

Phytochemical profiling, acute toxicity, antinociceptive and anti-inflammatory effects of Cypriot *Allium ampeloprasum* L. bulb extract in Wistar albino rats

Yakup Cem Yababa^{1,2,4,*}, Emmanuel Mshelia Halilu¹ and Ovgu Isbilen^{2,3}

¹Faculty of Pharmacy, Cyprus International University, Northern Cyprus, Mersin 10, Nicosia 99258, Turkey

²Biotechnology Research Center, Cyprus International University, Northern Cyprus, Mersin 10, Nicosia 99258, Turkey

³Department of Molecular Biology and Genetics, Faculty of Arts and Sciences, Cyprus International University, Northern Cyprus, Mersin 10, Nicosia 99258, Turkey

⁴Department of Biological Sciences, Faculty of Arts and Sciences, Eastern Mediterranean University, Northern Cyprus, Mersin 10, Famagusta 99628, Turkey

Abstract: Background: The genus *Allium* is a chemically diverse source of bioactive secondary metabolites, yet wild populations remain unevenly studied. Wild *Allium ampeloprasum* L. from Cyprus is underexplored despite evidence that the species contains phenolic, flavonoid and organosulfur constituents with diverse bioactivity. This study provides a locality-specific phytochemical and *in-vivo* evaluation of its methanolic bulb extract (AAB). **Objectives:** To characterise AAB and assess its acute toxicity, antinociceptive and local anti-inflammatory activity in Wistar albino rats. **Methods:** Dried bulb powder (50.0 g) was macerated in methanol, yielding 2.906 g extract (5.812%). Constituents were quantified by LC-MS/MS and screened using SwissADME and ProTox-III. Acute toxicity followed the two-phase Lorke design using 12 rats. Three independent efficacy cohorts (25 rats each; $n = 5/\text{group}$) received distilled water, AAB at 125, 250 or 500 mg/kg, or piroxicam 20 mg/kg. Antinociception was assessed by writhing, hot-plate and formalin tests and local anti-inflammatory activity by formalin-induced paw oedema. Biochemical, haematological, ESR and CRP measurements were performed after the final 5-h paw oedema assessment. **Results:** Thirteen constituents were quantified, with caffeic acid and *trans*-ferulic acid showing the highest relative abundances. No mortality occurred up to 5000 mg/kg, although transient clinical signs were observed. AAB reduced writhing by 45.8%, 69.5% and 91.5% at 125, 250 and 500 mg/kg, respectively (adjusted $p = 0.0035$, $p < 0.0001$ and $p < 0.0001$). Hot-plate latency increased significantly only at 500 mg/kg (adjusted $p = 0.0067$). Paw licking decreased at all concentrations in both formalin phases (adjusted $p < 0.0001$), with late-phase inhibition of 85.71-95.71%. Paw oedema inhibition at 5-h was 66.67-71.11%. Post-oedema blood analyses showed selected biochemical changes without a consistent concentration-related pattern. **Conclusion:** AAB demonstrated antinociceptive and local anti-inflammatory activity in rat models. Absence of mortality does not establish safety; further standardisation, mechanistic investigation and longer-duration toxicity studies are required.

Keywords: Analgesics; Anti-inflammatory; *Allium ampeloprasum*; Liquid chromatography-tandem mass spectrometry; Toxicity tests

Submitted on 16-05-2025 – Revised on 12-08-2026 – Accepted on 20-08-2026

INTRODUCTION

More than 900 species belong to the genus *Allium* L., many of which are consumed as foods and have also been used traditionally for medicinal purposes. Pharmacological research has primarily focused on *Allium sativum* L. (garlic) and *Allium cepa* L. (onion), which contain diverse secondary metabolites, including organosulfur compounds, phenolic acids, flavonoids and saponins. The presence of these constituents has encouraged continued investigation of *Allium* species for compounds potentially relevant to inflammation and pain (Alam *et al.*, 2023; Gorrepati *et al.*, 2024). This interest is reinforced by the close relationship between inflammation and nociception, which frequently coexist in acute and chronic pathological conditions and contribute substantially to impaired quality of life. Although non-steroidal anti-inflammatory drugs (NSAIDs), corticosteroids and opioid analgesics remain central to treatment, their prolonged use may be limited by

gastrointestinal, renal, hepatic and cardiovascular adverse effects, together with the risk of dependence associated with opioids (Alorfi, 2023). These limitations support the continued investigation of edible and traditionally used plants as sources of pharmacologically relevant constituents, while recognising that their efficacy and safety require experimental evaluation.

Allium ampeloprasum L., commonly known as wild leek, is a perennial bulbous species native to the Mediterranean region and is associated with several cultivated horticultural groups, including leek and elephant garlic (Shahrajabian *et al.*, 2021; Carullo *et al.*, 2024). The species occurs throughout the Eastern Mediterranean, including Cyprus, where it is consumed as a wild edible plant (Savvides *et al.*, 2023). Previous investigations of *A. ampeloprasum* collected from other geographical regions have demonstrated its nutritional and phytochemical relevance and have reported antioxidant, antimicrobial, antinociceptive and anti-inflammatory activities. In

*Corresponding author: e-mail: yakupyababa@gmail.com

particular, methanolic extracts prepared from the flowers, leaves and bulbs of Turkish material have been characterised by liquid chromatography-electrospray ionisation-tandem mass spectrometry (LC-ESI-MS/MS) and evaluated in biological assays (Emir *et al.*, 2022), while separate *in-vivo* studies have reported antinociceptive and anti-arthritic effects of other *A. ampeloprasum* preparations (Abbas, 2019; Kaur *et al.*, 2023). Collectively, these studies indicate that the species warrants further pharmacological investigation. However, they involved plant materials, extraction procedures and experimental models that differ from those employed in the present study.

Previous studies have demonstrated the bioactive potential of *A. ampeloprasum*; however, none have comprehensively investigated the phytochemical profile and *in-vivo* pharmacological activities of wild populations from Cyprus. Secondary-metabolite production may vary with geographical origin, climatic and soil characteristics, phenological stage and plant organ investigated, whereas differences in extraction and analytical procedures may further affect the chemical profile reported across studies (Heinrich *et al.*, 2022; Xu *et al.*, 2022; Horablagla *et al.*, 2023). Consequently, findings obtained from geographically distinct populations cannot be directly extrapolated to Cypriot plant material. This does not imply that the Cypriot accession contains unique compounds or higher concentrations of particular constituents; such conclusions would require a harmonised comparison of multiple populations collected, extracted and analysed under identical conditions. Rather, the current knowledge gap concerns the absence of an integrated, locality-specific chemical and *in-vivo* pharmacological assessment of wild Cypriot *A. ampeloprasum* bulb material.

The methanolic bulb extract of wild *A. ampeloprasum* collected in Cyprus was characterised and its preliminary *in-vivo* pharmacological activity was evaluated. The extract was examined by qualitative phytochemical screening, liquid chromatography-tandem mass spectrometry (LC-MS/MS) and all quantified constituents were subsequently assessed using SwissADME and ProTox-III to obtain preliminary compound-level pharmacokinetic and toxicological predictions. Acute oral toxicity was evaluated using the two-phase Lorke procedure, while antinociceptive activity was assessed using acetic acid-induced abdominal writhing, hot-plate and formalin-induced nociception tests. Local anti-inflammatory activity was investigated through formalin-induced paw oedema. Biochemical and haematological parameters, erythrocyte sedimentation rate and C-reactive protein were measured after completion of the paw oedema experiment to describe post-experimental systemic findings. Through this integrated approach, the study sought to establish a chemical and preliminary pharmacological baseline for the understudied Cypriot accession.

MATERIALS AND METHODS

Plant collection and authentication

Fresh specimens of *Allium ampeloprasum* L. were collected during May-June 2025 from the coastal area of Famagusta, Northern Cyprus. The plant material was authenticated by Assoc. Prof. Dr. Emmanuel Mshelia Halilu, Chair of the Department of Pharmaceutical Botany, Faculty of Pharmacy, Cyprus International University (CIU). Representative plant material was deposited in the Public Herbarium of CIU under the accession number CIU/PHARM/AMARY/002. No special permission was required from the Ministry of Agriculture and Natural Resources, Northern Cyprus for the collection of native plant species from the sampling area.

Preparation of the methanolic bulb extract

The bulbs of *Allium ampeloprasum* L. were separated from the collected plants, cut into small pieces and air-dried at room temperature. Once fully dried, the bulbs were ground into a powder using a mechanical grinder. The powdered bulb material (50.0 g) was extracted by maceration with 500 mL of analytical-grade methanol (100%; Merck, Darmstadt, Germany), giving a plant material-to-solvent ratio of 1:10 (w/v). Maceration was performed for 72 h at room temperature under continuous orbital agitation. The methanolic extract was filtered through Whatman No. 1 filter paper and concentrated under reduced pressure at 40°C using a rotary evaporator (Heidolph, Germany). The resulting dried methanolic bulb extract was designated AAB and stored at 4°C until analysis and administration. The extraction yielded 2.906 g of crude extract, corresponding to an extraction yield of 5.812%. Extraction yield was calculated using the following equation (Ozbil *et al.*, 2024).

$$\text{Extraction yield (\%)} = \frac{\text{Mass of dried crude extract (g)}}{\text{Mass of dried plant material (g)}} \times 100 \quad (1)$$

Preliminary phytochemical screening

The methanolic AAB extract was qualitatively screened for alkaloids, saponins, tannins, phenolics, flavonoids, triterpenoids and steroids using previously described procedures (Kebede *et al.*, 2021; Korkmazer and Halilu, 2023). The presence or absence of each phytochemical class was determined from the characteristic colour reaction or precipitate produced in the corresponding test.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used to identify and quantify phenolic and related constituents in AAB, following procedures adapted from Hamiti *et al.* (2025) and Oduncuoglu *et al.* (2026). To characterise the free constituents, the native methanolic extract was analysed without acidic or enzymatic hydrolysis. A 100 mg portion of dried AAB was dissolved in 1 mL of methanol. From this solution, 100 µL was mixed

with 900 μL of ultrapure water/methanol/formic acid (79:20:1, v/v/v), followed by vortexing for 30 s, sonication for 10 min and centrifugation at 9000 rpm for 5 min. The clear supernatant was transferred to an autosampler vial.

An Agilent 1260 Infinity liquid chromatograph interfaced with an Agilent 6460 triple-quadrupole mass spectrometer was used for the analysis; the mass spectrometer incorporated a Jet Stream electrospray-ionisation source (Agilent Technologies, Waldbronn, Germany). The mass spectrometer was operated in negative-ionisation mode. Analytes were separated on a Zorbax SB-C8 column (150 x 3.0 mm, 3.5 μm ; Agilent Technologies, Waldbronn, Germany), with the column temperature maintained at 35°C. Mobile phase A consisted of 5 mM ammonium acetate (Merck, Darmstadt, Germany) in ultrapure water, whereas mobile phase B consisted of 0.1% acetic acid (Merck, Darmstadt, Germany) in acetonitrile-methanol (50:50, v/v; Merck, Darmstadt, Germany). The flow rate was 0.7 mL/min. The gradient was maintained at 96% A from 0 to 0.7 min, decreased linearly to 2% A by 2.3 min and held at 2% A until 5.0 min, decreased to 0% A by 7.0 min and returned to 96% A at 7.10 min for re-equilibration. The total run time was 12 min. The injection volume was 10 μL and the autosampler was maintained at 4°C.

MassHunter software was used to acquire the data in dynamic multiple-reaction monitoring mode. The ion-source settings were as follows: drying-gas temperature, 300°C; drying-gas flow, 10 L/min; nebuliser pressure, 40 psi; sheath-gas temperature, 400°C; sheath-gas flow, 10 L/min; and capillary voltage, 3500 V. Compound identity was established by comparing retention times, precursor-to-product ion transitions and ion-ratio consistency with those of reference standards (Hamiti *et al.*, 2025; Oduncuoglu *et al.*, 2026).

Calibration and quantitative reporting

External calibration comprised six nominal levels (15.625, 31.25, 62.5, 125, 250 and 500 ng/mL), each of which was analysed three times. Calibration curves and quantitative processing were generated using MassHunter software. Depending on the analyte, linear or quadratic regression models were applied and individual calibration levels or points were retained in the final regression as reported by the instrument software. The instrument-generated calibration characteristics are provided in Supplementary Table S1. Analyte concentrations were calculated from the corresponding calibration models after application of the required dilution factors and expressed as ng/g of dry extract. Matrix-specific limits of detection (LOD) and quantification (LOQ) were not determined for AAB because dedicated matrix-dependent validation was not performed within the scope of the present analysis. Accordingly, LOD and LOQ values reported for other matrices or analytical applications were not extrapolated to

AAB. Relative abundance was calculated from the quantified concentrations using the following equation:

$$\text{Relative abundance (\%)} = \frac{\text{Concentration of an individual compound}}{\sum \text{Concentrations of all quantified compounds}} \times 100 \quad (2)$$

This quantitative approach provides a comparative representation of the phytochemical profile, with the calculated percentages reflecting the relative abundance of each compound within the pool of 13 quantified constituents and enabling identification of the predominant compounds in the extract.

In-silico pharmacokinetic and toxicological profiling

In-silico assessment included each of the 13 constituents quantified in AAB by LC-MS/MS. Inclusion of the complete quantified profile avoided subjective selection based on presumed pharmacological activity. Canonical simplified molecular-input line-entry system (SMILES) representations for the compounds were retrieved from PubChem (<https://pubchem.ncbi.nlm.nih.gov/>). SwissADME (<http://www.swissadme.ch>) was used to obtain predictions for gastrointestinal (GI) absorption, blood-brain barrier (BBB) penetrability, P-glycoprotein (P-gp) substrate status and compliance with Lipinski's rule of five. The same compound set was subsequently assessed with ProTox-III (<https://tox.charite.de/protox3/>) for predicted oral median lethal dose (LD_{50}), toxicity class, hepatotoxicity, carcinogenicity, mutagenicity, immunotoxicity and cytotoxicity.

The computational results were interpreted as preliminary compound-level predictions rather than experimental evidence of the pharmacokinetic behaviour, efficacy or safety of AAB. These tools evaluate individual compounds and do not fully reproduce the complexity of a crude extract, including constituent interactions, administered concentrations, metabolism and biological variability.

Ethical approval and experimental animals

The study protocol received approval from the Animal Research Ethics Committee, Department of Pharmacology and Toxicology, Usmanu Danfodiyo University, Sokoto, Nigeria (Approval No.: PTAC/As/(Me)/OT/66-26). Animal procedures complied with the institutional ethical requirements and the principles set out in the Guide for the Care and Use of Laboratory Animals (National Research Council, 2011). The study protocol was not prospectively registered in a public repository. Healthy adult Wistar albino rats (*Rattus norvegicus*) of both sexes, aged 7-10 weeks and weighing 180–200 g, were supplied by the Animal House of the Faculty of Pharmaceutical Sciences, Usmanu Danfodiyo University. Before experiments, the animals underwent a 14-day acclimatisation period and were maintained at $25 \pm 2^\circ\text{C}$ with a 12-h light/dark cycle. Standard laboratory feed and water were available *ad libitum*.

Experimental design and animal allocation

The study involved 87 rats in total, of which 12 were assigned solely to the acute oral toxicity experiments. The other 75 animals were divided among three separate efficacy cohorts, each containing 25 rats: one for the acetic acid-induced writhing test, one for the hot-plate test and one for the formalin experiment. Within each efficacy cohort, the 25 animals were distributed into five groups of five animals ($n = 5/\text{group}$): distilled water at 10 mL/kg served as the negative control, AAB was administered at 125, 250 or 500 mg/kg and piroxicam at 20 mg/kg served as the positive control. Formalin-induced nociception behaviour and paw oedema were assessed in the same formalin-treated cohort following the same formalin administration; therefore, no additional animals were used for the paw oedema assessment. Separate animals were used for the acute-toxicity, writhing, hot-plate and formalin experiments.

Sample-size rationale and bias control

Group allocation for acute toxicity followed the sequential Lorke procedure, using three animals at each concentration in Phase I and one animal at each concentration in Phase II. For the efficacy studies, an exploratory group size of five animals was selected with reference to established acute rodent pharmacological screening procedures while also limiting animal use. No formal a priori sample-size or statistical power calculation was performed. No formal randomisation procedure was used for group allocation. Accordingly, no primary outcome was designated for the purpose of sample-size determination. Treatment administration, outcome assessment and statistical analysis were not blinded. These design features are acknowledged as limitations of the present study. No specific strategy was used to control potential confounders related to cage location, treatment order or time of assessment beyond applying the same experimental procedures and environmental conditions across groups.

Preparation and oral administration of treatments

AAB extract was freshly prepared in distilled water immediately prior to oral administration to ensure consistency and stability of the administered preparation. Suspensions were prepared at 12.5, 25 and 50 mg/mL and administered at 10 mL/kg to deliver concentrations of 125, 250 and 500 mg/kg, respectively. The suspensions were mixed thoroughly during preparation and shaken immediately before each administration to maintain uniformity. The same vehicle and dosing volume were used in the acute oral toxicity study. Negative control groups received distilled water at 10 mL/kg. Piroxicam was administered orally at 20 mg/kg as the positive control treatment. All oral treatments were administered by gavage.

Acute oral toxicity study

Acute oral toxicity was evaluated using the two-phase procedure described by Lorke (1983) and Oduncuoglu and

Halilu (2023). In phase I, nine rats were assigned to three groups of three animals ($n = 3/\text{group}$) and each group received a single oral concentration of AAB at 10, 100 or 1000 mg/kg. The animals were observed for 24 h for mortality, behavioural alterations and physical signs of toxicity. In phase II, three additional rats were individually assigned to concentrations of 1600, 2900 or 5000 mg/kg ($n = 1/\text{group}$) and observed for a further 24 h. Thus, a total of 12 rats were used for the acute oral toxicity assessment. Where mortality permitted calculation, the LD_{50} was estimated as the geometric mean of the highest non-lethal concentration and the lowest concentration that produced mortality:

$$LD_{50} = \sqrt{D_0 \times D_{100}} \quad (3)$$

Where D_0 is the highest concentration that produced no mortality and D_{100} is the lowest concentration that produced mortality (Lorke, 1983). If no mortality occurred at the highest tested concentration, the LD_{50} was reported as >5000 mg/kg under the conditions of the acute study (Lorke, 1983; Oduncuoglu and Halilu, 2023).

Acetic acid-induced abdominal writhing test

A separate cohort of 25 rats was used for the acetic acid-induced abdominal writhing test. The animals were allocated to five groups of five animals ($n = 5/\text{group}$). The negative control group received distilled water at 10 mL/kg orally. The treatment groups received AAB at 125, 250 or 500 mg/kg orally, while the positive control group received piroxicam at 20 mg/kg orally. Forty-five minutes after treatment, each rat received an intraperitoneal injection of 1% acetic acid solution at 1 mL/kg. Following a 5-min latency period, the number of abdominal writhes was recorded over 10 min. Percentage inhibition was calculated as follows:

$$\text{Writhing inhibition (\%)} = \frac{W_c - W_t}{W_c} \times 100 \quad (4)$$

Where W_c is the mean number of writhes in the negative control group and W_t is the corresponding mean in a group treated with AAB or piroxicam.

Hot-plate test

A second independent cohort of 25 rats was used for the hot-plate test. Thermal nociceptive responses were evaluated using a hot-plate apparatus maintained at $55 \pm 0.5^\circ\text{C}$. Before group allocation, each animal was placed individually on the apparatus for eligibility screening. Animals that did not display a nociceptive response, defined as hind paw licking, paw shaking or escape jumping, within 15 s were excluded. The 15-s interval represented a pre-treatment eligibility criterion and not the maximum post-treatment exposure time. Eligible animals were allocated to five groups of five rats ($n = 5/\text{group}$) and received one of the following oral treatments: distilled

water at 10 mL/kg, AAB at 125, 250 or 500 mg/kg, or piroxicam at 20 mg/kg. Thirty minutes after treatment, each rat was placed on the hot-plate and the latency to the first nociceptive response was recorded in seconds. A maximum exposure time of 60 s was applied to prevent tissue injury. Piroxicam was selected as the common NSAID reference treatment across the nociceptive and inflammatory assays to facilitate internal comparison among the experimental models.

Formalin-induced nociceptive test

A third independent cohort of 25 rats was used for the formalin experiment. The same animals were assessed for both formalin-induced nociceptive behaviour and formalin-induced paw oedema. The animals were allocated to five groups of five rats ($n = 5/\text{group}$) and received distilled water at 10 mL/kg, AAB at 125, 250 or 500 mg/kg or piroxicam at 20 mg/kg orally. Thirty minutes after treatment, 0.05 mL of 2.5% formalin solution was injected into the plantar surface of the right hind paw. The duration of licking of the injected paw was recorded during the early phase (0-5 min) and the late phase (50-60 min). Percentage inhibition was calculated separately for each phase:

$$\text{Paw - licking inhibition (\%)} = \frac{L_c - L_t}{L_c} \times 100 \quad (5)$$

Where L_c is the mean paw-licking duration in the negative control group and L_t is the corresponding mean in a group treated with AAB or piroxicam.

Formalin-induced paw oedema test

Paw oedema was evaluated in the same 25 rats used for the formalin-induced nociception test following the same formalin injection described above. No additional animals or separate formalin administration were used for this assessment. Paw diameter was measured using a digital vernier calliper (Skole 0-150 mm, model 605035, Frits Pedersen, Denmark) immediately after formalin administration (0 h) and at 1, 2, 3, 4 and 5 h thereafter. Percentage inhibition was determined relative to the negative control group as follows:

$$\text{Paw - oedema inhibition (\%)} = \frac{E_c - E_t}{E_c} \times 100 \quad (6)$$

Where E_c is the mean paw diameter in the negative control group and E_t is the corresponding mean paw diameter in a group treated with AAB or piroxicam at the same time point. Formalin was selected to permit nociceptive behaviour and local oedema to be assessed using the same phlogistic stimulus.

Post-paw oedema analyses

Immediately after the final 5-h paw oedema measurement, blood samples were collected from three of the five animals in each group from the same formalin cohort. The three animals were randomly selected from each group. Approximately 1.2 mL of blood was collected from the tail vein of each selected animal without anaesthesia to avoid potential anaesthetic-related interference with the

measured biochemical and haematological parameters. Samples intended for biochemical and C-reactive protein (CRP) analyses were transferred to serum-separator tubes (SST), whereas samples intended for haematological and erythrocyte sedimentation rate (ESR) analyses were transferred to EDTA-containing tubes.

Serum biochemical parameters

Samples collected in SST were centrifuged at 3000 rpm for 5 min at room temperature. Serum alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin, total protein, total bilirubin and direct bilirubin were measured using a Raytochemray 120 automated biochemical analyser (Rayto Life and Analytical Sciences, Shenzhen, China) according to the manufacturer's assay procedures.

Haematological analyses

Haematological measurements were performed using EDTA-anticoagulated whole blood and a Genesis HA6000 automated haematology analyser (Perlong Medical Equipment, Nanjing, China). The evaluated leucocyte parameters included total white-blood-cell count, lymphocytes, mid-sized cells and granulocytes. Erythrocyte parameters included red-blood-cell count, haemoglobin, haematocrit, mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration and red-cell distribution width. Platelet-related measurements included platelet count, mean platelet volume, platelet distribution width, plateletcrit and platelet large-cell ratio.

Erythrocyte sedimentation rate (ESR)

ESR was determined using the Wintrobe method. EDTA-anticoagulated whole blood was gently mixed and introduced into a Wintrobe tube up to the zero mark while avoiding the formation of air bubbles. The tube was maintained vertically and left undisturbed at room temperature for 60 min. The height of the clear plasma column above the sedimented erythrocytes was recorded in mm/h.

C-reactive protein (CRP)

CRP was quantified in serum using the Anbio CRP Rapid Test Kit, a fluorescence-based immunoassay system (Anbio Biotechnology, Xiamen, China). For each determination, 10 μL of serum was combined with the kit diluent, after which 50 μL of the prepared mixture was dispensed into the assay cartridge. Following a 3-min incubation, the cartridge was inserted into the compatible fluorescence immunoassay analyser, which provided the CRP results in mg/L.

Statistical analysis

All analyses were performed using GraphPad Prism version 10.5.0 (GraphPad Software, Boston, MA, USA). Data are presented as mean $\pm$ SEM. Antinociceptive and anti-inflammatory experiments included five animals per group, whereas biochemical, haematological, ESR and

CRP analyses were based on three animals per group. Data from the acetic acid-induced abdominal writhing test and both phases of the formalin-induced nociception test were analysed separately by ordinary one-way ANOVA, followed by Tukey's test. Variance heterogeneity was observed in the hot-plate assay; therefore, these data were analysed using Brown-Forsythe and Welch ANOVA followed by Dunnett's T3 test. Formalin-induced paw oedema measurements were analysed by two-way repeated-measures ANOVA, with treatment and time included as the main factors and time treated as the repeated-measures factor. The Geisser-Greenhouse correction was applied to account for potential violations of the sphericity assumption. Tukey's test was used to compare treatment groups at individual time points. Variance homogeneity for the biochemical and haematological parameters was assessed using the Brown-Forsythe test. As no significant variance heterogeneity was detected, each parameter was analysed by ordinary one-way ANOVA followed by Dunnett's test, with each treatment group compared with the negative control. ESR and CRP data were assessed using the Kruskal-Wallis test followed by Dunn's test. All reported post-hoc *p*-values were adjusted for multiple comparisons. Statistical significance was accepted at an adjusted *p* < 0.05. Exact adjusted *p*-values are reported where available; in tables and figures, significance is additionally denoted using adjusted *p*-value thresholds of *p* < 0.05, *p* < 0.01, *p* < 0.001 and *p* < 0.0001. Formal effect-size estimates with confidence intervals were not prespecified for this exploratory study; percentage inhibition, mean ± SEM and adjusted *p*-values are reported for the relevant efficacy outcomes.

RESULTS

Extraction yield

The methanolic extraction of 50.0 g of dried AAB material yielded 2.906 g of crude extract, corresponding to an extraction yield of 5.812% (Table 1).

Table 1: The extraction yield of methanolic bulb extract of *Allium ampeloprasum* L.

Solvent	Dried bulb material (g)	Crude extract (g)	Extraction yield (%)
Methanol	50.0	2.906	5.812

Preliminary phytochemical screening

Qualitative phytochemical screening revealed presence of saponins, tannins, phenolics, flavonoids and triterpenoids in the methanolic AAB extract, whereas alkaloids and steroids were not detected (Table 2).

Table 2: Qualitative phytochemical constituent profile of the methanolic bulb extract of *Allium ampeloprasum* L.

Phytochemical Class	Methanolic AAB Extract
Alkaloids	-

Saponins	+
Tannins	+
Phenolics	+
Flavonoids	+
Triterpenoids	+
Steroids	-

Key: +, detected; -, not detected; AAB, *Allium ampeloprasum* bulb.

LC-MS/MS analysis

LC-MS/MS analysis identified and quantified 13 compounds in the AAB extract. Based on the summed concentrations of the quantified analytes, caffeic acid showed the highest relative abundance (49.29%), followed by *trans*-ferulic acid (28.83%), protocatechuic acid (6.84%), jaceosidin (3.83%), *p*-coumaric acid (3.78%), jasmonic acid (3.08%) and abscisic acid (3.04%). Salicylic acid, isorhamnetin, quercetin, gallic acid, apigenin and luteolin were present at lower relative abundances of 0.35% or less (Table 3).

In-silico pharmacokinetic and toxicity predictions

All 13 compounds quantified in the AAB extract were evaluated using SwissADME and ProTox-III. SwissADME predicted high gastrointestinal absorption and no Lipinski's rule-of-five violations for all compounds. None of the compounds was predicted to be a P-glycoprotein substrate. Blood-brain barrier permeation was predicted for *trans*-ferulic acid, *p*-coumaric acid, jasmonic acid, abscisic acid and salicylic acid, whereas the remaining compounds were predicted to be non-permeant (Table 4).

ProTox-III predicted LD₅₀ values ranging from 159 to 11,800 mg/kg. Predicted toxicity alerts varied among the compounds. Salicylic acid was predicted to be active for hepatotoxicity, while mutagenicity alerts were predicted for quercetin and luteolin. Carcinogenicity alerts were predicted for caffeic acid, protocatechuic acid, *p*-coumaric acid, quercetin, gallic acid and luteolin. Immunotoxicity alerts were predicted for *trans*-ferulic acid, jaceosidin and isorhamnetin. None of the compounds was predicted to be active for cytotoxicity (Table 4).

Acute oral toxicity

Oral administration of AAB caused no mortality at any tested concentration. In Phase I, all animals survived following administration of 10, 100 and 1000 mg/kg and no deaths occurred at 1600, 2900 or 5000 mg/kg in Phase II during the 24-h observation period. As no mortality occurred at the highest tested concentration, the oral LD₅₀ was considered to be >5000 mg/kg under the conditions of this acute study. Nevertheless, transient behavioural and physical signs, including calmness or drowsiness, piloerection, heavy breathing, mouth scratching and weakness/leaking of the legs were observed at selected concentrations (Table 5).

Table 3: LC-MS/MS identification and quantification of compounds in the methanolic bulb extract of *Allium ampeloprasum* L.

Compound	Molecular formula	Molecular weight (g/mol)	Retention time (min)	Concentration (ng/g dry extract)	Relative abundance (%) [*]	MS/MS transition (m/z)
Caffeic acid	C ₉ H ₈ O ₄	180.16	5.274	77072.32	49.29	179.0, 135.1
<i>Trans</i> -ferulic acid	C ₁₀ H ₁₀ O ₄	194.18	5.711	45081.40	28.83	193.0, 134.1
Protocatechuic acid	C ₇ H ₆ O ₄	154.12	3.490	10702.97	6.84	153.1, 109.1
Jaceosidin	C ₁₇ H ₁₄ O ₇	330.29	6.524	5984.37	3.83	329.0, 299.2
<i>p</i> -coumaric acid	C ₉ H ₈ O ₃	164.16	5.618	5912.26	3.78	163.1, 118.9
Jasmonic acid	C ₁₂ H ₁₈ O ₃	210.27	6.547	4822.63	3.08	209.0, 59.1
Abscisic acid	C ₁₅ H ₂₀ O ₄	264.32	6.267	4750.15	3.04	262.9, 153.0
Salicylic acid	C ₇ H ₆ O ₃	138.12	5.340	545.12	0.35	136.8, 93.1
Isorhamnetin	C ₁₆ H ₁₂ O ₇	316.26	6.524	538.17	0.34	314.9, 299.9
Quercetin	C ₁₅ H ₁₀ O ₇	302.24	6.246	453.70	0.29	301.0, 150.9
Gallic acid	C ₇ H ₆ O ₅	170.12	3.193	434.38	0.28	168.9, 125.0
Apigenin	C ₁₅ H ₁₀ O ₅	270.24	6.527	33.72	0.02	269.0, 117.0
Luteolin	C ₁₅ H ₁₀ O ₆	286.24	6.265	32.45	0.02	285.0, 133.0

^{*}Relative abundance (%) was calculated by dividing the concentration of each compound by the sum of the concentrations of all 13 quantified compounds and multiplying by 100.

Table 4: *In-silico* pharmacokinetic and toxicity predictions for compounds quantified in the methanolic bulb extract of *Allium ampeloprasum* L.

Compound	GI absorption	BBB permeant	P-gp substrate	Lipinski violations	Predicted LD ₅₀ (mg/kg)	Toxicity class	Predicted toxicity alerts
Caffeic acid	High	No	No	0	2980	5	Carcinogenicity
<i>Trans</i> -ferulic acid	High	Yes	No	0	1772	4	Immunotoxicity
Protocatechuic acid	High	No	No	0	2000	4	Carcinogenicity
Jaceosidin	High	No	No	0	3919	5	Immunotoxicity
<i>p</i> -coumaric acid	High	Yes	No	0	2850	5	Carcinogenicity
Jasmonic acid	High	Yes	No	0	11800	2	-
Abscisic acid	High	Yes	No	0	5000	5	-
Salicylic acid	High	Yes	No	0	1034	4	Hepatotoxicity
Isorhamnetin	High	No	No	0	5000	5	Immunotoxicity
Quercetin	High	No	No	0	159	3	Carcinogenicity Mutagenicity
Gallic acid	High	No	No	0	2000	4	Carcinogenicity
Apigenin	High	No	No	0	2500	5	-
Luteolin	High	No	No	0	3919	5	Carcinogenicity Mutagenicity

GI, gastrointestinal; BBB, blood–brain barrier; P-gp, P-glycoprotein; LD₅₀, median lethal dose; -, no alert. All values and toxicity alerts are computational predictions generated using SwissADME and ProTox-III and do not represent experimentally confirmed pharmacokinetic or toxicological effects.

Table 5: Acute oral toxicity of the methanolic bulb extract of *Allium ampeloprasum* L. in Wistar albino rats during the 24-h observation period.

Extract	Phase	Concentration (mg/kg)	Mortality (dead/total)	Survival at 24 h (%)
<i>Allium ampeloprasum</i> bulb	I	10	0/3	100
		100	0/3	100
		1000	0/3	100
<i>Allium ampeloprasum</i> bulb	II	1600	0/1	100
		2900	0/1	100
		5000	0/1	100

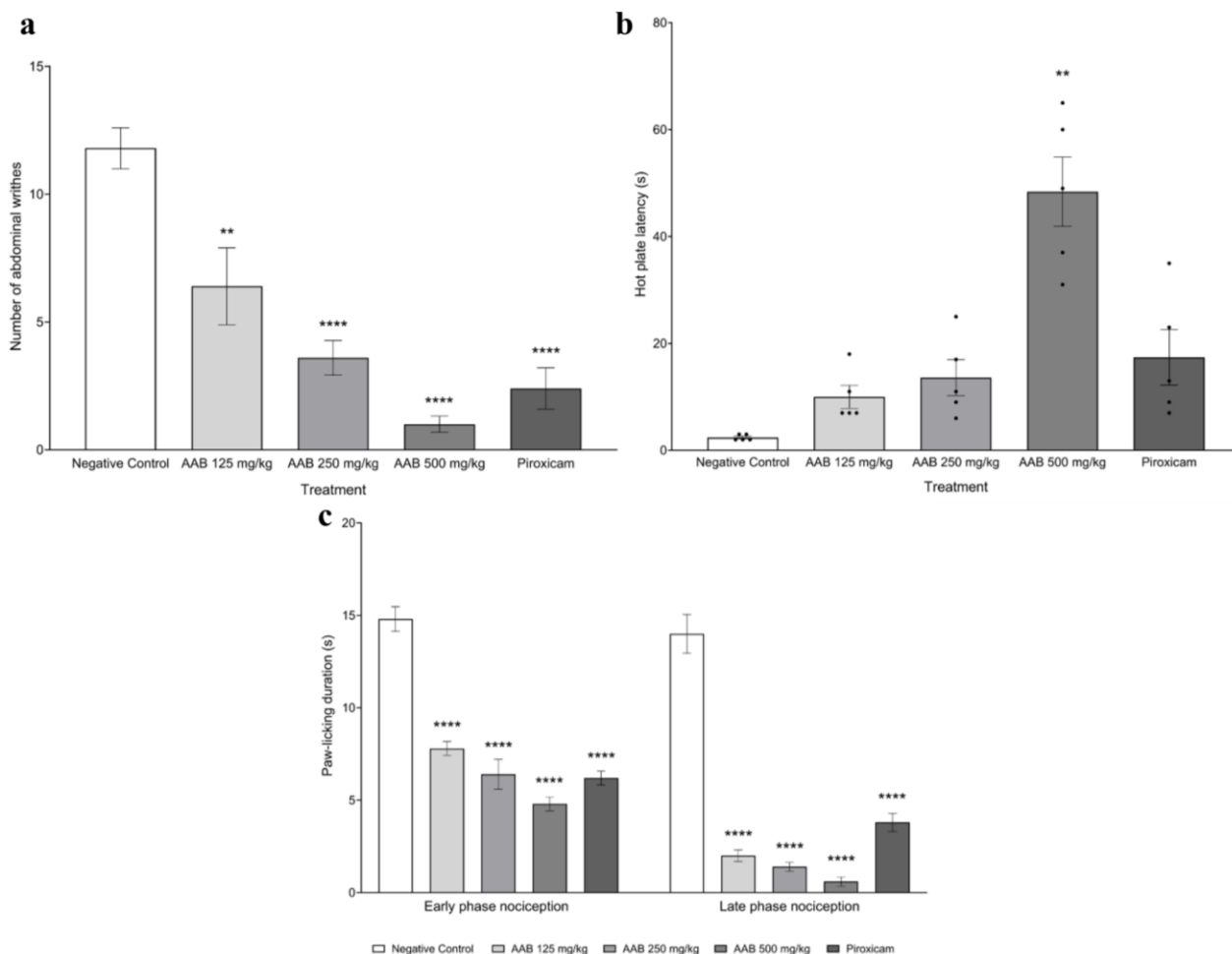


Fig. 1: Antinociceptive effects of the methanolic bulb extract of *Allium ampeloprasum* L. in Wistar albino rats. (a) Number of abdominal writhes in the acetic acid-induced writhing test; (b) Reaction latency in the hot-plate test; (c) Paw-licking duration during the early and late phases of the formalin-induced nociceptive test. Animals received distilled water as the negative control, AAB extract at 125, 250 and 500 mg/kg or piroxicam at 20 mg/kg as the reference drug. Data are mean $\pm$ SEM ($n = 5$ /group), with individual data points shown. Panel (a) and the early and late phases of panel (c) were analysed separately using ordinary one-way ANOVA followed by Tukey's test. Panel (b) was analysed using Brown-Forsythe and Welch ANOVA followed by Dunnett's T3 test. Asterisks indicate significant differences from the negative control based on multiplicity-adjusted p -values: ** $p < 0.01$ and **** $p < 0.0001$. AAB, *Allium ampeloprasum* bulb.

Antinociceptive activity

Acetic acid-induced abdominal writhing test

In the acetic acid-induced writhing test, AAB extract significantly reduced the number of abdominal writhes at all tested concentrations compared with the negative control. The mean number of writhes decreased from 11.80 ± 0.80 in the negative control group to 6.40 ± 1.50 , 3.60 ± 0.68 and 1.00 ± 0.32 at 125, 250 and 500 mg/kg, respectively. These values corresponded to inhibitions of 45.8%, 69.5% and 91.5%. The reductions were significant at 125 mg/kg (adjusted $p = 0.0035$) and at 250 and 500 mg/kg (adjusted $p < 0.0001$). Piroxicam reduced the mean number of writhes to 2.40 ± 0.81 , corresponding to 79.7% inhibition compared with the negative control (adjusted $p < 0.0001$). Although inhibition increased numerically with increasing AAB concentration, the response at 500 mg/kg

did not differ significantly from that produced by piroxicam (adjusted $p = 0.8093$) (Fig. 1a).

Hot-plate test

Hot-plate reaction latency increased numerically across the AAB-treated groups, with the greatest response observed at 500 mg/kg. Mean latency was 2.40 ± 0.24 s in the negative control group and 10.00 ± 2.14 , 13.60 ± 3.37 and 48.40 ± 6.49 s in the AAB 125, 250 and 500 mg/kg groups, respectively. However, only the 500 mg/kg concentration produced a significant increase compared with the negative control (adjusted $p = 0.0067$). The increases observed at 125 and 250 mg/kg were not statistically significant (adjusted $p = 0.0749$ and adjusted $p = 0.0899$, respectively). Piroxicam produced a mean latency of 17.40 ± 5.19 s, which did not differ significantly from the negative control (adjusted $p = 0.1336$) (Fig. 1b).

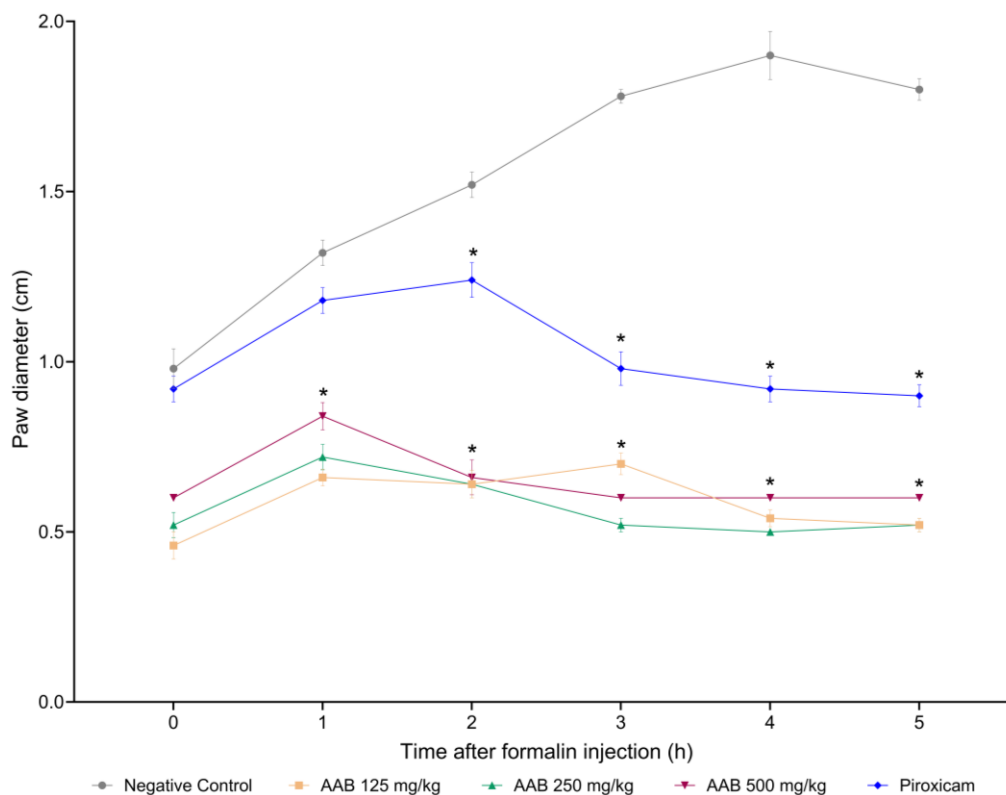


Fig. 2: Effect of the methanolic bulb extract of *Allium ampeloprasum* L. on formalin-induced paw oedema. Paw diameter was measured at 0-5 h following formalin injection. Animals received distilled water as the negative control, AAB extract at 125, 250 and 500 mg/kg or piroxicam at 20 mg/kg as the reference drug. Data are mean $\pm$ SEM ($n = 5/\text{group}$). Statistical analysis was performed using two-way repeated-measures ANOVA with Geisser-Greenhouse correction followed by Tukey's test. Asterisks indicate significant differences versus the negative control at the post-formalin time points from 1 to 5 h (adjusted $p < 0.05$); exact adjusted p -values are reported in the Results. AAB, *Allium ampeloprasum* bulb.

Formalin-induced nociception test

All tested concentrations of AAB significantly reduced paw-licking duration during both phases of the formalin test compared with the negative control (adjusted $p < 0.0001$ for all comparisons). During the early phase, paw-licking duration decreased from 14.80 ± 0.66 s in the negative control group to 7.80 ± 0.37 , 6.40 ± 0.81 and 4.80 ± 0.37 s at 125, 250 and 500 mg/kg, respectively. These reductions corresponded to inhibitions of 47.30%, 56.76% and 67.57%. Piroxicam produced a paw-licking duration of 6.20 ± 0.37 s, corresponding to 58.11% inhibition. Among the AAB-treated groups, only the difference between 125 and 500 mg/kg was significant (adjusted $p = 0.0079$).

During the late phase, paw-licking duration decreased from 14.00 ± 1.05 s in the negative control group to 2.00 ± 0.32 , 1.40 ± 0.24 and 0.60 ± 0.24 s at 125, 250 and 500 mg/kg, respectively. These values corresponded to inhibitions of 85.71%, 90.00% and 95.71%. Piroxicam produced a paw-licking duration of 3.80 ± 0.49 s, corresponding to 72.86% inhibition. Paw-licking durations at 250 and 500 mg/kg were significantly lower than those in the piroxicam group (adjusted $p = 0.0455$ and adjusted $p = 0.0050$, respectively) (Fig. 1c).

Formalin-induced paw oedema test

Following formalin administration, paw diameter increased progressively in the negative control group, from 1.32 ± 0.04 cm at 1 h to a maximum of 1.90 ± 0.07 cm at 4 h, before decreasing slightly to 1.80 ± 0.03 cm at 5 h. In contrast, all tested concentrations of AAB produced a rapid and sustained reduction in paw diameter throughout the 1-5 h observation period. Each AAB-treated group differed significantly from the negative control at every post-formalin time point (all adjusted $p \leq 0.0003$). At 3 h, paw diameter values were 0.70 ± 0.03 , 0.52 ± 0.02 and 0.60 ± 0.00 cm at 125, 250 and 500 mg/kg, respectively, corresponding to inhibitions of 60.67%, 70.79% and 66.29%. The inhibitory effect reached 71.58%, 73.68% and 68.42% at 4 h and remained pronounced at 5 h, with respective inhibitions of 71.11%, 71.11% and 66.67%. Piroxicam did not significantly reduce paw diameter at 1 h (adjusted $p = 0.1506$), but its effect became significant from 2 h onwards. Piroxicam produced 44.94%, 51.58% and 50.00% inhibition at 3, 4 and 5 h, respectively. Overall, the AAB-induced reduction in paw oedema was maintained throughout the observation period, with the greatest inhibition generally observed at 250 mg/kg (Fig. 2).

Table 6: Serum biochemical parameters measured following the formalin-induced paw oedema test in Wistar albino rats. Animals received distilled water as the negative control, methanolic *Allium ampeloprasum* L. bulb (AAB) extract at 125, 250 and 500 mg/kg, or piroxicam at 20 mg/kg as the reference drug. Data are mean $\pm$ SEM ($n = 3$). Statistical analysis was performed using one-way ANOVA followed by Dunnett's test versus the negative control. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

Groups	Negative Control	AAB 125 (mg/kg)	AAB 250 (mg/kg)	AAB 500 (mg/kg)	Positive Control
ALP (U/L)	203.00 $\pm$ 30.29	535.00 $\pm$ 121.97*	278.00 $\pm$ 47.72	288.00 $\pm$ 22.30	302.00 $\pm$ 35.79
ALT (U/L)	6.67 $\pm$ 1.76	20.00 $\pm$ 6.43	10.67 $\pm$ 3.18	13.00 $\pm$ 6.00	5.67 $\pm$ 2.33
AST (U/L)	38.67 $\pm$ 1.45	13.67 $\pm$ 3.38***	11.67 $\pm$ 5.24***	8.33 $\pm$ 0.33****	3.67 $\pm$ 1.20****
Albumin (g/dL)	3.50 $\pm$ 0.06	2.53 $\pm$ 0.13**	2.80 $\pm$ 0.25*	2.57 $\pm$ 0.09**	2.70 $\pm$ 0.12**
Total protein (g/dL)	7.10 $\pm$ 0.25	9.57 $\pm$ 0.13*	8.90 $\pm$ 0.10	6.67 $\pm$ 0.79	8.00 $\pm$ 0.72
Total bilirubin (mg/dL)	0.92 $\pm$ 0.01	1.24 $\pm$ 0.04**	1.16 $\pm$ 0.10*	0.97 $\pm$ 0.04	1.00 $\pm$ 0.01
Direct bilirubin (mg/dL)	0.16 $\pm$ 0.01	0.24 $\pm$ 0.01*	0.25 $\pm$ 0.04*	0.21 $\pm$ 0.01	0.20 $\pm$ 0.01

Table 7: Haematological parameters measured following the formalin-induced paw oedema test in Wistar albino rats. Animals received distilled water as the negative control, methanolic *Allium ampeloprasum* L. bulb (AAB) extract at 125, 250 and 500 mg/kg or piroxicam at 20 mg/kg as the reference drug. Data are mean $\pm$ SEM ($n = 3$). Statistical analysis was performed using one-way ANOVA followed by Dunnett's test versus the negative control. * $p < 0.05$ and ** $p < 0.01$.

Groups	Negative control	AAB 125 (mg/kg)	AAB 250 (mg/kg)	AAB 500 (mg/kg)	Positive control
WBC ($10^9/L$)	16.70 $\pm$ 6.65	19.23 $\pm$ 2.41	14.10 $\pm$ 2.03	12.40 $\pm$ 0.80	13.40 $\pm$ 3.20
Lymph ($10^9/L$)	12.07 $\pm$ 5.29	14.90 $\pm$ 2.80	9.70 $\pm$ 1.31	8.40 $\pm$ 0.74	7.90 $\pm$ 1.27
Mid ($10^9/L$)	1.23 $\pm$ 0.43	1.07 $\pm$ 0.15	0.83 $\pm$ 0.19	0.87 $\pm$ 0.07	1.10 $\pm$ 0.23
Gran ($10^9/L$)	3.40 $\pm$ 1.07	3.27 $\pm$ 0.57	3.57 $\pm$ 0.58	3.13 $\pm$ 0.23	4.40 $\pm$ 1.85
Lymph (%)	70.10 $\pm$ 5.40	76.10 $\pm$ 5.86	68.87 $\pm$ 1.22	67.63 $\pm$ 2.32	61.17 $\pm$ 5.46
Mid (%)	7.50 $\pm$ 0.35	5.37 $\pm$ 0.07**	5.70 $\pm$ 0.46*	6.90 $\pm$ 0.12	8.43 $\pm$ 0.58
Gran (%)	22.40 $\pm$ 5.42	18.53 $\pm$ 5.90	25.43 $\pm$ 1.31	25.47 $\pm$ 2.32	30.40 $\pm$ 6.01
RBC ($10^{12}/L$)	6.02 $\pm$ 0.27	6.45 $\pm$ 0.36	6.28 $\pm$ 0.37	6.14 $\pm$ 0.30	7.20 $\pm$ 0.19
HGB (g/dL)	11.80 $\pm$ 0.76	12.10 $\pm$ 0.40	11.63 $\pm$ 0.74	11.67 $\pm$ 0.38	13.90 $\pm$ 0.42
HCT (%)	37.17 $\pm$ 1.57	38.90 $\pm$ 2.06	36.80 $\pm$ 1.76	36.17 $\pm$ 1.42	44.57 $\pm$ 1.39*
MCV (fL)	61.80 $\pm$ 0.17	60.37 $\pm$ 0.65	58.60 $\pm$ 0.90	58.93 $\pm$ 0.94	62.03 $\pm$ 3.04
MCH (pg)	19.60 $\pm$ 0.40	18.77 $\pm$ 0.43	18.47 $\pm$ 0.07	19.03 $\pm$ 0.27	19.37 $\pm$ 0.94
MCHC (g/dL)	31.73 $\pm$ 0.70	31.10 $\pm$ 0.65	31.57 $\pm$ 0.58	32.27 $\pm$ 0.23	31.20 $\pm$ 0.06
RDW-CV (%)	17.37 $\pm$ 1.31	19.67 $\pm$ 1.24	18.53 $\pm$ 0.43	17.77 $\pm$ 0.59	18.70 $\pm$ 1.62
RDW-SD (fL)	34.27 $\pm$ 2.64	38.07 $\pm$ 2.81	34.77 $\pm$ 1.37	33.43 $\pm$ 1.68	37.17 $\pm$ 4.42
PLT ($10^9/L$)	594.33 $\pm$ 76.38	511.67 $\pm$ 56.42	430.33 $\pm$ 7.86	442.33 $\pm$ 32.27	542.00 $\pm$ 17.56
MPV (fL)	6.53 $\pm$ 0.19	6.73 $\pm$ 0.18	6.53 $\pm$ 0.12	6.47 $\pm$ 0.13	6.67 $\pm$ 0.13
PDW	14.47 $\pm$ 0.09	14.53 $\pm$ 0.03	14.40 $\pm$ 0.00	14.43 $\pm$ 0.03	14.57 $\pm$ 0.09
PCT (mL/L)	3.87 $\pm$ 0.47	3.42 $\pm$ 0.37	2.82 $\pm$ 0.10	4.19 $\pm$ 1.32	3.62 $\pm$ 0.17
P-LCR (%)	6.67 $\pm$ 0.90	7.90 $\pm$ 0.85	6.80 $\pm$ 0.35	6.57 $\pm$ 0.62	7.50 $\pm$ 0.91

Biochemical, haematological and inflammatory parameters

Serum biochemical parameters

Serum biochemical parameters measured after completion of the formalin-induced paw oedema test are presented in Table 6. Compared with the negative control, ALP was significantly increased only at 125 mg/kg (535.00 $\pm$ 121.97 versus 203.00 $\pm$ 30.29 U/L; adjusted $p = 0.0129$), whereas ALT did not differ significantly among the groups. AST was significantly lower at 125, 250 and 500 mg/kg, with values of 13.67 $\pm$ 3.38, 11.67 $\pm$ 5.24 and 8.33 $\pm$ 0.33 U/L, respectively, compared with 38.67 $\pm$ 1.45 U/L in the negative control group (adjusted $p = 0.0004$, $p = 0.0002$ and $p < 0.0001$, respectively). Albumin was also

significantly lower at all AAB concentrations (adjusted $p = 0.0028$, $p = 0.0215$ and $p = 0.0036$, respectively). Total protein was significantly increased only at 125 mg/kg (adjusted $p = 0.0142$). Total bilirubin was significantly increased at 125 and 250 mg/kg (adjusted $p = 0.0041$ and $p = 0.0244$, respectively). Direct bilirubin was also significantly increased at these concentrations (adjusted $p = 0.0287$ and $p = 0.0185$, respectively). The piroxicam group showed significantly lower AST and albumin levels than the negative control group (adjusted $p < 0.0001$ and $p = 0.0098$, respectively), whereas the remaining biochemical parameters did not differ significantly. The observed changes did not follow a consistent concentration-dependent pattern.

Table 8: Erythrocyte sedimentation rate and C-reactive protein levels measured following the formalin-induced paw oedema test in Wistar albino rats. Animals received distilled water as the negative control, methanolic *Allium ampeloprasum* L. bulb (AAB) extract at 125, 250 and 500 mg/kg or piroxicam at 20 mg/kg as the reference drug. Data are mean $\pm$ SEM ($n = 3$). Statistical analysis was performed using the Kruskal-Wallis test followed by Dunn's test versus the negative control.

Group	Erythrocyte sedimentation rate (mm/h)	C-reactive protein (mg/L)
Negative Control	1.33 $\pm$ 0.33	1.42 $\pm$ 0.05
AAB 125 (mg/kg)	2.67 $\pm$ 1.20	1.36 $\pm$ 0.03
AAB 250 (mg/kg)	2.33 $\pm$ 0.67	1.67 $\pm$ 0.18
AAB 500 (mg/kg)	2.00 $\pm$ 1.00	1.64 $\pm$ 0.12
Positive Control	1.00 $\pm$ 0.00	1.64 $\pm$ 0.07

Haematological parameters

Most haematological parameters measured after the formalin-induced paw oedema test did not differ significantly between the AAB-treated groups and the negative control (Table 7). The percentage of mid-sized cells (Mid%) was significantly lower at 125 mg/kg (5.37 $\pm$ 0.07%, adjusted $p = 0.0075$) and 250 mg/kg (5.70 $\pm$ 0.46%, adjusted $p = 0.0208$), but not at 500 mg/kg. No significant differences were observed in the absolute MID count or in the remaining leucocyte, erythrocyte and platelet indices following AAB treatment. In the positive control group, haematocrit was significantly higher than in the negative control group (44.57 $\pm$ 1.39% compared with 37.17 $\pm$ 1.57%, adjusted $p = 0.0324$). Overall, the observed haematological changes were limited and did not show a consistent concentration-dependent pattern.

Erythrocyte sedimentation rate and C-reactive protein

Following completion of the formalin-induced paw oedema test, the mean ESR values were 1.33 $\pm$ 0.33 mm/h in the negative control group and 2.67 $\pm$ 1.20, 2.33 $\pm$ 0.67 and 2.00 $\pm$ 1.00 mm/h in the AAB 125, 250 and 500 mg/kg groups, respectively. The positive control group showed an ESR value of 1.00 $\pm$ 0.00 mm/h. Mean CRP levels were 1.42 $\pm$ 0.05 mg/L in the negative control group and 1.36 $\pm$ 0.03, 1.67 $\pm$ 0.18 and 1.64 $\pm$ 0.12 mg/L upon treatment with AAB at 125, 250 and 500 mg/kg, respectively. The corresponding value in the positive control group was 1.64 $\pm$ 0.07 mg/L. Neither ESR [$H(4)=3.654$, $p = 0.5091$] nor CRP [$H(4)=6.059$, $p = 0.2009$] differed significantly among the groups. Dunn's test also identified no significant differences between the negative control and any treatment group (Table 8).

DISCUSSION

This study provides an integrated locality-specific phytochemical and *in-vivo* pharmacological assessment of the methanolic bulb extract of wild *Allium ampeloprasum* L. collected in Cyprus. LC-MS/MS analysis identified and quantified 13 phenolic and related constituents, while the *in-vivo* experiments demonstrated significant antinociceptive activity across chemical and thermal nociception models together with marked inhibition of formalin-induced paw oedema. In addition, no mortality

occurred during acute oral toxicity testing at concentrations up to 5000 mg/kg. Collectively, these findings establish a chemical and pharmacological profile for the Cypriot accession and provide experimental evidence supporting the antinociceptive and local anti-inflammatory potential of AAB.

The phytochemical findings expand the available information on *A. ampeloprasum* by characterising wild bulb material collected specifically in Cyprus. Caffeic acid and *trans*-ferulic acid represented the predominant quantified constituents, followed by protocatechuic acid, jaceosidin, *p*-coumaric acid, jasmonic acid and abscisic acid, while several additional phenolic acids and flavonoids were detected at lower relative abundances. Previous investigations of *A. ampeloprasum* from other geographical origins have similarly reported phenolic acids and flavonoids, including gallic acid, *p*-coumaric acid, luteolin, quercetin and isorhamnetin (Emir *et al.*, 2022). The contribution of the present work therefore lies not in proposing that these compounds are unique to Cyprus but in establishing the phytochemical composition of this locally collected accession and linking the same characterised extract with its *in-vivo* pharmacological activities. As geographical origin, plant organ, environmental conditions, extraction procedure and analytical methodology can influence reported phytochemical profiles, direct quantitative comparisons among geographically distinct populations would require studies conducted under harmonised conditions (Heinrich *et al.*, 2022; Xu *et al.*, 2022; Horablaga *et al.*, 2023).

The predominance of phenolic acids and the presence of several flavonoids are pharmacologically relevant to the biological responses observed in the present study. Caffeic acid and its derivatives, *trans*-ferulic acid, quercetin and luteolin have previously demonstrated anti-inflammatory and/or antinociceptive activities in experimental systems (Stompor-Gorący and Machaczka, 2021; Ntalouka and Tsirivakou, 2023; Takeda *et al.*, 2024; Muheyuddeen *et al.*, 2026). Their occurrence in AAB therefore provides a plausible phytochemical context for the observed *in-vivo* activity. Nevertheless, because the individual contributions of these constituents were not examined experimentally, the pharmacological effects observed in the present study

are most appropriately attributed to the crude extract rather than to any single constituent or molecular pathway.

The quantitative LC-MS/MS analysis further defined the chemical characteristics of AAB and enabled comparison of the relative contributions of the 13 quantified analytes. External calibration was performed across defined concentration ranges and the corresponding instrument-generated calibration characteristics are reported to provide transparency regarding the quantitative analysis. Matrix-specific LOD and LOQ values were not determined because dedicated matrix-dependent validation was outside the scope of the present analysis. Calibration performance varied among analytes; therefore, the reported concentrations and relative abundances should be interpreted within the analytical conditions of the present study rather than as fully validated absolute compositional values. The relative abundance values represent the contribution of each compound to the summed concentration of the 13 quantified analytes rather than the absolute composition of the crude extract. Further matrix-specific validation and extract standardisation would strengthen future quantitative investigations and facilitate reproducibility between preparations.

The *in-silico* analysis complemented the phytochemical findings by providing compound-level pharmacokinetic and toxicological predictions for all 13 quantified constituents. SwissADME predicted high gastrointestinal absorption and no Lipinski's rule-of-five violations for all compounds, while several constituents were predicted to cross the blood-brain barrier. ProTox-III additionally indicated differing predicted toxicological profiles among the constituents. These findings provide useful information for prioritising compounds for further pharmacokinetic, toxicological and mechanistic investigations. As the computational platforms evaluate individual molecular structures, the predictions are most appropriately regarded as complementary compound-level information rather than direct measurements of the pharmacokinetic or toxicological behaviour of the complete AAB extract.

Acute oral toxicity testing produced an important preliminary toxicological finding, as no mortality occurred following administration of AAB at any tested concentration up to 5000 mg/kg, corresponding to an oral LD₅₀ > 5000 mg/kg under the conditions of the study. Transient behavioural and physical signs, including calmness or drowsiness, piloerection, heavy breathing, mouth scratching and weakness/leaking of the legs were nevertheless observed at selected concentrations. These findings demonstrate the absence of mortality under the acute experimental conditions while indicating that further toxicological characterisation is warranted. As the Lorke procedure evaluates acute exposure, longer-duration studies involving repeated administration will be required to characterise the consequences of prolonged exposure (Lorke, 1983).

In the acetic acid-induced abdominal writhing test, AAB produced a pronounced antinociceptive response, with inhibition increasing from 45.8% at 125 mg/kg to 69.5% at 250 mg/kg and 91.5% at 500 mg/kg. All tested concentrations differed significantly from the negative control and the response obtained at 500 mg/kg did not differ significantly from that produced by piroxicam. The acetic acid-induced writhing test is sensitive to chemically induced visceral nociception involving peripheral nociceptive and inflammatory processes and is widely used for the evaluation of antinociceptive activity (da Fonsêca *et al.*, 2025). The present findings therefore demonstrate pronounced antinociceptive activity of AAB in this model and are consistent with an effect on peripheral nociceptive processes. Previous studies have similarly reported antinociceptive activity for *A. ampeloprasum* and for individual constituents identified in the present extract, providing further biological context for the observed activity (Abbas, 2019; Stompor-Gorący and Machaczka, 2021; Takeda *et al.*, 2024).

The hot-plate test provided complementary information on the response of AAB to thermal nociceptive stimulation. Reaction latency increased across AAB-treated groups, although statistical significance compared with the negative control was reached only at 500 mg/kg. This finding indicates that AAB influenced thermal nociceptive responsiveness at the highest tested concentration. The hot-plate model is commonly used to investigate responses involving supraspinal processing of thermal nociceptive stimuli; however, the present experiment alone does not identify the neuronal or receptor pathways involved (Modi *et al.*, 2023). Piroxicam was selected as a common NSAID reference treatment across the nociceptive and inflammatory experiments allowing internal comparison among the models. Although opioid analgesics are more conventional positive controls for the hot-plate test, the use of piroxicam does not detract from the significant response observed with AAB at 500 mg/kg, but it limits conclusions regarding any specific centrally mediated or opioidergic mechanism.

The formalin-induced nociception experiment further demonstrated the antinociceptive activity of AAB across both phases of the biphasic formalin response. All tested concentrations significantly reduced paw-licking duration during both the early and late phases, with late-phase inhibition reaching 85.71-95.71%. At 250 and 500 mg/kg, late-phase paw-licking durations were also significantly lower than those observed in the piroxicam-treated group. The early phase of the formalin response is predominantly associated with direct nociceptor activation, whereas the late phase involves a substantial inflammatory component together with ongoing nociceptive processing (Hirota *et al.*, 2023; Elgendy *et al.*, 2025). The marked inhibition observed in both phases therefore demonstrates activity across different components of formalin-induced

nociception, while the particularly pronounced late-phase response is consistent with the activity observed in the writhing and paw oedema experiments. Specific involvement of cytokines, cyclooxygenase enzymes, oxidative-stress pathways or particular receptors cannot be established from the present experiments and remains an appropriate subject for further mechanistic investigation.

Formalin-induced paw oedema was assessed in the same animals used for the formalin-induced nociception experiment following the same formalin injection, thereby allowing nociceptive behaviour and local inflammatory swelling to be evaluated within a single experimental cohort without the use of additional animals. AAB significantly reduced paw diameter compared with the negative control throughout the post-formalin observation period and inhibition remained pronounced through 5 h. The greatest inhibition was generally observed at 250 mg/kg, while the magnitude of inhibition did not increase uniformly with increasing AAB concentration. Formalin produces local inflammatory swelling through vascular and inflammatory processes and therefore provides a useful complementary endpoint to formalin-induced nociceptive behaviour (Teixeira and Faria, 2021; Alabi *et al.*, 2024; Elgendy *et al.*, 2025). Although carrageenan-induced paw oedema is more commonly used for anti-inflammatory screening, formalin was selected in the present study to permit the nociceptive and oedematous components of the same inflammatory stimulus to be evaluated together. Overall, the sustained reduction in paw diameter supports the local anti-inflammatory activity of AAB under the conditions of this model.

Biochemical, haematological, ESR and CRP analyses were performed after completion of the formalin-induced nociception and 5-h paw oedema assessments in animals from the same formalin cohort. Within this post-formalin assessment, serum biochemical analysis revealed a mixed pattern of changes rather than a uniform concentration-related response. The increase in ALP and bilirubin at selected AAB concentrations, together with the reduction in albumin, indicate that hepatic and metabolic parameters were affected and warrant further investigation. However, ALT remained unchanged, AST decreased rather than increased across the AAB-treated groups and the alterations did not become progressively greater with increasing AAB concentration. Moreover, reductions in AST and albumin were also observed in the piroxicam group, suggesting that some of the biochemical variation may not be specific to AAB. The influence of the formalin challenge, treatment and acute experimental conditions therefore cannot be separated completely. Taken together, the biochemical findings do not demonstrate a clear concentration-dependent pattern of hepatic injury but the changes in ALP, bilirubin and albumin justify further evaluation in dedicated longer-duration toxicity studies.

The haematological findings were comparatively stable, with most leucocyte, erythrocyte and platelet-related parameters remaining unchanged following AAB administration. The isolated reductions in Mid% at the lower concentrations were not accompanied by corresponding changes in the absolute MID count or by broader alterations in the measured blood-cell indices and no consistent pattern was evident across increasing AAB concentrations. This suggests that the observed differences were limited rather than indicative of a generalised haematological response. However, because the measurements were obtained from three animals per group after the formalin experiment, more extensive repeated-administration studies will be required to characterise any longer-term haematological effects.

The absence of significant differences in ESR and CRP indicates that no detectable treatment-related differences in these systemic inflammatory markers were present at the post-formalin sampling time. This finding should be viewed alongside, rather than in opposition to the marked reduction in paw oedema, because the endpoints reflect different aspects of the inflammatory response. Paw diameter directly assessed the local response to formalin, whereas ESR and CRP provide broader systemic measures (Mouliou, 2023; Tishkowski and Zubair, 2025). The results therefore suggest that the local anti-inflammatory activity observed in the paw was not accompanied by measurable changes in the systemic markers under the conditions and timing of the present experiment.

Taken together, the pharmacological findings demonstrate a coherent pattern of activity across complementary experimental models. AAB reduced chemically induced visceral nociception, increased thermal-response latency at the highest tested concentration, suppressed both phases of formalin-induced nociception and produced a sustained reduction in formalin-induced paw oedema. The particularly pronounced responses observed in the writhing and formalin models, together with the reduction in local oedema, support the antinociceptive and local anti-inflammatory potential of the Cypriot *A. ampeloprasum* bulb extract. The identified phenolic acids, flavonoids and related constituents provide relevant phytochemical context for these biological effects, while the relative contribution of individual constituents and the molecular pathways involved remain to be investigated.

Several limitations should be considered when interpreting the present findings. The efficacy experiments were exploratory, with five animals per group and no formal power calculation, randomisation or blinding, while post-formalin blood analyses were based on three animals per group. Complete matrix-specific LC-MS/MS validation and batch-to-batch reproducibility were not established. Piroxicam was used as a common reference treatment, although an opioid comparator would be more

conventional for the hot-plate test and formalin-induced paw oedema is less commonly used than the carrageenan model. Finally, the study did not directly investigate specific molecular mechanisms or the effects of repeated AAB administration. Further repeated-concentration toxicity studies incorporating histopathological evaluation of major organs would provide a more comprehensive assessment of the toxicological profile of AAB.

Overall, the present study established a locality-specific phytochemical and pharmacological baseline for wild Cypriot *A. ampeloprasum* bulb extract. The combined phytochemical, computational and *in-vivo* findings demonstrate significant antinociceptive and local anti-inflammatory activity and support further investigation of AAB as a source of pharmacologically relevant constituents. Further studies incorporating reproducible extract standardisation, matrix-validated quantitative analysis, targeted mechanistic evaluation and longer-duration toxicological assessment will further define the biological basis, reproducibility and broader pharmacological significance of these findings.

CONCLUSION

This study provides an integrated locality-specific phytochemical and *in-vivo* pharmacological assessment of Cypriot *Allium ampeloprasum* L. bulb extract. LC-MS/MS identified and quantified 13 constituents and AAB demonstrated antinociceptive activity in acute chemical and thermal nociception models and reduced formalin-induced paw oedema. No mortality occurred following single oral concentrations up to 5000 mg/kg; however, the transient clinical signs observed during acute toxicity testing and selected post-oedema biochemical alterations preclude a conclusion of safety from the present study. The *in-silico* findings and reported biological activities of individual constituents provide biological plausibility but do not establish the mechanisms responsible for the effects of the crude extract. Further studies should address marker-based extract standardisation, batch-to-batch reproducibility, matrix-specific analytical validation, targeted mechanistic endpoints and repeated-concentration toxicity.

Acknowledgments

The authors gratefully acknowledge the Trakya University Technology Research and Development Application and Research Center (TÜTAGEM), Edirne, Türkiye, for conducting the LC-MS/MS analyses of the submitted extract samples and for providing the associated analytical data.

Author's contributions

YCY: Conducted the research, collected the data, performed data analysis and interpretation and drafted the manuscript; EMH: Contributed to data interpretation and

critically reviewed the manuscript; OI: Conceptualised and designed the study, contributed to data analysis and interpretation and critically revised the manuscript. All authors contributed to manuscript preparation and approved the final version for publication.

Funding

This research received no specific funding.

Data availability statement

The datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethical approval

All animal experiments were approved by the Animal Research Ethics Committee, Department of Pharmacology and Toxicology, Usmanu Danfodiyo University, Sokoto, Nigeria (Approval No.: PTAC/As/(Me)/OT/66-26). All procedures were conducted in accordance with institutional ethical requirements and the principles described in the Guide for the Care and Use of Laboratory Animals. This study was performed in adherence with the ARRIVE guidelines. See supplementary file for the ARRIVE checklist.

Conflict of interest

The authors declare no conflict of interest.

REFERENCES

- Abbas MA (2019). Analgesic effect of *Allium ampeloprasum*: Evidence for the involvement of beta-adrenergic system. *J. Funct. Foods*, **57**: 1-6.
- Alabi MA, Adigun TO, Ajagun EJ, Adeegbe JF, Ibrahim TH, Na'Allah A, Afolabi FJ, Aladodo RA, Abdulsalam TA, Kareem FA, Aransiola SA, Maddela NR and Prasad R (2024). *Syzygium aromaticum* ethanol extract mitigates formalin-induced inflammatory oedema: *In-vivo* evaluation and molecular mechanism exploration. *S. Afr. J. Bot.*, **172**: 598-608.
- Alam A, Al Arif Jahan A, Bari MS, Khandokar L, Mahmud MH, Junaid M, Chowdhury MS, Khan MF, Seidel V and Haque MA (2023). Allium vegetables: Traditional uses, phytoconstituents and beneficial effects in inflammation and cancer. *Crit. Rev. Food Sci. Nutr.*, **63**(23): 6580-6614.
- Alorfi NM (2023). Pharmacological methods of pain management: Narrative review of medication used. *Int. J. Gen. Med.*, **16**: 3247-3256.
- Carullo G, Borghini F, Fusi F, Saponara S, Fontana A, Pozzetti L, Fedeli R, Panti A, Gorelli B, Aquino G, Basilicata MG, Pepe G, Campiglia P, Biagiotti S, Gemma S, Butini S, Pianezze S, Loppi S, Cavaglioni A, Perini M and Campiani G (2024). Traceability and authentication in agri-food production: A multivariate

- approach to the characterization of the Italian food excellence elephant garlic (*Allium ampeloprasum* L.), a vasoactive nutraceutical. *Food Chem.*, **444**: 138684.
- da Fonsêca DV, Rocha JS, da Silva PR, de Sa Novaes Pereira HN, dos Santos LVN, de Santana MAD, Alves AF, Pontes AHO, de Souza Gomes J, Felipe CFB, de Sousa DP, Scotti MT and Scotti L (2025). 4-Hydroxycoumarin exhibits antinociceptive and anti-inflammatory effects through cytokine modulation: An integrated *in-silico* and *in-vivo* study. *Int. J. Mol. Sci.*, **26**(6): 2788.
- Elgendy E, Zaky A, Wahby M, Landry M and Bassiouny A (2025). Targeting APE1/Ref-1 to alleviate formalin-induced pain and spinal neuro-inflammation in rats: A promising therapeutic approach. *Front. Neurosci.*, **19**: 1542264.
- Emir C, Coban G and Emir A (2022). Metabolomics profiling, biological activities and molecular docking studies of elephant garlic (*Allium ampeloprasum* L.). *Process Biochem.*, **116**: 49-59.
- Gorrepati K, Krishna R, Singh S, Shirsat DV, Soumia PS and Mahajan V (2024). Harnessing the nutraceutical and therapeutic potential of *Allium* spp.: Current insights and future directions. *Front. Nutr.*, **11**: 1497953.
- Hamiti X, Shallari G, Pupuleku B, Yücel A, Çelik S, Sulejmani E and Lazo P (2025). Phenolic profiling of Albanian honeys by LC-MS/MS: Gallic acid as a predictive marker of antioxidant potential. *Molecules*, **30**(20): 4037.
- Heinrich M, Jalil B, Abdel-Tawab M, Echeverria J, Kulic Z, McGaw LJ, Pezzuto JM, Potterat O and Wang JB (2022). Best practice in the chemical characterisation of extracts used in pharmacological and toxicological research - The ConPhyMP-Guidelines. *Front. Pharmacol.*, **13**: 953205.
- Hirota I, Koyama Y and Shimada S (2023). Histochemical analysis of the biphasic properties of formalin pain-induced behavior. *Biochem. Biophys. Rep.*, **34**: 101467.
- Horablaga NM, Cozma A, Alexă E, Obistioiu D, Cocan I, Poiana MA, Lalescu D, Pop G, Imbrea IM and Buzna C (2023). Influence of sample preparation/extraction method on the phytochemical profile and antimicrobial activities of 12 commonly consumed medicinal plants in Romania. *Appl. Sci.*, **13**(4): 2530.
- Kaur B, Kumar N, Patel MK, Chopra K and Saxena S (2023). Validation of traditional claims of anti-arthritis efficacy of trans-Himalayan snow mountain garlic (*Allium ampeloprasum* L.) extract using adjuvant-induced arthritis rat model: A comparative evaluation with normal garlic (*Allium sativum* L.) and dexamethasone. *J. Ethnopharmacol.*, **303**: 115939.
- Kebede T, Gadisa E and Tufa A (2021). Antimicrobial activities evaluation and phytochemical screening of some selected medicinal plants: A possible alternative in the treatment of multidrug-resistant microbes. *PLoS One*, **16**(3): e0249253.
- Korkmazer C and Halilu EM (2023). Biological activities of crude leaf extract and fractions of *Scutellaria sibthorpii* (Benth.) Halácsy: An endemic plant of North-cyprus. *Pak. J. Pharm. Sci.*, **36**(6 Special): 1837-1847.
- Lorke D (1983). A new approach to practical acute toxicity testing. *Arch. Toxicol.*, **54**(4): 275-287.
- Modi AD, Parekh A and Pancholi YN (2023). Evaluating pain behaviours: Widely used mechanical and thermal methods in rodents. *Behav. Brain Res.*, **446**: 114417.
- Mouliou DS (2023). C-reactive protein: Pathophysiology, diagnosis, false test results and a novel diagnostic algorithm for clinicians. *Diseases*, **11**(4): 132.
- Muheyyudeen G, Verma S, Yadav P, Khan MY, Ahmad T and Khushtar M (2026). Caffeic acid: A comprehensive review of its traditional use and modern pharmacological applications by 2025. *Rev. Colomb. Cienc. Quím. Farm.*, **55**(2): 336-382.
- National Research Council (2011). *Guide for the Care and Use of Laboratory Animals*. 8th ed. National Academies Press, Washington, DC.
- Ntalouka F and Tsirivakou A (2023). Luteolin: A promising natural agent in management of pain in chronic conditions. *Front. Pain Res.*, **4**: 1114428.
- Oduncuoglu G, Cavusoglu M, Kayak N and Halilu EM (2026). Phytochemical characterization and wound healing potential of *Salvia veneris* Hedge extract-based ointment. *Nat. Prod. Res.*, in press.
- Oduncuoglu G and Halilu EM (2023). *In-vivo* evaluation of the traditional uses of *Salvia veneris* Hedge and development of a topical formulation for localized pain and inflammation. *Pak. J. Pharm. Sci.*, **36**(5 Special): 1637-1647.
- Ozbil E, Ilktac M, Ogmen S, Isbilen O, Duran Ramirez JM, Gomez J, Walker JN and Volkan E (2024). *In-vitro* antibacterial, antibiofilm activities and phytochemical properties of *Posidonia oceanica* (L.) Delile: An endemic Mediterranean seagrass. *Heliyon*, **10**(15): e35592.
- Savvides AM, Stavridou C, Ioannidou S, Zoumides C and Stylianou A (2023). An ethnobotanical investigation into the traditional uses of Mediterranean medicinal and aromatic plants: The case of troodos mountains in cyprus. *Plants*, **12**(5): 1119.
- Shahrajabian MH, Sun W and Cheng Q (2021). A review of leek (*A. ampeloprasum* L.), an important vegetable and food ingredient with remarkable pharmaceutical activities. *Pharmacogn. Commun.*, **11**(1): 9-12.
- Stompor-Gorący M and Machaczka M (2021). Recent advances in biological activity, new formulations and prodrugs of ferulic acid. *Int. J. Mol. Sci.*, **22**(23): 12889.
- Takeda M, Sashide Y, Toyota R and Ito H (2024). The phytochemical, quercetin, attenuates nociceptive and

- pathological pain: Neurophysiological mechanisms and therapeutic potential. *Molecules*, **29**(16): 3957.
- Teixeira G and Faria R (2021). Inflammatory mediators leading to edema formation through plasma membrane receptors. In: Neri V, Huang L and Li J (eds.), *Infections and Sepsis Development*. IntechOpen, London, pp. 281-302.
- Tishkowski K and Zubair M (2025). Erythrocyte sedimentation rate. In: *StatPearls [Internet]*. StatPearls Publishing, Treasure Island (FL). Updated 7 July 2025. Available at: <https://www.ncbi.nlm.nih.gov/books/NBK557485/> (Accessed 9 August 2026).
- Xu W, Cheng Y, Guo Y, Yao W and Qian H (2022). Effects of geographical location and environmental factors on metabolite content and immune activity of *Echinacea purpurea* in China based on metabolomics analysis. *Ind. Crops Prod.*, **189**: 115782.

Article in Press