

Research Article

Safety Evaluation of *Barleria lupulina* Lindl. Leaf Extract via Toxicity Assessment and Analysis of Chemical Constituents

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ABSTRACT

Barleria lupulina Lindl. is a perennial herb commonly referred to as the hophead Philippine violet and “Penawar Seribu Bisa” or “Bisa Ular” in Malaysia. It belongs to the Acanthaceae family and holds a significant place in the traditional pharmacopeia of indigenous communities in Malaysia. This study aims to assess the safety profile of the leaf extract, focusing on its elemental composition and potential cytotoxicity. The concentrations of heavy metals were quantified using Inductively Coupled Plasma-Mass Spectrometry (ICP-MS), while cytotoxicity was assessed through the Brine Shrimp Lethality Assay (BSLA). Additionally, Ultra-High-Performance Liquid Chromatography coupled with Quadrupole Time-of-Flight Mass Spectrometry (UHPLC-QTOF/MS) was utilized to characterize the chemical constituents of the extract. Results indicate that the heavy metal concentrations in *B. lupulina* leaf are within the permissible limits set by the Codex Alimentarius, suggesting they are safe for consumption. The BSLA assessment demonstrated no significant mortality in brine shrimp, even at the highest extract concentration of 1000 µg/mL, further supporting the safety of the extract. UHPLC-QTOF/MS analysis revealed the presence of several compounds—paeonolide, sibiricaxanthone B, scroneoside A, roseoside, and cornuside II—reported for the first time in this species.

Key words: *Barleria lupulina*, Brine Shrimp Lethality Assay (BSLA), ICP-MS, QTOF/MS, UHPLC

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INTRODUCTION

The exploration of natural products for their medicinal properties has garnered considerable attention due to their potential therapeutic benefits. The proven efficacy of medicinal compounds sourced from plants not only ensures safety but also enhances effectiveness while remaining cost-efficient, thus elevating the global importance of plants within the medical domain (Chaachouay & Zidane 2024; Vaou *et al.*, 2025). Phytochemicals, bioactive compounds synthesized by plants for their defense mechanisms, are ubiquitous in numerous sources, including whole grains, fruits, vegetables, nuts, and herbs (Kumar *et al.*, 2023). Over a thousand phytochemicals have been cataloged, encompassing carotenoids, polyphenols, phytoosterols, and specific polysaccharides (Edo *et al.*, 2025). These compounds exhibit potent antioxidant capabilities and demonstrate efficacy against microbes, parasites, viruses, allergies, diarrhea, and muscle spasms. Furthermore, their influence extends to regulating

gene expression, facilitating cellular communication, bolstering immunity, and defense against cancer (Kumar *et al.*, 2023; Paul *et al.*, 2024; Edo *et al.*, 2025).

Barleria lupulina Lindl., belonging to the Acanthaceae family, has attracted increasing attention in recent years for its potential medicinal properties. This species is commonly distributed in tropical and subtropical regions across the world, with a particular prevalence in Africa and Asia (Balkwill & Balkwill, 1998; Darbyshire *et al.*, 2019). It is known by many names in different regions: Malaysia (Penawar seribu bias or Bisa ular), Indonesia (Landik or Sujen trus), Thailand (Salet phangphong or Phimsen ton), Vietnam (Gai kim vàng), India (Kanta bishalyakarani, mullanangai) and China (Hua je jia du juan) (Aguilar, 2001; Samuel *et al.*, 2010; Quattrocchi, 2012). Documented research reveals that different parts of the plant harbor a rich assortment of phytoconstituents, ranging from terpenes, terpenoid compounds, iridoid glucosides, iridoid digluco-

sides, phenylpropanoid glucosides, and phenylethanoid glucosides. Isolated compounds and extracts of *B. lupulina* exhibit a spectrum of pharmacological activities, including antibacterial, antiarthritic, central nervous system (CNS) depressant, anti-osteoporotic, antioxidant, anti-diabetic, and anti-inflammatory properties (Gangaram *et al.*, 2021; Sawarkar *et al.*, 2023).

Globally, concerns over plant safety and quality have intensified due to the escalating threat of heavy metal contamination (Ozyigit *et al.*, 2018; Karahan *et al.*, 2020). The ubiquitous presence of heavy metals in the environment stems from numerous human activities such as industrial processes, metallurgy, mining operations, agricultural practices, medical applications, technological uses, and traffic-related activities. This widespread distribution raises significant apprehensions concerning potential implications for human health and environmental integrity (Tchounwou *et al.*, 2012; Lajayer *et al.*, 2017; Karahan *et al.*, 2020). Dense metals and metalloids, collectively termed heavy metals, are characterized by their high density (often greater than 5 g/cm³) and pose a threat to organisms due to their propensity to bioaccumulate in biological systems and be transmitted along the food chain (Järup, 2003; Witkowska *et al.*, 2021). These metals are non-biodegradable and possess extended biological half-lives, allowing them to persist within the body for prolonged durations (Akoury *et al.*, 2022). They can enter the human body via ingestion, skin contact, and inhalation. Once inside, these metal ions can wreak havoc at the cellular level, acting as cofactors or inhibitors within enzymatic pathways (Jaishankar *et al.*, 2014; Witkowska *et al.*, 2021). As plants absorb these heavy metals, they can accumulate in the food chain, posing potential health hazards for animals and humans (Shahid *et al.*, 2017). The severity of this threat depends on various factors, including dosage, exposure route, chemical composition, as well as demographic variables such as age, gender, genetic predisposition, and nutritional state of the exposed individuals (Tchounwou *et al.*, 2012; Ahmad *et al.*, 2021).

Given the gravity of these concerns, toxicity assessment becomes crucial in determining the safety of plant extracts intended for medicinal use. Such assessments provide valuable insights into the safety margin of plant extracts, guiding regulatory decisions and ensuring consumer safety. Commonly employed methods in toxicity assessment include Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) and the Brine Shrimp Lethality Assay (BSLA). ICP-MS enables the quantitative analysis of elemental composition, facilitating the detection of heavy metals or other potentially harmful substances in the sample (Choi *et al.*, 2019). By quantifying heavy metals or toxic elements, ICP-MS identifies the potential contaminants that may pose health risks upon consumption. This analytical technique complements other toxicity assays, contributing to a comprehensive safety assessment of the plant extract. Meanwhile, the BSLA serves as a preliminary screening tool for evaluating the cytotoxicity of natural products (Hamidi *et al.*, 2014). This technique is cost-effective, easy to perform, and requires only a minimal quantity of sample. By exposing brine shrimp nauplii to the sample, researchers can assess its lethality and infer its potential toxicity to higher organisms (Simorangkir *et al.*, 2021). It has been reported to exhibit predictive value for cytotoxic and pesticidal activities (Ghisalberti, 1993), evaluating the acute toxicity of medicinal plants from Kenyan biodiversity (Nguta *et al.*, 2012), comparing bioactivity among different plant parts (Urmi *et al.*, 2013), and assessing the lethality of Thai medicinal plants in the Meliaceae family.

The increasing interest in the medicinal potential of *B. lupulina* Lindl., particularly regarding its diverse pharmacological properties, warrants a safety evaluation of its leaf extract. While the exploration of its therapeutic benefits holds promise, ensuring the

safety of its use is equally paramount, especially considering its potential application in various health conditions. Therefore, an in-depth assessment of its toxicity becomes imperative to ascertain its safety profile for human consumption.

MATERIALS AND METHODS

Sample collection and preparation

Barleria lupulina Lindl. leaves were collected from Serdang, Selangor, Malaysia (3°00'13.6"N 101°44'11.5"E) and identified by a botanist at the Herbarium of the Laboratory of Natural Products, Institute of Bioscience, Universiti Putra Malaysia, based on a voucher specimen (MFI 0047/19). Leaves were washed with water and dried in an oven at 40°C. The extraction was performed using microwave-assisted extraction with 80% ethanol at 600 W for 30 sec (Ismail-Suhaimy *et al.*, 2021).

Analysis of metal content

A 0.5 g sample of plant material was mixed with 10 mL of 65% v/v nitric acid (HNO₃) and 8 mL of 30% v/v hydrogen peroxide (H₂O₂) before undergoing digestion in triplicate using a microwave digestion system (ETHOS UP, Milestone Srl, Sorisole (BG), Italy) at 180°C for 15 min. Following digestion, the colorless solution of the sample was filtered using 0.45 µm filter paper (Advantec MFS, Inc., Pleasanton (CA), USA) and subsequently diluted to 50.0 mL with distilled water. Multi-element/trace metal analysis was conducted using a Perkin-Elmer Sciex Elan 9000 quadrupole-based ICP-MS system (Coetzee *et al.*, 2005; Siti Salwa *et al.*, 2023).

Toxicity bioassay

The experiment utilized *Artemia salina* hatching eggs premixed with sea salt (Ocean Nutrition Europe, Rijkmakerlaan, Essen, Belgium) following the methodology outlined by (Baruah *et al.*, 2015; Balqis-Wong *et al.*, 2025). Seawater collected from Port Dickson, Negeri Sembilan, Malaysia, was filtered and autoclaved for sterilization. In a 50 mL Falcon tube, 200 mg of *Artemia* eggs were hydrated with 18 mL of potable water. After an hr, a solution composed of 660 µL of 32% sodium hydroxide and 10 mL of 5% sodium hypochlorite was gently introduced to the egg suspension and mixed. Subsequently, 14 mL of sodium thiosulfate (10 g/L) was added, followed by filtration through a sieve and washing with 20 mL of sterilized seawater. The eggs were then resuspended in 30 mL of sterilized seawater in a new Falcon tube and allowed to hatch and develop as nauplii on an incubator shaker at room temperature for one day. To enhance visibility, the nauplii were extracted from the Falcon tube using a pipette and diluted with sterile seawater. The experiments were performed with potassium dichromate as the positive control and autoclaved seawater as the negative control. The concentrations of *B. lupulina* leaf extracts were set from 0.00195 to 1 mg/mL. Each experiment utilized 10 nauplii, and all analyses were conducted in triplicate at room temperature for 24 hr.

Analysis of chemical constituents

Ultra-high-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UHPLC-QTOF/MS) and a cutting-edge ultra-performance LC system (Waters, Milford, MA, USA) were employed for the identification of chemical constituents. Separation of chromatographic compounds was achieved using a specialized column (ACQUITY UPLC HSS T3, 100 mm × 2.1 mm × 1.8 µm, Waters, Manchester, UK). A gradient elution strategy involving mobile phases A (0.1% formic acid) and B (acetonitrile) was utilized, transitioning from 1% B and 99% A at the start to 35% B and 65% A at 16.00 min, reaching 100% B and 0% A at 18.00 min, and finally returning to 1% B and 99% A at 20.00 min. A sample injection volume of 1 µL was introduced at a flow rate of 0.6 mL/min. The spectrometer was coupled with electrospray ionization (ESI) operating in negative ion mode. Data acquisition was performed using independent data acquisition (IDA) over the m/z range of 50–1500 at a scan rate of 0.1 sec/scan, employing high-definition mass spectrometry elevated energy (HDMSE) mode, incorporating both

low-energy (LE) and high-energy (HE) scans with varying collision energies (CE) ranging from 4 eV to 40 eV (Zulkifli *et al.*, 2020; Bang *et al.*, 2023).

RESULTS AND DISCUSSION

The selection of heavy metal analysis and the BSLA in the present study was based on their suitability as preliminary screening tools in toxicity assessment. Heavy metal analysis was conducted to detect the presence of potentially harmful elemental contaminants and to provide an initial evaluation of sample safety. In parallel, BSLA was applied as a rapid and cost-effective bioassay, which has been widely used to indicate general cytotoxic and biological activity.

While more established toxicological approaches, such as MTT assays, acute oral toxicity, and sub-chronic studies, offer more detailed and specific evaluations, these methods are typically employed at later stages of research. They require greater resources, time, and experimental complexity. Therefore, the present study focused on preliminary screening methods to generate baseline toxicity data, which can inform and support subsequent in-depth toxicological analyses.

Analysis of metal content

Inductively coupled plasma mass spectrometry (ICP-MS) is a highly effective elemental detection method, adept at discerning an extensive array of substances, encompassing organic compounds, organometallic compounds, and complex biomolecules such as nucleic acids, phospholipids, and proteins (Pröfrock & Prange, 2012; Wilschefske & Baxter, 2019). This study employed ICP-MS to assess the concentrations of various heavy metals in the leaves of *B. lupulina* against the permissible limits established by the Codex Alimentarius, a collection of internationally recognized standards, codes of practice, and guidelines related to food safety and quality (Table 1).

An analysis of 12 heavy metals in *B. lupulina* leaf extracts yielded promising results for its safety. Levels of well-established toxic metals like arsenic (As: 0.0071 mg/kg), cadmium (Cd: 0.0007 mg/kg), lead (Pb: 0.0392 mg/kg), and mercury (Hg: 0.00006 mg/kg) all fell well within the permissible limits set by Codex Alimentarius, indicating minimal health risks associated with these common heavy metals. Barium (Ba: 0.4759 mg/kg), beryllium (Be: 0.0002 mg/kg), bismuth (Bi: 0.0007 mg/kg), and silver (Ag: 0.0003 mg/kg) were also

detected at low levels, further supporting the safety of *B. lupulina* consumption.

However, the absence of Codex limits for cobalt (Co: 0.0031 mg/kg), gallium (Ga: 0.0049 mg/kg), lithium (Li: 0.0021 mg/kg), and vanadium (V: 0.0014 mg/kg) necessitates caution. While the measured concentrations were relatively low, long-term exposure to these elements warrants further investigation. Cobalt, for instance, can cause cardiotoxicity and thyroid dysfunction at high concentrations (Packer, 2016; Chen & Lee, 2023) despite being essential in small amounts. Similarly, prolonged exposure to gallium and vanadium may lead to kidney problems (Rehder, 2013; White & Shine, 2016). Lithium, though used therapeutically in psychiatry, can result in tremors and kidney dysfunction with excessive intake (Gitlin, 2016; Medić *et al.*, 2020).

Toxicity bioassay

Numerous plant species contain bioactive compounds that are becoming indispensable for traditional medicine and pharmaceutical development. However, these substances can have adverse effects when their concentrations exceed critical thresholds in vulnerable biological systems. Therefore, it is necessary to conduct toxicity assessments to acquire relevant data applicable to human health.

The BSLA is well known to be the simplest test for screening toxicity of plant extracts; it requires a short time and low cost and was employed to assess the toxicity of *B. lupulina* leaf extracts *in vivo*. Table 2 provides the mortality rates observed in brine shrimp following exposure to varying concentrations of *B. lupulina* leaf extracts over 24 hr, alongside positive control groups. The results reveal significant correlations between the concentration of potassium dichromate and the mortality rate of brine shrimp. Conversely, *B. lupulina* leaf extracts did not induce mortality in brine shrimp at any concentration tested, indicating the lack of acute toxic effects associated with *B. lupulina* leaf extracts. The current finding is consistent with the results of Sirisidithi *et al.* (2016), which showed that raising the concentration of the extract up to a maximum of 1000 µg/mL does not cause any deaths in brine shrimp.

Analysis of chemical constituent

The UHPLC-QTOF/MS analysis was performed in negative ionization mode to profile leaf extract compounds. All detected constituents were assigned molecular formulas based on high-resolution MS data, and the subsequent MS/MS analysis provided precise mass information on fragment ions, enabling the identifi-

Table 1. Metal content in *B. lupulina* leaf extract.

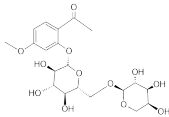
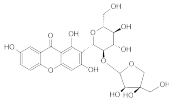
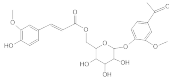
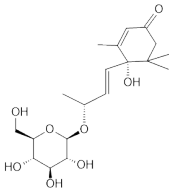
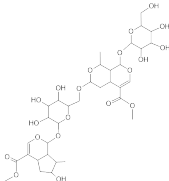
Heavy metal	Concentration (mg/kg)	Codex Alimentarius
Arsenic (As)	0.0071 ± 0.96	0.35
Barium (Ba)	0.4759 ± 23.11	1.3
Beryllium (Be)	0.0002 ± 0.14	NM
Bismuth (Bi)	0.0007 ± 0.02	0.01
Cadmium (Cd)	0.0007 ± 0.14	0.2
Cobalt (Co)	0.0031 ± 0.25	NM
Gallium (Ga)	0.0049 ± 0.3	NM
Lithium (Li)	0.0021 ± 0.23	NM
Lead (Pb)	0.0392 ± 0.32	0.3
Mercury (Hg)	0.00006	0.1
Silver (Ag)	0.0003	NM
Vanadium (V)	0.0014 ± 0.53	NM

Adapted from Codex Alimentarius International Food Standards. NM: Not mentioned.

Table 2. Cytotoxicity of *B. lupulina* leaf extracts on brine shrimp.

Concentration (mg/mL)	Mortality Rate, % Positive control	Mortality Rate, % <i>B. lupulina</i> leaf extracts
0.00195	3	0
0.00391	10	0
0.00781	17	0
0.0156	23	0
0.03125	50	0
0.0625	100	0
0.125	100	0
0.25	100	0
0.5	100	0
1	100	0

Table 3. Chemical constituents of *B. lupulina* leaf extracts assessed by UHPLC-QTOF/MS.

Compound name	Formula	Retention time (min)	Natural mass (Da)	Observed m/z	Ion fragment	Chemical structure
Paeonolide	C ₂₀ H ₂₈ O ₁₂	5.63	460.15808	459.1514	265.07292	
Sibiricaxanthone B	C ₂₄ H ₂₆ O ₁₄	8.43	538.13226	537.1253	160.04086 / 399.09405	
Scroneoside A	C ₂₅ H ₂₈ O ₁₁	9.99	504.1635	503.1562	149.06031 / 161.02506 / 323.07759	
Roseoside	C ₁₉ H ₃₀ O ₈	10.13	386.19407	385.1873	203.05845	
Cornuside II	C ₃₄ H ₅₀ O ₂₀	10.25	778.2895	777.2822	593.20621	

cation of specific compounds through comparison with authentic standards or literature data within the chromatograms (Table 3). MS/MS spectra of these compounds obtained at low and high collision energy are shown in Figure 1-5. Compounds tentatively identified in the leaf extract were cross-referenced using the Waters library, ensuring acquisition mass accuracy of less than 5 ppm with theoretical fragments consisting of more than one ion.

The analysis revealed the presence of several bioactive compounds, notably paeonolide, sibiricaxanthone B, scroneoside A, roseoside, and cornuside II. This is the first report of these compounds in this particular species, potentially contributing to its unique chemical profile. Paeonolide is recognized for its medicinal benefits in treating bone diseases such as osteoporosis and periodontitis by promoting osteoblast development and bone mineralization,

primarily via the ERK1/2-RUNX2 signaling pathway (Park *et al.*, 2021). The presence of sibiricaxanthone B has been associated with therapeutic benefits, especially for neurological disorders (Chen *et al.*, 2024; Garg *et al.*, 2024). Meanwhile, scroneoside A has been associated with antimalarial properties (Rokaya *et al.*, 2020). Roseoside has been discovered to exhibit many bioactivities, including anti-allergy, antioxidant, insulinotropic, antitumor-promoting, and an inhibitory effect on G-6-Pase activity as evaluated in intact microsomes (Ito *et al.*, 2002; Yajima *et al.*, 2009; Frankish *et al.*, 2010; Tan *et al.*, 2024), while cornuside II has been implicated with potential in Type 1 Diabetes treatment due to its anti-inflammatory and anti-apoptotic properties (Sharp-Tawfik *et al.*, 2019).

The identification of these constituents signifies a novel discovery within the chemical profile of this species, and quantifying the

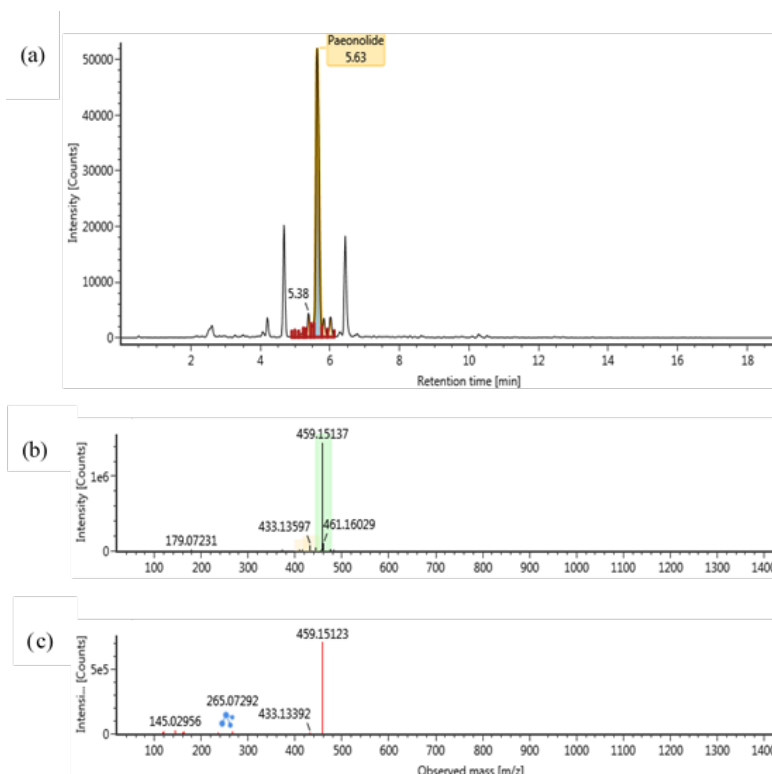


Fig. 1. UHPLC-QTOF/MS chromatograms of paeonolide from *B. lupulina* leaf extract. (a) chromatogram mass spectrum; (b) low-energy mass spectrum; (c) high-energy mass spectrum.

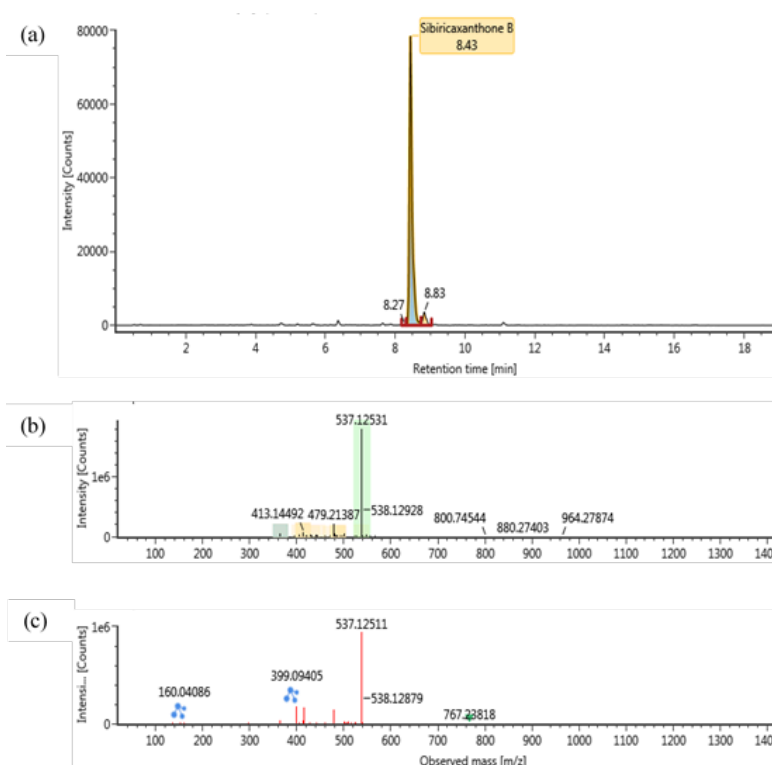


Fig. 2. UHPLC-QTOF/MS chromatograms of sibiricaxanthone B from *B. lupulina* leaf extract. (a) chromatogram mass spectrum; (b) low-energy mass spectrum; (c) high-energy mass spectrum.

abundance of these novel compounds would be valuable for understanding their potential contribution to the overall pharmacological activity of the extract. Further investigation should consider elucidating the biosynthetic pathways responsible for their production, which would provide insights into the plant's metabolic processes and potential ecological adaptations. Additionally, elucidating the

structural features and bioactivities of these newfound compounds could pave the way for the development of novel therapeutic agents and contribute to a more comprehensive understanding of the plant's ecological importance.

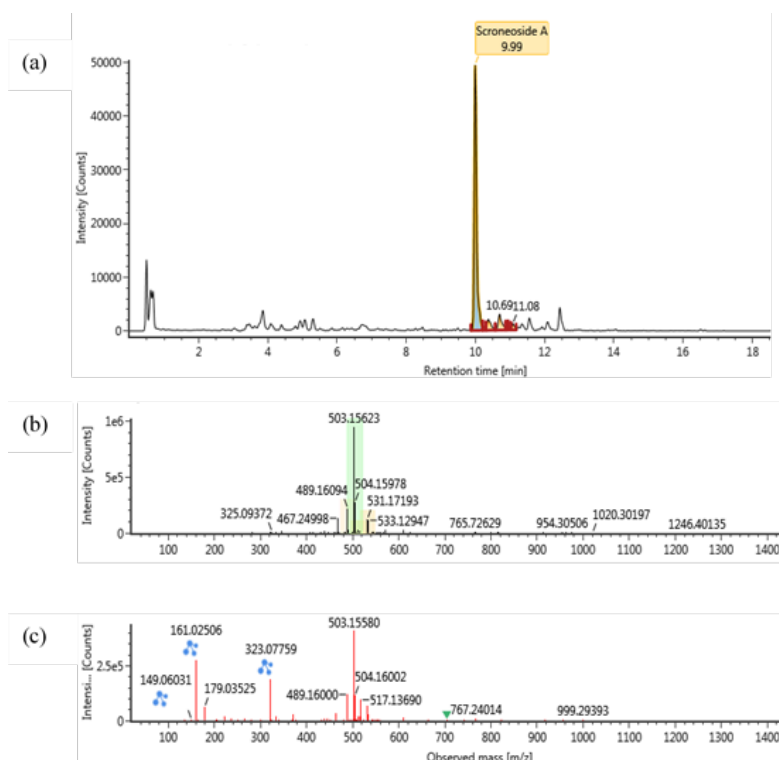


Fig. 3. UHPLC-QTOF/MS chromatograms of scroneoside A from *B. lupulina* leaf extract. (a) chromatogram mass spectrum; (b) low-energy mass spectrum; (c) high-energy mass spectrum.

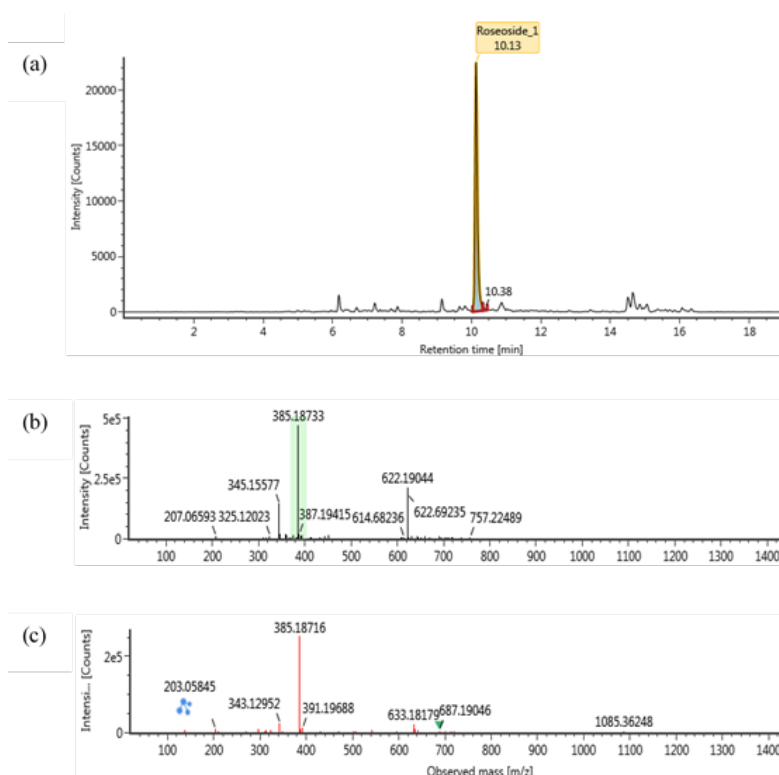


Fig. 4. UHPLC-QTOF/MS chromatograms of roseoside from *B. lupulina* leaf extract. (a) chromatogram mass spectrum; (b) low-energy mass spectrum; (c) high-energy mass spectrum.

CONCLUSION

Plants employ various mechanisms to mitigate the accumulation of heavy metals within the cell, thereby preventing the harmful effects of heavy metal toxicity. The findings of this study indicate that the concentration of metals in *B. lupulina* Lindl. falls within the permissible limits as defined by the Codex Alimentarius, indicating

the safety of consuming the leaves with respect to these metals. However, continuous monitoring and further research are recommended to ensure long-term safety, particularly for metals with no established Codex limits. Concurrently, the BSLA assessment reveals no mortality in brine shrimp at concentrations up to 1000 µg/mL, indicating that the extract exhibited no detectable toxicity

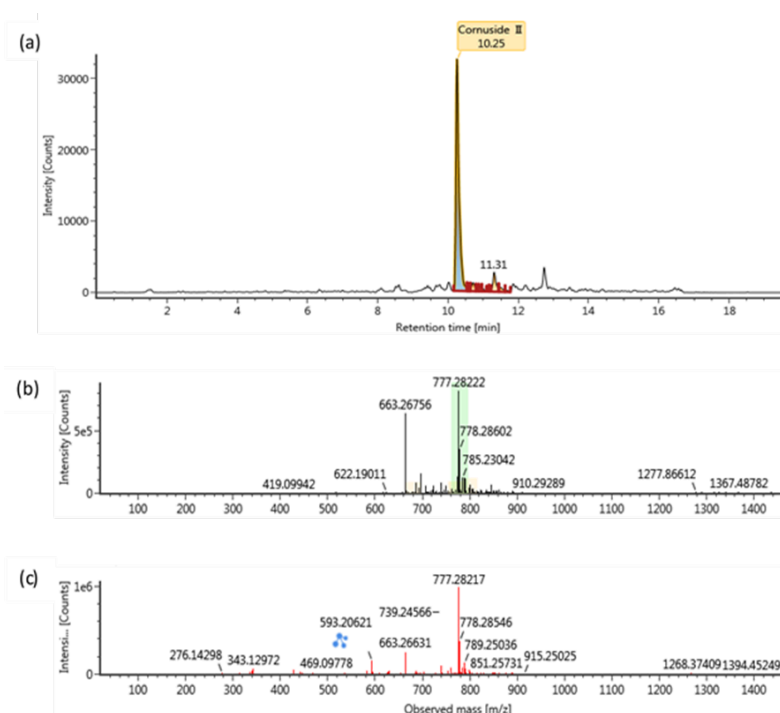


Fig. 5. UHPLC-QTOF/MS chromatograms of cornuside II from *B. lupulina* leaf extract. (a) chromatogram mass spectrum; (b) low-energy mass spectrum; (c) high-energy mass spectrum.

under the test conditions. Furthermore, UHPLC/QTOF-MS analysis has identified for the first time in this species the presence of the following compounds: paeonolide, sibiricaxanthone B, scroneoside A, roseoside, and cornuside II, further enriching our understanding of its chemical profile.

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ETHICAL STATEMENT

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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