inflammatory cells in the peritoneal cavity⁴⁰.

Conclusion

Qualitative and semi-quantitative profile analysis of ethanol extract of *G. microphylla* thoroughly using LC-HRMS technique. The findings showed that *G. microphylla* ethanolic extract is rich in polyphenols, terpenoid groups, coumarins, and fatty acids. This is supported by the high TPC and TFC tests and the results of antioxidant activity tests that are very strong against DPPH and ABTS radicals. In vitro, anti-inflammatory tests showed that all extracts showed robust inhibition, as did in vivo tests. Finally, it was found that the anti-inflammatory effects of the *G. microphylla* extracts tested were solely related to the content of phenolic compounds and could be explained by various mechanisms involving polyphenols and radical scavenging. In short, *G. microphylla* extract is a very beneficial phytochemical mixture and can be a candidate for anti-inflammatory and antioxidant drugs. For the initially time, the phytochemical analysis and anti-inflammatory and antioxidant properties of *G. microphylla* extract are reported.

Declarations

Competing interests

The authors declare no competing interests.

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Author Contribution

D.D.W planned the research, carried out the experiments, analyzed the findings, and composed the paper. Y.P contributed to providing samples, S.F outlined the content, A.F edited the manuscript and planned the study and experiments, and M.S wrote the manuscript and contributed knowledge, crucial information, and input. All authors have read and approved the final manuscript.

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Data Availability

The corresponding author will provide data upon reasonable request.

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Figures

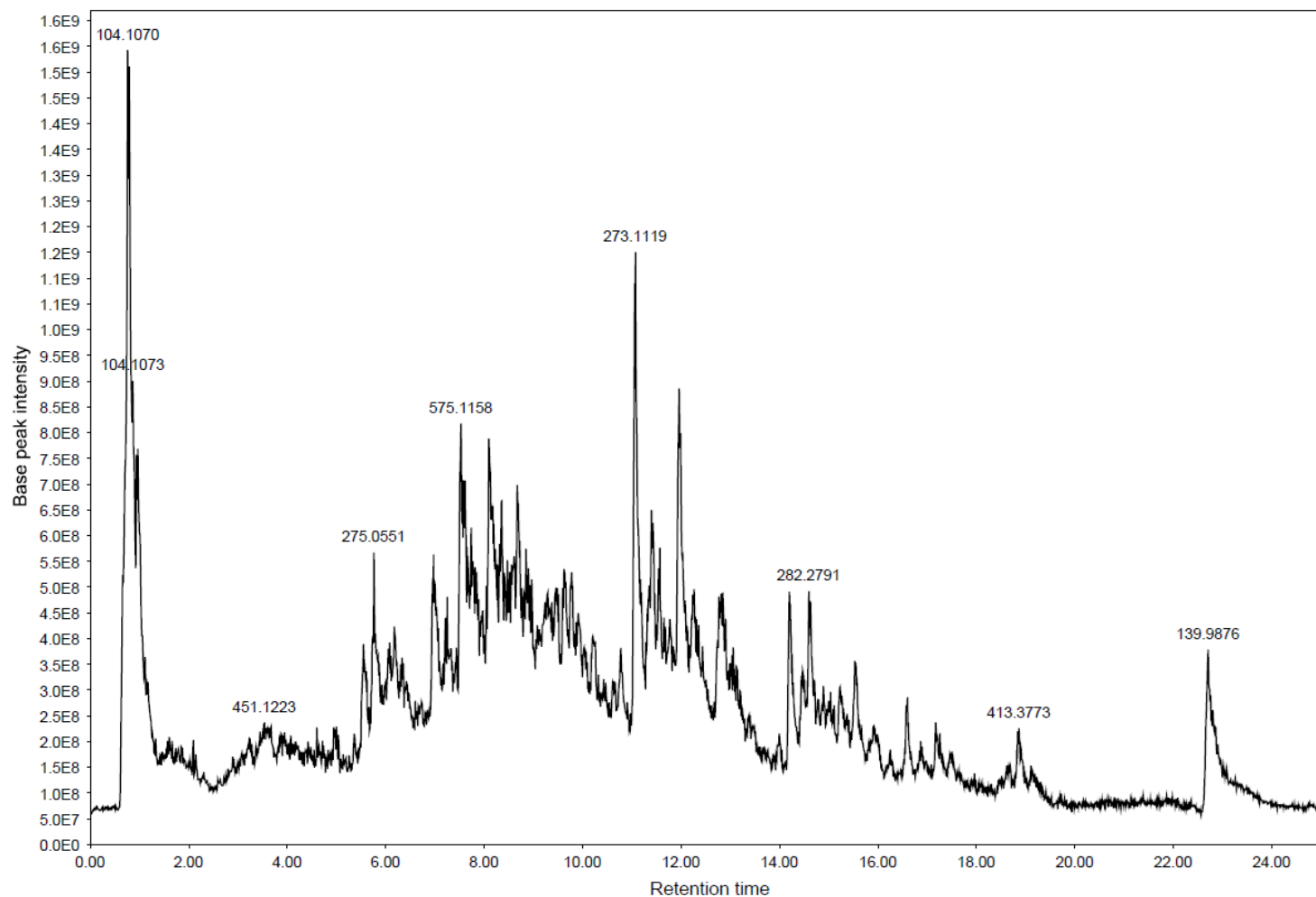


Figure 1

Total Ion Chromatograms of LC-HRMS

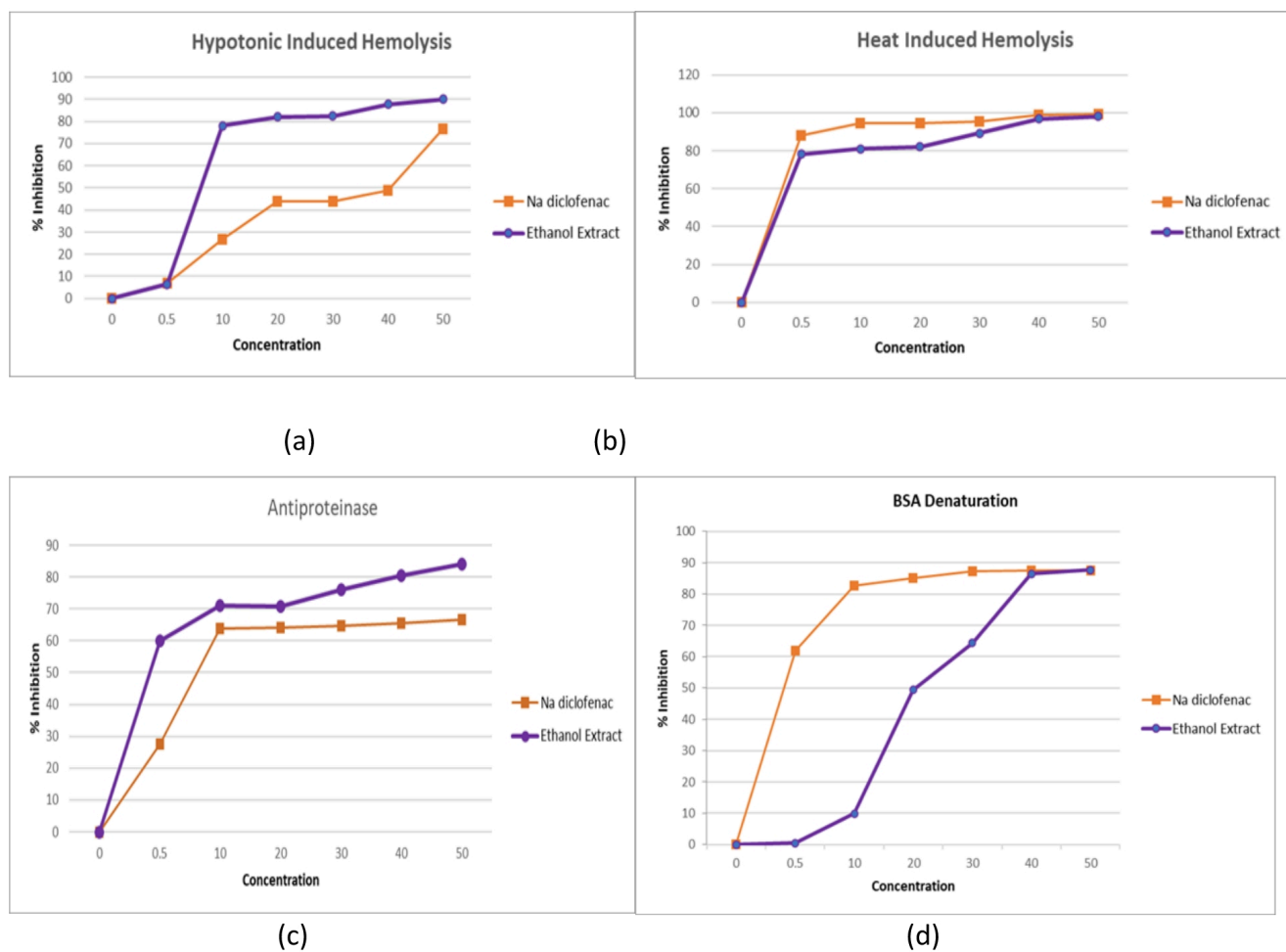


Figure 2

Graph of the relationship between concentration and % inhibition of each extract in (a) heat-induced hemolysis test; (b) hypotonic-induced hemolysis test; (c) antiproteinase test; (d) BSA denaturation test

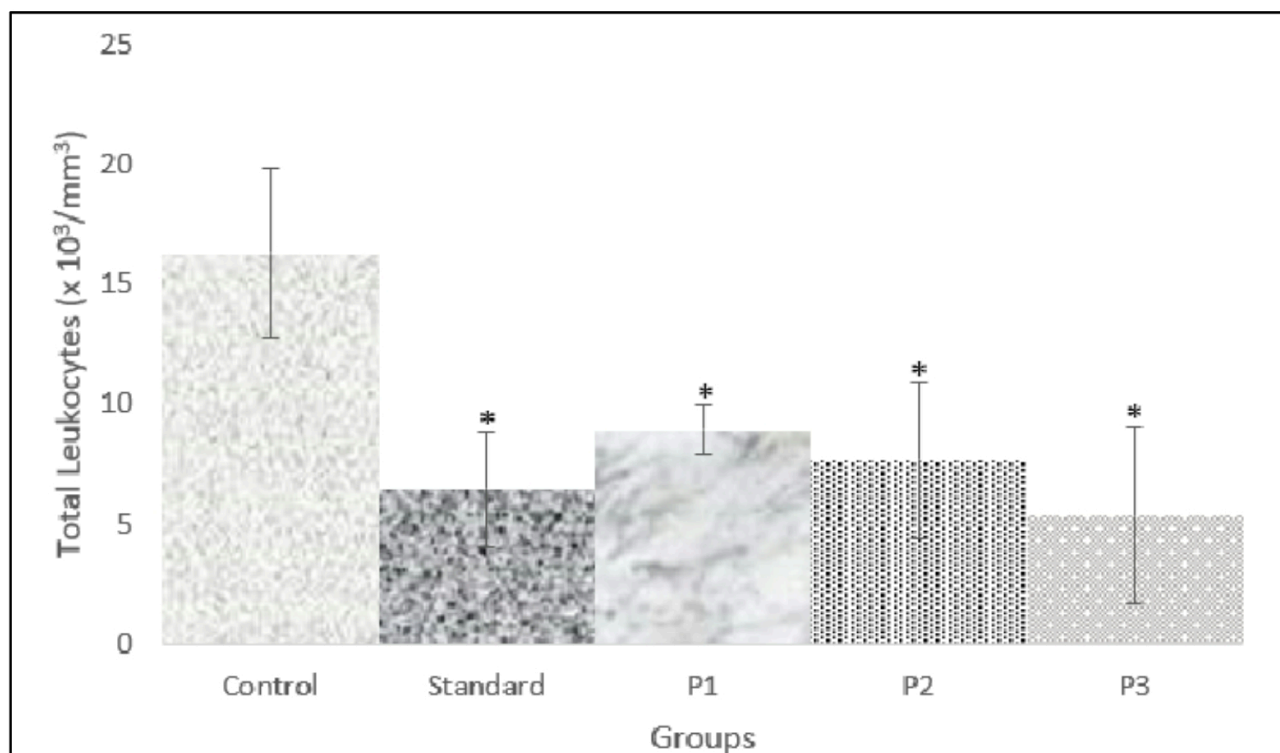


Figure 3

Graph of the relationship between giving *G. microphylla* ethanol extract and total leukocyte count in the control group, the standard drug group sodium diclofenac, treatment group GME 1 (P1), treatment group GME 2 (P2), and treatment group GME 3 (P3). The mean $\pm$ Sd with the asterisk * $P < 0.001$ when compared with a control. A one-way ANOVA analysis was followed by Tukey's post hoc analysis.