

Insights into Metabolites Profiling and Pharmacological Investigation of *Aconitum heterophyllum* wall ex. Royle Stem through Experimental and Bioinformatics Techniques

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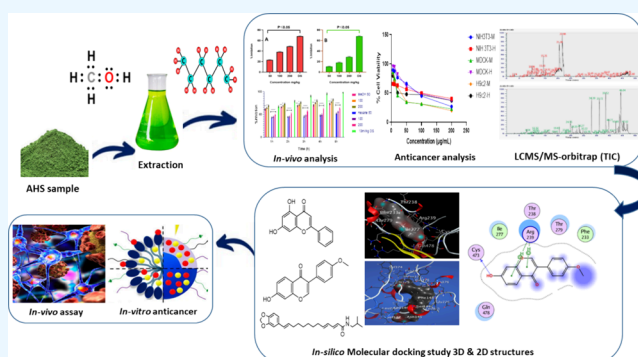
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ABSTRACT: The *Aconitum* genus is a leading source of a wide range of structurally diverse metabolites with significant pharmacological implications. The present study investigated metabolite profiling, pharmacological investigation, anticancer potential, and molecular docking analysis of the stem part of *Aconitum heterophyllum* (AHS). The metabolite profiling of the AHS extract was experimentally examined using LC-MS/MS-orbitrap in both modes (ESI⁺/ESI⁻) and GC-MS in EI mode. The *in vitro* MTT model was used to study the anticancer potential, while the *in vivo* animal model was used to study the anti-inflammatory and antinociceptive activities. The MOE software was used for the molecular docking study. A total of 118 novel and previously known metabolites, among 44 metabolites (26 in ESI⁺

positive mode and 18 in ESI⁻ negative mode) in the MeOH extract, while 74 metabolites (46 in ESI⁺ and 28 in ESI⁻ mode) were identified in the *n*-hexane extract via LCMS/MS. The identified metabolites include 24 phenolic compounds, 18 alkaloids, 10 flavonoids, 24 terpenoids, 2 coumarins, 2 lignans, and 38 other fatty acids and organic compounds. The major bioactive metabolites identified were hordenine, hernagine, formononetin, chrysin, *N*-methylhernagine, guinesine, shogaol, kauralexin, colneate, zerumbone, medicarpin, boldine, miraxinthin-*v*, and larciresinol-4-*O*-glucoside. Furthermore, the GC-MS study helped in the identification of volatile and nonvolatile chemical constituents based on the mass spectrum and retention indices. The methanol extract significantly inhibited tumor progression in H9c2 and MDCK cancer cells with IC₅₀ values of 186.39 and 199.63 μg/mL. In comparison, the positive control aconitine exhibited potent IC₅₀ values (132.32 and 141.58 μg/mL) against H9c2 and MDCK cell lines. The anti-inflammatory (carrageenan-induced hind paw edema) and antinociceptive (acetic acid-induced writhing) effects were significantly dose-dependent, ($p < 0.001$) and ($p < 0.05$), respectively. In addition, a molecular docking study was conducted on identified ligands against the anti-inflammatory enzyme (COX-2) (PDB ID: 5JVZ) and the cancer enzyme ADAM10 (PDB ID: 6BDZ) which confirmed the anti-inflammatory and anticancer effects in an *in silico* model. Among all ligands, L2, L3, and L7 exhibit the most potent potential for inhibiting COX-2 inflammation with binding energies of -7.3424, -7.0427, and -8.3562 kcal/mol. Conversely, against ADAM10 cancer protein, ligands L1, L4, L6, and L7, with binding energies of -8.0650, -7.7276, -7.0454, and -7.2080 kcal/mol, demonstrated notable effectiveness. Overall, the identified metabolites revealed in this AHS research study hold promise for discovering novel possibilities in the disciplines of chemotaxonomy and pharmacology.



1. INTRODUCTION

Natural products (NP) and their structural analogues are isolated compounds from medicinal plants and microorganisms. They are an important source of novel drugs for the prevention and treatment of various diseases.^{1–3} A huge number of metabolites of varying structure and abundance, which play important roles in plant growth, development, and feedback to the environment have been isolated from the medicinal plants.⁴ These medicinal plants, since time immemorial, have been exploited as a source of novel pharmacophores in the production of lead compounds in the pharmaceutical industry.^{5,6}

The FDA finds that most approved drugs have pharmacophore links to natural products. These comprise antihypertensive drugs like enalapril and captopril, as well as antitumor agents camptothecin, taxol, and docetaxel.⁷ The WHO reports 21,000 different medicinal plants used for therapeutic purposes which surges its demand globally.⁸ The increased utilization of

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natural products is attributed to their chemical novelties, diversity, and potential as lead drug candidates for complex targets, surpassing other sources. Despite their complex structures, these metabolites exhibit high absorption and efficient metabolism within the body.⁹

The genus *Aconitum* has various pharmacological potential such as cardiotoxic, anesthetic, antiplasmodial and hypertensive.^{10,11} Among the 250 aconite species globally, a few are recorded in Pakistan, and these specific species are known to have poisonous characteristics.^{12,13}

Aconitum heterophyllum (*A. heterophyllum*) wall ex. Royle belongs to the family *Ranunculaceae*. It is also known as Asian Monkshood or/and Ativisha.¹⁴ *A. heterophyllum* is a perennial herb composed of dried bulbous roots, the stem is erect/clasping, and the leaves are ovate, heart-shaped. The flowers are helmet-shaped, greenish-blue in color. It is found in Pakistan in various regions including Kashmir, Chitral, Swat, Shangla, and Gilgit-Baltistan, at altitudes of about 4000–4500 m above sea level.¹⁵

Numerous ailments are treated by *A. heterophyllum*, including joint pain, rheumatic fever, bronchial asthma and endocrine disorders such as irregular menstruation.^{16,17} The root of this plant shows great antimicrobial and antibiotic effects and is used in the treatment of skin, blood diseases, diarrhea and urinary tract infections.^{18–20} It is also used as an expectorant and has been shown to be responsible for promoting hepatoprotective activity.^{14,21,22} The phytochemical components reported in this species include aconitine, N-diethyl-N-formylaconitine, N-succinoylanthranyl, anthonine, 12-secohetisan-2-ol and atesinol-6-benzoylheterastine.^{23,24} In Ayurveda, the stem part was renowned for its better flavor and has been recommended as an alternative treatment for diabetic patients.²⁵ Traditionally, the powdered forms of the stem part are mixed with honey and juice to address bronchitis and cough irritations. A recent study of the root component of *A. heterophyllum* has qualitatively confirmed the presence of diverse phytochemicals.¹⁴

Based on phytochemistry and pharmacognosy, the stem part of the *A. heterophyllum* (AHS) was selected to explore its potent medicinal properties in the treatment of various disorders. The stem extract was screened to analyze its metabolites profiling, aiming to identify additional metabolites, especially novel ones, using LC-MS/MS and GC-MS techniques. In addition, *in vitro* and *in vivo* activities were evaluated to investigate the biological and pharmacological potential of the AHS extract. Additionally, the extract's anticancer potential was assessed against diverse cancer cell lines. The experimental study was supported by a bioinformatics approach using molecular docking studies.

2. MATERIALS AND METHODS

2.1. Chemical Reagents and Experimental Instrumentations. All of the chemicals used in this study were of analytical grade. Methanol ($\text{CH}_3\text{OH} \geq 99.9\%$), chloroform ($\text{CHCl}_3 \geq 99\%$), *n*-hexane ($\text{C}_6\text{H}_{14} \geq 99.7\%$), DMSO ($\text{C}_2\text{H}_6\text{SO} \geq 99.5\%$), ethanol ($\text{C}_2\text{H}_6\text{O} \geq 95\%$), ethyl acetate ($\text{C}_4\text{H}_8\text{O}_2 \geq 99.9\%$), sulfuric acid (H_2SO_4 98%), hydrochloric acid ($\text{HCl} \geq 36\%$), standard aconitine ($\text{C}_3\text{H}_4\text{NO}_{11} \geq 95\%$ HPLC, crystalline A8001–5MG, SLCJ6844), and UHPLC/MS grade acetonitrile ($\text{CH}_3\text{CN} \geq 99.92\%$) and formic acid ($\text{HCOOH} \geq 95\%$), were purchased from Sigma-Aldrich Lab (St. Paul MN, USA). The Dulbecco's Modified Eagles-medium (DMEM: 0.11 g/L sodium pyruvate, 4.5 g/L glucose,

2 mM l-glutamine, 100 U/ml penicillin, 10% fetal bovine serum (FBS), and 100 U/ml streptomycin in a humidified incubator at 5% (v/v) CO_2 ; Gibco; Invitrogen, Carlsbad, CA), streptomycin and penicillin (100 $\mu\text{g}/\text{mL}$), Cells counting Kit-8 (CCK-8; Dojindo, Shanghai-China), BioTek Instruments EL \times 808, Absorbance Microplate Reader (AMR) [96-well, wavelength 380–900 nm, 8s reading speed, model number 6880-G11EA, ASIN-B00GN23LB4, Inc., Winooski, VT, USA], and cancer cell lines, i.e., MDCK (Madin-Darby dog kidney), H9c2 (rat embryonic ventricular-myocardial), and NIH3T3 (fibroblast cell) were purchased from the American-Type Culture-Collection (ATCC, Manassas, VA-USA). Using a Thermo Scientific system (Waltham, MA, USA), ISQ 9000 single quadrupole MS coupled with a Trace GC Ultra gas chromatograph and Tri Plus autosampler, and the Fusion Tribrid LC-MS/MS-orbitrap Instruments (Thermo Scientific, Waltham, MA, USA).

2.2. Plant Material Collection and Authentication.

Aconitum heterophyllum stem (AHS) fresh plants were collected during July to August 2021 at high altitude, above sea level 4000–4500 m from District Shangla Hill Top, Khyber Pakhtunkhwa, Pakistan. This region is situated in the Himalayan mountain ranges and notable for its diverse flora. The District exists between 72°-33' to 73°-01' East longitude, and 33°-31' to 34°-08' North latitudes. The study zone remains snow-covered over half of the year (snow is 8–9-yard-deep). During winter, the extreme temperature drops from -2 to -5 °C. The authenticity of the collected plant was validated by a taxonomist affiliated with the Department of Botany at the University of Peshawar, Pakistan. The voucher for biological authentication (BOT-20301 PUP-AHS) has been deposited for future reference.

2.3. Extraction and Fractionation. First, 500 g of AHS was washed well, cut into small pieces, and dried in the laboratory in a clean, shaded place at 25 °C and 60% relative humidity for 2 weeks. The dried plant material was crushed/ground with an electric mill (Yigan, model/WF-130) and extracted with 99.9% methanol (3.0 L), Sigma-Aldrich. The solvent was evaporated using a rotary evaporator (BUCHI-300 Japan; model SJ29/32) at 40–45 °C, yielding 71 g of the crude MeOH extract. The methanol extract was fractionated with *n*-hexane (3.5 L), yielding 22 g of *n*-hexane fraction. Both the methanol and *n*-hexane fractions were stored in the refrigerator at 4 °C for further analysis.²⁶

2.4. LC-MS/MS-Orbitrap Profiling of AHS Extracts of *A. heterophyllum* to Identify Major Metabolites.

Untargeted metabolites profiling of AHS extracts was performed at the Mass Spectrometry Laboratory, Masonic Cancer Center, CCRB, University of Minnesota, USA. Using a Fusion Tribrid LC-MS/MS-orbitrap Instruments (Thermo Scientific, Waltham, MA, USA) mass spectrometer (MS) coupled to a Dionex UltiMate 3000 RSLC nano UPLC and using the system's loading pump. Chromatographic separations of 10 μL sample injections were performed on an Acquity HSS (Waters, Milford, MA) C_{18} reverse phase column (100 2.1 mm, 1.8 μm particle size) using water containing 0.1% formic acid (A) and acetonitrile (B) as mobile phases. Two LC conditions were used: 1) Initial conditions were 2% B for 3 min, followed by a linear gradient to 95% B in 40 min with a hold at 95% B for 2 min, followed by 2% B to re-equilibrate the column for the next run. 2) Initial conditions were 2% B for 3 min followed by a linear gradient to 95% B in 15 min with a hold at 95% B for 2 min followed by 2% B to re-equilibrate the

column for the next run. MS data acquisition was performed using electrospray ionization (ESI) with full-scan orbitrap detection (m/z 100–1000, resolution 120,000) and data-dependent HCD fragmentation (stepped 20, 35, 60%) with one cycle time of 1 s, dynamic exclusion of 6 s, quadrupole isolation width of 1.6 Da, exclusion width of 10 ppm, and orbitrap detection (resolution of 15,000). Analysis of each sample was performed separately in positive ($M+H^+$) and negative ($M - H^+$) mode. The data obtained were granted access to the Personal Compound Database Library (PCDL) database (Thermo Scientific, Waltham, MA, USA) and METLIN Gen2 based on high-resolution mass (MS/MS).²⁷

2.5. GC-MS Profiling of AHS Extracts of *A. heterophyllum* to Identify Major Phytochemicals. The phytochemical analysis of AHS extracts was conducted via gas chromatography–mass spectrometry (GC-MS) at the Mass Spectrometry Laboratory, Masonic Cancer Center, CCRB, University of Minnesota, USA. Using a Thermo Scientific system (Waltham, MA) consisting of an ISQ 9000 single quadrupole mass spectrometer coupled with a Trace GC Ultra gas chromatograph and a Tri Plus autosampler. The GC was equipped with a 30 m (0.25 mm inner diameter, 0.25 m film thickness) DB-5MS fused silica capillary column (Agilent Technologies, Santa Clara, CA) and a 2 m, 0.53 mm deactivated fused silica guard column. The injection port temperature was 280 °C, injecting one microliter of 10 g of methanol solutions and a split ratio of 20:1. The constant flow rate was 1.0 mL/min of He for a total GC run time of 69 min. The oven temperature was programmed as follows: 3 min at 60 °C, ramping at 5 °C/min to 280 °C, followed by 20 min Hold at 280 °C. The MS was operated in positive (EI) mode with an ion source temperature of 230 °C, an emission current of 50 A, and a filament voltage of –70 eV. The instrument was scanned from 40 to 550 Da with a scan time of 0.3 s, and the filament was turned off for the first 5 min. The electron impact spectra of the base peak chromatograms of each sample were searched for putative analyte identification in the National Institute of Standard Technology (NIST) 2017 MS Library (R) spectral database using Thermo Scientific FreeStyle software (version 1.8).²⁸

2.6. Anticancer Activity (Cytotoxicity Assay).

2.6.1. Cell Culture, Growth Condition and Treatment. A panel of various cancer cell lines NIH3T3, MDCK, and H9c2 were purchased from the American Type Culture Collection (ATCC, Manassas, VA-USA). Cells were cultured in Dulbecco's Modified Eagles-medium (DMEM; Gibco; Invitrogen, Carlsbad, CA), incubated at 37 °C in a humidified incubator/atmosphere (5% CO₂) in 95% air. RPMI-1640 medium supplemented with 10% heat inactivated fetal bovine-serum (FBS, Gibco, Invitrogen), streptomycin and penicillin (100 µg/mL) were used for the typical sub culturing. According to the instructions, the viability of cells was measured using a cell counting Kit-8 (CCK-8; Dojindo, Shanghai-China). Trypan crystal violet blue-exclusion experiment was used to count the number of cells viable.^{29,30}

2.6.2. In Vitro Cell Viability Assay. The anticancer effect of AHS extracts on various cancer cells were screened by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric method. The cells (2×10^4 cells/well) were seeded at 37 °C in 96-well plates (humidified incubator, 5% CO₂) and treated with different concentrations (200, 100, 50, 20, 10, 5) µg/mL for 24, 48, and 72 h. Aconitine, a standard drug with reported anticancer properties,³¹ served as

a positive control. DMSO with 0.1% final concentration was utilized as a negative control. Cell viability was assessed using MTT solution, and the OD was measured at 562 nm with EL × 808 AMR. The concentration causing a 50% reduction in cancer cell proliferation (IC₅₀) was determined using concentration–response curves.³² The percentage of cell viability were calculated as follows: (A = absorbance).

$$\% \text{cell viability} = (A_{\text{treatment}} - A_{\text{blank}}) / (A_{\text{control}} - A_{\text{blank}}) \times 100\%$$

2.7. Ethical Consideration (Ethics Approval and Consent to Participate).

The experimental protocol for the *in vivo* animal study was approved by the Ethics Committee, The Animal Care & Use Committee (approval no.7198/AHS/UAP) of the University of Agriculture Peshawar, KP, Pakistan in accordance with guidelines given in Act. of Animal Scientific Protocol, National Institute of Health (UK, 1986) for Ethical Principles and Protection of Safe Use of Laboratory Animals.³³

2.8. Animals for In Vivo Study. Healthy Balb-c mice (*Mus domesticus*) of both sex (male and female), weighing 25–30 g and ages (5–6 weeks) were purchased from Animal Center Veterinary Research Institute (VRI) Peshawar, KP, Pakistan. The animals were housed under laboratory conditions (temperature: 25.0 ± 2 °C) and humidity (55 ± 10%) in a 12 h light/dark cycle with free access to food and water. A standard drug, Diclofenac sodium (DS) NSAIDs were used for both anti-inflammatory and antinociceptive studies. The Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely employed medications to relieve pain and reduce inflammation.³⁴

2.9. Antinociceptive Activity. The antinociceptive study of AHS extract was determined using an induced abdominal constriction test.^{25,35} The mice were divided into five groups (each, $n = 6$) and fasted overnight. The extract was administered orally at different doses (50, 100, and 200 mg/kg). The diclofenac sodium (DS) was used as a standard drug in a dose range of 10 mg/kg. After a 60 min interval, mice were administered intraperitoneally with 1% acetic acid at a volume to mass ratio of 10 mL/kg. After a 20 min latency with continuous acetic acid injection, acid-induced writhing, abdominal constriction (hind-limb extension) was counted for 30 min. Percent antinociceptive (inhibition) was calculated as follows:

$$\% \text{Inhibition} = (1 - C1/C2) \times 100$$

C1 = the number of writhes in treated groups, C2 = the number of writhes in vehicle (DMSO 5% and 1% Tween80). Followed by one-way ANOVA, performed Dennett's posthoc test, using Graph Pad prism 8.0 package.

2.10. Anti-inflammatory Activity. To determine the anti-inflammatory potential of AHS extract with a slight modification was made in the protocol.³⁶ A total of 5 groups (each, $n = 6$) were treated with doses (50, 100, 200 mg/kg), standard drug, diclofenac sodium (10 mL/kg). After 1 h interval of the administration of various agents, the edema was induced by the injection of freshly prepared carrageenan (0.1 mL, 3%, w/v in saline) into the subplanter tissue of the right hind-paw of each mouse. The inflammation was noted by measuring the volume displayed by paw, using a plethysmometer (Beijing Zhong-shidichuang Science Technology Development Co., Ltd., model TLS-7C, China) after a

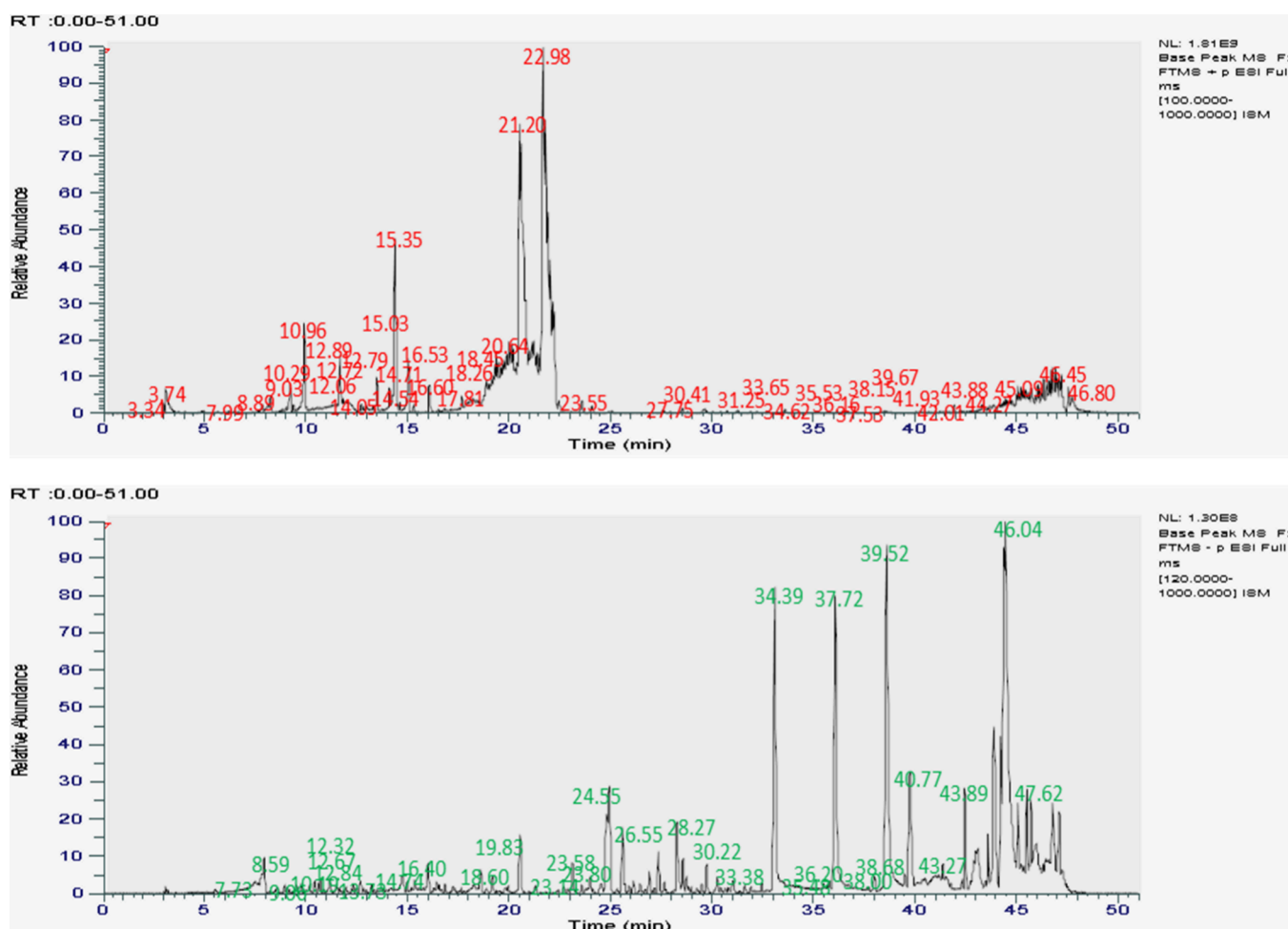


Figure 1. LC-MS/MS-orbitrap metabolites profiling of AHS methanol extract in ESI⁺ mode (red) and ESI⁻ mode (green). A total ion chromatogram (TIC) in both positive and negative ions based on UPLC-ESI-MS/MS analysis.

carrageenan injection at 0, 1, 2, 3, 4, 5 h, respectively. The percentage inhibition (each mass and group) described as follows:

$$\text{Percentage Inhibition} = C - T/C \times 100$$

C = Increases in paw edema (control), T = Increases in paw edema (test)

2.11. Bioinformatics Approach. **2.11.1. Molecular Docking In Silico Study.** The *in silico* molecular study of the identified metabolites in the AHS through LC-MS/MS-orbitrap were studied against the inflammatory enzyme cyclooxygenase-2 (COX2) (PDB-ID: 5JVZ), and cancer enzyme ADAM10 (PDB ID: 6BDZ) to support the *in vitro* and *in vivo* activities. COX-2 enzyme is the mediator of prostaglandin, the prostaglandin is responsible for the cause of pain and inflammation, and ADAM10 is a key matrix remodeling cancer enzyme and has an important role in causing cancer.^{37,38}

2.11.2. Preparation of Ligands. The three-dimensional (3D) chemical structures of the identified compounds were retrieved from the Pub-chem Data Base (<https://pubchem>) and saved in PDB format. The crystal structure of COX-2 and ADAM10 enzymes were retrieved from Protein-Data-Bank (<http://www.rcsb.org/pdb>). The structures of both enzymes were prepared in the Molecular Operating Environment (MOE) software.

2.11.3. Retrieval and Preparation of Target-Proteins. The hetero atoms and water molecules were removed followed by the addition of polar hydrogen to maintain cellular pH. The correct state of hybridization was assigned to each atom in each residue. The MOE software was used for locating active residues at the active sites of enzymes. The selecting phytochemicals were docked inside the active pocket of COX-2 (5JVZ) and ADAM10 (6BDZ) enzymes; and employing the docking program from software. The energy minimization was performed, and the largest chain of amino acids of both enzymes were selected and isolated as dummy atoms for docking. The prepared protein was saved for further analysis. A library of the tentatively 7 ligands was prepared, and their structures were energy minimized. For docking of the both ligands and prepared protein the number of possess were selected as 3 in number, their affinity was calculated on London-Dock score using rigid system.³⁹

2.12. Analysis of In Silico Toxicity. The toxicity prediction of the compounds was conducted using the ProTox-II version 3.0 web online server (<https://tox-new.charite.de/protoxII/>). Additionally, evaluations were performed for the hepatotoxicity, immunogenicity, mutagenicity, cytotoxicity, and carcinogenicity of the compounds.

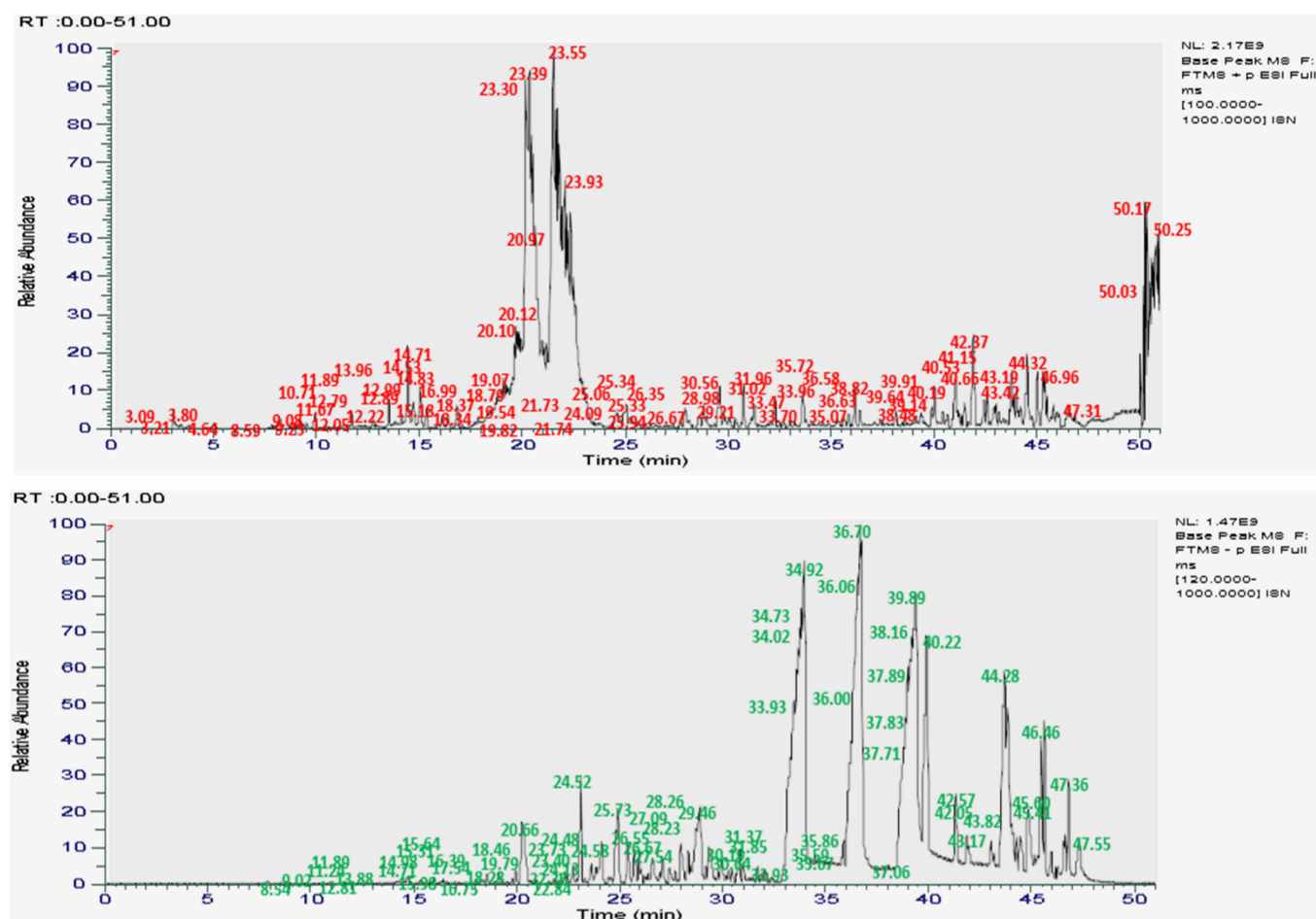


Figure 2. LC-MS/MS-orbitrap metabolites profiling of AHS *n*-hexane extract in ESI⁺ mode (red), and ESI⁻ mode (green). A total ion chromatogram (TIC) was performed in both positive and negative ions based on UPLC-ESI-MS/MS analysis.

3. RESULTS AND DISCUSSION

3.1. Metabolites Profiling via LC-MS/MS-Orbitrap. In the present research study, an untargeted metabolite profiling of AHS was investigated for the first time, showing satisfactory data quality with high sensitivity and specificity. The base-peak chromatograms (BPCs) of analyzed AHS extract ESI⁺ (red) and ESI⁻ (green) with elution areas of each metabolite were efficiently detected and separated, as depicted in Figure 1 and Figure 2. AHS extract (methanol and *n*-hexane) were analyzed via LC-MS/MS-orbitrap to interpret immense diversity of available bioactive metabolites (phytochemicals) and provided detailed chromatographic profile. Accordingly, metabolites were identified by matching the retention time (RT), $[M + H]^+$ and $[M - H]^-$ m/z precise molecular mass including MS/MS fragments ion with database (PCDL) & METLIN. Relating with literature their structure was elucidated, along with some unidentified extrolites mass peaks in both ESI⁺ and ESI⁻. However, only peaks of 90–99% confidence level were selected in this study Table 1–4. A total of 44 metabolites, 26 in ESI⁺ positive mode and 18 in ESI⁻ negative mode from MeOH extract, while 74 metabolites (46 in ESI⁺ and 28 in ESI⁻ mode) were identified in the *n*-hexane extract Table 1–4. The significant bioactive metabolites were identified including alkaloids, flavonoids, organic acids, amino acids, fatty acids, phytosterols, terpenoids, carbohydrates, steroids, phenols, lignans, ketone, cyclic hydrocarbons, and nitrogenous compounds.

In MeOH extract (ESI⁺/ESI⁻ mode) of AHS, prominent metabolites reported were hernagine, shogaol, (s)-boldine, kauralexin, and salidroside. Likewise, in the *n*-hexane extract the following important metabolites were observed in the (ESI⁺/ESI⁻) mode such as zerumbone, antheraxanthin, cohumulone, *N*-methylhernagine and formononetin correspondingly.

The identified metabolites including shogaol, coumarin, kaempferol, erucamide, monolinolenin, oleamide, kaempferol 3,7,4'-trimethyl ether, hordenine and hernagine have been investigated for various biological and pharmacological activities^{40,41} including anticancer, analgesic, cardiotoxic, anti-inflammatory, anthelmintic, antimalarial, antimicrobial and anticonvulsant potential. These compounds are also used in the synthesis of many products, and its higher exposure in mice has been linked to adverse effects.^{42–44} In addition, other alkaloids and phenolic metabolites reported in this study also exhibited remarkable pharmacological potency.^{45,46}

Interestingly, to the best of our knowledge some metabolites including hernagine, shogaol, formononetin, cohumulone, colneleate, medicarpin, zerumbone, boldine has not been reported previously from this specie.⁴⁷ This change might be due to multigene responses in the primary and secondary metabolites accumulation influenced by abiotic stresses.⁴⁸ Formononetin is reported to exhibit anticancer effects on lungs, breast, prostate, colon and nasopharyngeal cancer cells.⁴⁹ Formononetin and chrysin are naturally flavone, and

Table 1. LC-MS/MS-Orbitrap Metabolites Profiling of AHS Methanol Extract in ESI⁺ Positive Ions Mode^a

No.	Proposed Metabolites	Compound Formulas	RT (min)	[M + H] ⁺ (m/z)	Molecular Weight	Major secondary MS/MS ion fragments
Phenolic compounds						
1	Shogaol	C ₁₇ H ₂₄ O ₃	34.62	277.17944	276.17215	149.79648, 173.55020, 96.14411, 91.59561
2	4-Coumaric acid	C ₉ H ₈ O ₃	10.50	165.05712	164.04712	147.04353, 149.80675, 137.09528, 128.11758, 119.04871
3	Thymol (2-ethyl-4,5-dimethylphenol)	C ₁₀ H ₁₄ O	12.60	151.11131	150.10431	91.05379, 105.06936, 117.06932
4	Phloracetophenone	C ₈ H ₈ O ₄	4.05	169.04902	168.04202	123.04364
5	N-Acetyldopamine	C ₁₀ H ₁₃ NO ₃	10.26	196.09648	195.08920	137.05922, 113.96326
6	Ferulate (Ferulic acid)	C ₁₀ H ₁₀ O ₄	11.21	195.06774	194.05774	145.02805, 120.36972, 113.96287
7	2,3-dihydroxy-p-cumate	C ₁₀ H ₁₂ O ₄	12.53	197.08032	196.07332	105.03305, 128.23633, 179.10612
8	Tricoumaroyl spermidine	C ₃₄ H ₃₇ N ₃ O ₆	18.26	584.27496	583.26769	162.89926, 292.20197
Alkaloid compounds						
9	Hordeanine	C ₁₀ H ₁₅ NO	3.74	166.12241	165.11513	121.06427, 150.11224, 149.79932, 96.20840
10	(S)-Boldine	C ₁₉ H ₂₁ NO ₄	12.72	328.15402	327.14675	265.08536, 177.49207, 153.64423, 145.11467, 127.99976
11	N-Methyltyramine	C ₉ H ₁₃ NO	3.34	152.10687	151.09960	121.06443, 119.17963, 105.04449, 102.20107, 134.05914
12	Miraxanthin V	C ₁₇ H ₁₈ N ₂ O ₆	10.96	347.12329	346.11605	193.97574, 211.08521
13	Glandicoline A	C ₂₂ H ₂₁ N ₃ O ₃	14.05	404.16946	403.16246	242.11705
Flavonoid compounds						
14	Taxifolin (dihydroxyquercetin)	C ₁₅ H ₁₂ O ₇	10.29	305.06794	304.05794	149.79730, 128.01340, 82.86884
15	Rutin (quercetin-3-O-rutinoside)	C ₂₇ H ₃₀ O ₁₆	12.89	611.16003	610.15276	287.05467, 311.05609, 355.07147, 286.04737, 233.11487
16	Kaempferol (3,4,5,7-tetrahydroxy-flavone)	C ₁₅ H ₁₀ O ₆	12.79	287.05466	286.04738	153.01770, 165.01816, 213.05396, 121.02798, 111.04381
17	Quercetin (3,3,4,5,7-pentahydroxy-flavone)	C ₁₅ H ₁₀ O ₇	12.06	303.0495	302.04222	153.01759, 165.01765, 187.03781, 201.05411, 137.02272
Terpenoid compounds						
18	α -Pinene-2-oxide	C ₁₀ H ₁₆ O	13.09	153.12687	152.11987	107.08504, 95.08511, 93.06946
19	Dehydroabietate	C ₂₀ H ₂₈ O ₂	21.20	301.21597	300.20860	81.06951, 105.06937, 119.08508, 135.04349, 145.10049
20	Ionone	C ₁₃ H ₂₀ O	12.60	193.16119	192.15119	135.11639, 109.10075, 99.08004
Other organic compounds						
21	Estradiol	C ₁₈ H ₂₄ O ₁₂	10.96	433.13368	432.12660	127.03845
22	Acetonylacetone	C ₆ H ₁₀ O ₂	3.15	115.07532	114.06804	102.38411, 96.96056
23	Loliolide	C ₁₁ H ₁₆ O ₃	14.71	197.11681	196.10953	91.05388, 95.04881, 105.06949, 118.07711, 173.54370,
24	Androsta-1,4-diene-3,17-dione	C ₁₉ H ₂₄ O ₂	33.68	285.18415	284.17715	191.10632, 177.09090, 151.07491, 217.12192
25	Monolinolenin	C ₂₁ H ₃₆ O ₄	31.25	353.2681	352.26088	261.22089, 263.23334, 279.22989, 243.21010, 233.22569
26	8-Hydroxyquinoline	C ₉ H ₇ NO	9.03	146.05983	145.05255	91.05380, 101.74202, 117.05676,

^aRT: retention time.

flavonoids have been used as drugs and documented as anti-inflammatory, anticancer, antibacterial, antiviral and antioxidant properties.⁵⁰

Some of the mass values in AHS methanol fraction such as m/z [M + H]⁺ 403.28218, 181.04896, 285.18415 and [M - H]⁻ 311.22168, 477.13843 did not showed any match with METLIN & PCDL library (LC-MS/MS-orbitrap) database. Similarly, in *n*-hexane fraction m/z [M + H]⁺ 273.257, 311.2215, 289.25177, 399.31012, 267.19485, 347.29422 and m/z [M - H]⁻ 293.2124, 295.228, 311.22287, 309.20691, 265.18097 did not show any match with the available database. However, the identification of these unknown m/z peaks could lead to the exploration of new compounds in this specie.

This research study explores the variation of diverse metabolites in the AHS extract, which will support researchers in determining the chemotaxonomy and bioactive as well as the toxic potential of *A. heterophyllum*. The identified metabolites (chemotypes) in this mass spectrometry study

will also facilitate the scientist to minimize the efforts' loss to avoid (dereplication) in the future.

3.2. Gas Chromatography–Mass Spectrometry (GC-MS) Analysis. The volatile and nonvolatile chemical composition of both MeOH and *n*-hexane extracts of AHS were analyzed employing a GC-MS technique. These results were obtained based on matching of molecular masses with NIST library record number Tables 5 and 6. Only peaks having molecular masses between 90 and 99% confidence level were selected in this study. Similarly, a well-defined chromatogram depicted the chemical profile of both extracts of AHS in Figure 3. The GC-MS identified phytochemicals were classified into organic acids, fatty acids, nitrogenous compounds, phenols, alcohols, phytosterols, myristin aldehyde, saccharides, long chain hydrocarbons along with some typical some GC impurities.

The principal identified phytochemicals were furan-2-carbaldehyde, 2,4-dihydroxy-2,5-dimethyl-3(2H)-furan-3-one, 4H-pyran-4-one, 2,3-dihydroxy-6-methyl, benzoic acid, (+)-di-

Table 2. LC-MS/MS-Orbitrap Metabolites Profiling of AHS Methanol Extract in ESI⁻ Negative Ions Mode^a

No.	Proposed Metabolites	Compound Formulas	RT (min)	[M-H] ⁻ (m/z)	Molecular Weight	Major secondary MS/MS ion fragments
Phenolic compounds						
1	Salidroside	C ₁₄ H ₂₀ O ₇	9.06	299.11243	300.11963	101.02321, 119.02324, 143.03438, 174.94678
2	2,5-Dihydroxybenzaldehyde	C ₇ H ₆ O ₃	8.59	137.0239	138.03117	127.02981, 108.02098
3	Phloracetophenone	C ₈ H ₈ O ₄	12.84	167.03442	168.04170	108.02098
4	1-O-(4-Coumaroyl)-β-D-glucose	C ₁₅ H ₁₈ O ₈	10.19	325.09177	326.09903	197.69624, 145.02878, 163.03937, 187.02234
Alkaloid and derivatives compounds						
5	4-Indolecarbaldehyde (3-hydroxyquinoline)	C ₉ H ₇ NO	14.74	144.04479	145.05206	126.03415, 116.04984, 111.75036
6	4-Methoxycarbonylindole	C ₁₀ H ₉ NO ₂	19.83	174.05547	175.06274	115.04206, 126.81171, 142.02905
7	Pheophorbide A	C ₃₅ H ₃₆ N ₄ O ₅	35.48	591.26983	592.26710	156.94370, 149.75351
Terpenoid compounds						
8	Oleanolic acid	C ₃₀ H ₄₈ O ₃	38.00	455.35226	456.35957	348.07373
9	Kauralexin A2	C ₂₀ H ₃₀ O ₄	23.14	333.20609	334.21337	255.17455, 217.21831, 173.51964, 123.08077
10	3-Hydroxyurs-12-en-23-oic acid	C ₃₀ H ₄₈ O ₃	37.72	455.35226	456.35956	365.76349, 173.51904
Fatty acids, esters						
11	Palmitoleate	C ₁₆ H ₃₀ O ₂	33.38	253.21672	254.22399	193.78717, 149.77524, 91.00308
12	Stearate	C ₁₈ H ₃₆ O ₂	43.27	283.26398	284.27126	173.51840, 149.78381
13	Colneleate	C ₁₈ H ₃₀ O ₃	23.58	293.21124	294.21864	173.52242, 218.81137, 149.75681
14	Oleate	C ₁₈ H ₃₄ O ₂	39.52	281.24805	282.25530	111.71655, 173.49643, 244.09117
15	Palmitate	C ₁₆ H ₃₂ O ₂	47.62	255.2327	256.23958	216.90852, 199.17119, 149.78563, 118.98807
Other organic compounds						
16	11(Z),14(Z)-Eicosadienoic acid	C ₂₀ H ₃₆ O ₂	40.77	307.26395	308.27122	173.49602, 149.57520, 136.59280, 111.81728
17	Arachidic acid	C ₂₀ H ₄₀ O ₂	36.20	311.29498	312.30226	173.49750, 163.59361, 118.37830, 206.17496
18	16-Hydroxyhexadecanoic acid	C ₁₆ H ₃₂ O ₃	28.27	271.22723	272.23452	225.22150, 221.19011, 197.19037

^aRT: retention time.

benzoyl-L-tartaric acid anhydride, 5-hydroxymethylfurfural, 1,2,3-propanetriol 1-acetate, glycerol 1,2-diacetate, ketone methyl 2-methyl-1,3-oxothiolan-2-yl, 2-methoxy-4-vinylphenol and n-hexadecanoic acid. These metabolites are known for strong pharmacological activities, including hypocholesterolemic, analgesic, antiandrogenic, nematocidal, hemolytic inhibitor, pesticide, lubricant, and antioxidant activity.⁵¹ Gamma-sitosterol is reported to possess anticancer, anti-inflammatory and antidiabetic properties.⁵² However, the ethyl/methyl esters of the fatty acids and phenol such as 2-methoxy-4-vinylphenol, phytol linoleate, neophytadiene, phytol, linolenic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester were reported as cytotoxic, antimicrobial, antimicrobial, anti-inflammatory and antioxidant.^{53–55} (+)-ascorbic acid 2,6-dihexadecanoate, 9,12,15-Octadecatrienoic acid, phenyl methyl ester, furfural and 5-hydroxymethylfurfural was also reported in scientific literature to possess cytotoxic anticancer activity.^{54,56}

Previous studies on genus *aconitum* have reported the same chemical profile of the metabolites as reported in our investigation.^{57,58} This will help in chemotaxonomic studies and phytochemical profiling of medicinal plants in the genus *aconitum*.

3.3. Disease-Relevant *In Vitro* and *In Vivo* Activities of *A. heterophyllum* Stem Extracts. The justification that selected metabolites identified in *Aconitum heterophyllum* stem (AHS) extracts are predicted to bind to COX-2 (5JVZ) and ADAM10 (6BDZ) suggests that these extracts may contain components capable of altering the activities of crucial enzymes involved in mediating human disease. Consequently,

the extracts are expected to exhibit effects in some commonly used models of human disease. Accordingly, we have investigated both the methanol and *n*-hexane extracts for *in vitro* toxicity, and in model experimental systems for *in vivo* anti-inflammatory and analgesic activities.

3.3.1. *In Vitro* Anticancer Cell Viability Assay. Cancer is the second largest cause of mortality globally owing to uncontrolled-growth and proliferation of cells.^{59,60} The high cost and adverse effects of drugs with long-term process are not fulfilling the desired expectations of researchers.^{61,62} In recent decades, synthetic chemotherapy approach is used globally to treat various types of cancers disease.^{24,63} In this study, the anticancer activities of AHS extracts were investigated against various cancer cell lines, MDCK (Madin-Darby canine kidney), NIH3T3 (fibroblast cell), and H9c2 (rat embryonic ventricular-myocardial) in 96-well plates by measuring the percent cell viability. The IC₅₀ μg/mL data showed that treatment of both extracts in 24 h potently suppressed the cell viability. The most significant IC₅₀ μg/mL (186.39 and 199.63) was recorded for MeOH extract against H9c2 and MDCK cells, respectively. No significant difference was found between the IC₅₀ of *n*-hexane extract against the cancer cell lines H9c2 and MDCK. Although no selective effect was observed in any of the extracts, as both extracts displayed optimal anticancer activities against all the tested cancer cell lines (Table 7).

Similarly, the study also showed the dose-dependent behavior, because it was evident that the % cell viability decreases as the dose concentration increases. It was

Table 3. LC-MS/MS-Orbitrap Metabolites Profiling of AHS *n*-Hexane Extract in ESI⁺ Positive Ions Mode^a

No.	Proposed Metabolites	Compound Formulas	RT (min)	[M + H] ⁺ (<i>m/z</i>)	Molecular Weight	Major secondary MS/MS ion fragments
Phenolic compounds						
1	2-Anisic acid	C ₈ H ₈ O ₃	23.39	153.04393	152.04721	92.02522, 95.04869, 105.04425
2	<i>p</i> -Coumaric acid	C ₉ H ₈ O ₃	15.18	165.05712	164.04717	91.05383
3	4-Hydroxybenzyl alcohol	C ₇ H ₈ O ₂	28.77	125.05951	124.05223	95.04872, 105.04424, 83.17227
4	(-)-Medicarpin	C ₁₆ H ₁₄ O ₄	27.71	271.09585	270.08885	147.04347, 105.06942, 91.05383
5	Traumatin	C ₁₂ H ₂₀ O ₃	20.12	213.14805	212.14105	-
6	Tricoumaroyl spermidine	C ₃₄ H ₃₇ N ₃ O ₆	18.79	584.27518	583.26818	147.04362
7	3-Hydroxyanthranilic acid	C ₇ H ₇ NO ₃	25.06	154.04968	153.04241	-
8	2,4-Dimethylcinnamic acid	C ₁₁ H ₁₂ O ₂	33.65	177.09074	176.08347	91.05389, 116.06164
Alkaloid compounds						
9	<i>N</i> -Methylhernagine	C ₂₀ H ₂₃ NO ₄	11.83	342.16959	341.16231	191.08493, 207.0795, 222.0668, 179.08495, 239.070
10	Hernagine	C ₁₉ H ₂₁ NO ₄	11.67	328.15411	327.14678	222.06693, 207.07979, 191.08499, 179.08492
11	Norharman	C ₁₁ H ₈ N ₂	11.89	169.07553	168.06853	115.05395
12	<i>N</i> -(5-Methoxy-1H-indol-3-yl)acetamide	C ₁₁ H ₁₂ N ₂ O ₂	18.53	205.08066	204.07366	129.05684, 145.05173, 117.05685
13	<i>N</i> -(1H-Indol-3-ylacetyl)isoleucine	C ₁₆ H ₂₀ N ₂ O ₃	14.83	289.15427	288.14727	144.10185, 186.12776
14	Solasodine	C ₂₇ H ₄₃ NO ₂	41.69	414.33606	413.32878	93.06982, 105.0698, 131.0853, 145.10103, 173.1323
15	Guineesine	C ₂₄ H ₃₃ NO ₃	33.70	384.25354	383.24628	149.05968, 131.04913, 103.05423, 91.05426
Flavonoid compounds						
16	Formononetin	C ₁₆ H ₁₂ O ₄	21.73	269.0805	268.07350	197.05921
17	Chrysin	C ₁₅ H ₁₀ O ₄	24.25	255.06497	254.05770	103.05383, 153.01772
18	Kaempferol	C ₁₅ H ₁₀ O ₆	12.79	287.05463	286.04759	153.01822, 121.02828
19	Quercetin	C ₁₅ H ₁₀ O ₇	12.05	303.04941	302.04241	153.01813, 137.02307
20	Rutin (quercetin-3-O-rutinoside)	C ₂₇ H ₃₀ O ₁₆	12.89	611.16046	610.15319	287.05493
Terpenoid compounds						
21	Zerumbone	C ₁₅ H ₂₂ O	31.02	219.17415	218.16687	91.05385, 105.04429, 117.06947, 129.06938
22	Cibacic acid	C ₁₈ H ₂₈ O ₅	27.58	325.20014	324.19314	147.11606
23	18-β-Glycyrrhetic acid	C ₃₀ H ₄₆ O ₄	32.15	471.34598	470.33898	119.08508, 133.10074, 189.1003, 253.19490, 107.08
24	Kauralexin B3	C ₂₀ H ₂₈ O ₃	33.96	317.21063	316.20348	253.19443, 225.16435, 197.13165, 271.20508
25	(+)-Myrtenal	C ₁₀ H ₁₄ O	14.71	151.11142	150.10442	109.10083, 123.08016, 93.06938
26	Antheraxanthin	C ₄₀ H ₅₆ O ₃	40.53	585.42952	584.42252	105.06939, 119.0850, 145.1005, 173.54510, 93.0694
27	Abieta-7,13-dien-18-ol	C ₂₀ H ₃₂ O	42.76	289.25177	288.24477	110.05949, 123.06729, 154.50533, 82.06472
28	(±)-Abscisic acid (ABA)	C ₁₅ H ₂₀ O ₄	17.63	265.14301	264.13601	187.11121, 173.13194, 163.07491, 149.05916
29	<i>p</i> -Cymene (1-methyl-4-(1-methylethyl)-benzene)	C ₁₀ H ₁₄	30.07	135.11633	134.10933	91.05389, 105.06942, 84.95943
Coumarin compounds						
30	Coumarin (1-benzopyran-2-one)	C ₉ H ₆ O ₂	13.96	147.04372	146.03672	91.05386
31	Methoxsalen (UVADEX)	C ₁₂ H ₈ O ₄	20.41	217.04913	216.04213	118.04098
Lignan compounds						
32	Lariciresinol-4-O-glucoside	C ₂₆ H ₃₄ O ₁₁	12.99	523.21683	522.20983	219.10133
33	Sedanolid	C ₁₂ H ₁₈ O ₂	28.70	195.13773	194.13045	109.06436, 91.05385, 81.06953
Other organic compounds						
34	Acetanilide (<i>N</i> -phenylacetamide)	C ₈ H ₉ NO	19.07	136.07491	135.06791	95.04868, 105.04433
35	Jasmonoyl-phenylalanine (JA-Phe)	C ₂₁ H ₂₇ NO ₄	20.97	358.20081	357.19381	254.11717, 143.08517, 119.08535, 93.06950
36	Erucamide	C ₂₂ H ₄₃ NO	46.96	338.34119	337.33391	223.20660, 170.15308, 142.12201, 109.10062
37	Stearoylethanolamide (SEA)	C ₂₀ H ₄₁ NO ₂	42.37	328.32063	327.31325	236.32623, 151.14711, 139.11136, 111.11632
38	(9S,13R)-12-oxo-phytodienoic acid	C ₁₈ H ₂₈ O ₃	29.21	293.21075	292.20354	229.19479, 187.11115, 145.10068, 107.08505
39	Rhodiocetane	C ₁₉ H ₃₆ O ₁₀	16.99	425.23749	424.23049	145.04942, 223.05981, 295.10199, 127.03886
40	Dihydropanaxacol (marcrocetone)	C ₁₇ H ₂₈ O ₃	31.96	281.21076	280.20351	135.11658, 147.1165, 189.1136, 231.17426, 121.009
41	Eicosatetraenoic acid (ETYA)	C ₂₀ H ₂₄ O ₂	29.58	297.18415	296.17715	105.06944, 143.0849, 179.0848, 193.1006, 91.05383
42	tetranor-12(R)-HETE	C ₁₆ H ₂₆ O ₃	25.79	267.19485	266.18785	91.05387, 105.06950, 118.06472, 81.06958

Table 3. continued

No.	Proposed Metabolites	Compound Formulas	RT (min)	[M + H] ⁺ (m/z)	Molecular Weight	Major secondary MS/MS ion fragments
Other organic compounds						
43	3-Dehydrosphinganine	C ₁₈ H ₃₇ NO ₂	41.74	300.29194	299.28194	173.54608, 145.10074, 107.085, 93.06941, 83.08509
44	Linoleyl alcohol	C ₁₈ H ₃₄ O	36.02	267.26758	266.26058	95.08510, 109.10067, 123.11629
45	Cohumulone	C ₂₀ H ₂₈ O ₅	26.67	349.20071	348.19339	275.20026, 257.18970, 229.19533, 147.11630
46	Erucic acid (omega-9-fatty acid)	C ₂₂ H ₄₂ O ₂	42.23	339.32513	338.31786	223.2040, 191.1783, 163.14705, 135.11580, 83.0848

^aRT: retention time.

Table 4. LC-MS/MS-Orbitrap Metabolites Profiling of AHS *n*-Hexane Extract in ESI⁻ Negative Mode^a

No.	Proposed Metabolites	Compounds Formula	RT (min)	[M - H] ⁻ (m/z)	Molecular weight	Major secondary MS/MS ion fragments
Phenolic compounds						
1	Salidroside	C ₁₄ H ₂₀ O ₇	9.02	299.11258	300.11978	119.03434, 101.02379, 89.02379
2	Ferulic acid	C ₁₀ H ₁₀ O ₄	15.98	193.05025	194.05753	133.02881, 160.01610, 108.02110
3	Protocatechuic aldehyde	C ₇ H ₆ O ₃	8.54	137.02388	138.03116	108.02099, 92.02606
4	Methyl 4-hydroxycinnamate	C ₁₀ H ₁₀ O ₃	18.76	177.05559	178.06286	117.03395
Alkaloid compounds						
5	4-Indolecarbaldehyde	C ₉ H ₇ NO	14.71	144.04537	145.05237	126.03419, 116.04987
6	4-Methoxycarbonylindole	C ₁₀ H ₉ NO ₂	19.79	174.05592	175.06320	115.04205, 142.02911
7	Pheophorbide A	C ₃₅ H ₃₆ N ₄ O ₅	35.67	591.26184	592.26912	178.98854, 149.74893, 134.14359
Flavonoid compounds						
8	Kievitone	C ₂₀ H ₂₀ O ₆	30.52	355.11874	356.12602	253.05013
Terpenoid compounds						
9	Asiatic acid	C ₃₀ H ₄₈ O ₅	28.23	487.34247	488.34985	139.02373, 169.67679, 378.50348
10	Kauralexin A1	C ₂₀ H ₃₂ O ₂	35.59	303.23276	304.24003	173.51834, 223.70741, 105.77166
11	3-Hydroxyurs-12-en-23-oic acid	C ₃₀ H ₄₈ O ₃	38.16	455.35315	456.36044	375.27051, 162.97017
12	Kauralexin A2	C ₂₀ H ₃₀ O ₄	23.40	333.20663	334.21391	123.08083, 255.17456, 175.11220
13	(3R)-Linalool (2,6-dimethyl-2,7-octadien-6-ol)	C ₁₀ H ₁₈ O	22.84	153.12852	154.13580	118.78210, 85.17933
14	Kauralexin A3	C ₂₀ H ₃₀ O ₃	27.09	317.21244	318.21944	173.52000, 123.08104
15	Dehydroabietate	C ₂₀ H ₂₈ O ₂	31.37	299.20148	300.20875	144.60588, 169.12080, 178.16594
16	Kauralexin B2	C ₂₀ H ₂₈ O ₄	30.64	331.19138	332.19873	101.99514
17	Kauralexin B3	C ₂₀ H ₂₈ O ₃	29.46	315.19666	316.20366	173.49675, 97.06527
Other organic compounds						
18	Corchorifatty acid F	C ₁₈ H ₃₂ O ₅	15.31	327.21729	328.22429	171.10239, 97.06534, 211.13385
19	α -Linolenate	C ₁₈ H ₃₀ O ₂	31.01	277.21707	278.22435	127.07572, 177.39499, 197.37523, 118.84630
20	5-Androstene-3,17-dione	C ₁₉ H ₂₆ O ₂	19.43	285.18603	286.19303	173.52113, 149.77179, 93.04300, 183.04449
21	Lignoceric acid	C ₂₄ H ₄₈ O ₂	45.60	367.35785	368.36513	252.46346, 119.67238, 178.4710, 127.1274, 90.10276
22	Gibberellin A12-aldehyde	C ₂₀ H ₂₈ O ₃	23.73	315.19632	316.20363	173.51968
23	Colneleate	C ₁₈ H ₃₀ O ₃	26.23	293.21246	294.21972	175.45064, 135.29364, 112.66634, 81.43077
24	Eicosapentaenoate (EPA)	C ₂₀ H ₃₀ O ₂	36.06	301.21725	302.22453	94.56732
25	Ricinoleic acid	C ₁₈ H ₃₄ O ₃	26.80	297.24316	298.25048	183.01167, 173.51744
26	Pyridoxal	C ₈ H ₉ NO ₃	20.92	166.05096	167.05824	135.04440, 121.02885, 107.04945
27	Jasmonic acid (JA)	C ₁₂ H ₁₈ O ₃	13.88	209.11808	210.12508	173.52335, 149.76279
28	Arachidic acid	C ₂₀ H ₄₀ O ₂	47.55	311.29535	312.30263	173.49533, 192.1789, 224.4843, 149.7538, 112.56179

^aRT: retention time.

documented that in comparison, the concentration of 200 μ g/mL was highly active against all cancer cell lines. The lowest anti proliferative effect was observed in the doses of *n*-hexane extract against NIH3T3 cell line. Correspondingly, the doses of methanol extract were highly cytotoxic against H9c2 cell lines (Figure 4).

While comparing our result of AHS with the standard drug aconitine, it was observed that at low doses, the difference between the doses of extracts and aconitine was nonsignificant. But as the concentration of doses increase, a significant

difference in the antiproliferative effect was noted against cancer lines except MDCK. The methanol extract of AHS showed almost the equivalent anti proliferative effect as compared to aconitine at higher doses (Figure 5).

Natural products (NPs) are receiving unprecedented attention around the globe owing to bioactive phytochemical constituents especially in the anti-infection and antitumor areas. About 67% effective cancer drugs are derived from NPs such as vincristine and vinblastine from various plant species.^{5,64,65}

Table 5. Identified Phytocompounds through the GC-MS Spectrum of AHS Methanol Extract

Proposed Phytocompounds	Formula	RT (min)	Molecular weight	NIST record No.
Furan-2-carbaldehyde	C ₅ H ₄ O ₂	5.16	96.02	228304
2,4-Dihydroxy-2,5-dimethyl-3(2 <i>H</i>)-furan-3-one	C ₆ H ₈ O ₄	8.28	144.04	413166
4 <i>H</i> -Pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl	C ₆ H ₈ O ₄	12.71	144.04	156511
Benzoic acid	C ₇ H ₆ O ₂	13.23	122.04	290514
(+)-Dibenzoyl-L-tartaric acid anhydride	C ₁₈ H ₁₂ O ₇	13.13	340.06	244836
5-Hydroxymethylfurfural	C ₆ H ₆ O ₃	15.10	126.03	231276
1,2,3-Propanetriol, 1-acetate	C ₈ H ₁₀ O ₄	15.72	134.06	76112
Glycerol 1,2-diacetate	C ₇ H ₁₂ O ₅	15.72	176.07	385652
Ketone,methyl 2-methyl-1,3-oxothiolan-2-yl	C ₆ H ₁₀ O ₂ S	15.72	146.04	138885
2-Methoxy-4-vinylphenol	C ₉ H ₁₀ O ₂	17.4	150.07	135956
<i>n</i> -Hexadecanoic acid	C ₁₆ H ₃₂ O ₂	32.36	256.24	428595
(+)-Ascorbic acid 2,6-dihexadecanoate	C ₃₈ H ₆₈ O ₈	32.36	652.49	233167
9,12,15-Octadecatrienoic acid(<i>Z,Z,Z</i>)	C ₁₈ H ₃₀ O ₂	35.65	278.22	333201
Benzoic acid hydrazide	C ₇ H ₈ N ₂ O	40.47	136.06	231448
Benzeneethanol,4-methyl	C ₉ H ₁₂ O	40.47	136.09	236084
Phytyl linoleate	C ₃₈ H ₆₈ O ₂	54.22	556.52	465220
9,12,15-Octadecatrienoic acid, 1-methyl ester	C ₂₁ H ₃₆ O ₂	54.22	320.27	336802

Table 6. Identified Phytocompounds through the GC-MS Spectrum of AHS *n*-Hexane Extract

Proposed Phytocompounds	Formula	RT (min)	Molecular Weight	NIST record No.
Neophytadiene	C ₂₀ H ₃₈	29.9	278.3	412348
3,7,11,15-Tetramethyl-2-hexadecen-1-ol	C ₂₀ H ₄₀ O	29.9	296.31	114703
<i>n</i> -Hexadecanoic acid	C ₁₆ H ₃₂ O ₂	32.42	256.24	36484
(+)-Ascorbic acid 2,6-dihexadecanoate	C ₃₈ H ₆₈ O ₈	32.42	652.49	233167
Diterpenoid hexadec-2-en-1-ol	C ₂₀ H ₄₀ O	35.18	296.31	108727
9,12,15-Octadecatrienoic acid(<i>Z,Z,Z</i>)	C ₁₈ H ₃₀ O ₂	35.73	278.22	230588
Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	C ₁₉ H ₃₈ O ₄	41.94	330.28	414397
Linolenic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester(<i>Z,Z,Z</i>)	C ₂₁ H ₃₆ O ₄	44.71	352.26	15958
Butyl 9,12,15-octadecatrienate	C ₂₂ H ₃₈ O ₂	44.71	334.29	336546
γ -sitosterol	C ₂₉ H ₅₀ O	55.86	414.39	151559

The plants of the genus *aconitum* chemically include steroids, glycosides, alkaloids, and flavonoids. Diterpenoid alkaloids are considered toxic as well as the main efficient anticancer components of these plants. They have been reported for the significant anticancer activities against various cancer cell lines by inducing apoptosis and altering the effect on multidrug resistant (MDR) carcinomas.⁶⁶ *Taipeinine*, *Lappaconitine* have been studied for inhibiting the cell cycle of cancer cell lines HepG2 and A549. Similarly, various recent studies conducted on the genus *aconitum* has evaluated the significant effect of its metabolites as a potent anticancer.^{47,67,68}

Our LC-MS/MS analysis revealed the interesting phytochemical profile of this plant, which will help in the understanding of the mechanism of the bioactive potential of this genus.

3.3.2. Antinociceptive Activity on Acetic Acid-Induced Writhes. The writhing-model induced by acetic acid in mice, considered as classical peripheral animal pain model for the evaluation of anti-inflammatory and analgesic drugs.⁶⁹ The analgesic activity of the AHS extract was investigated, using the acetic acid induced writhing mice assay. Both MeOH and *n*-hexane extract of doses (50, 100, and 200 mg/kg) evoked a dose dependent inhibition. The AHS extract significantly ($P < 0.05$) inhibited the acetic acid-induced writhing response in a dose dependent manner, with a maximum inhibitory response of MeOH (48.85%) and *n*-hexane (28.85%) at a dose concentration of 200 mg/kg, while lowest inhibitory response was observed at 50 mg/kg (23.23%) and (10.56%) Table 8.

At all doses level, efficient antinociceptive response was shown by MeOH extract compared to *n*-hexane. As compared to standard Diclofenac Sodium (DS) and *n*-hexane extract, the 200 mg/kg of MeOH extract exhibited a significant decrease in writhing, Figure 6.

The LC-MS/MS and GC-MS analyses carried out in this study identified various metabolites with bioactive potential. The alkaloids and phenolic metabolites such as hordenine, colneleate, flavone, rutin (quercetin-3-O-rutinoside), shogaol, hernagine, (s)-boldine (2,9-Dihydroxy-1,10-dimethoxy-aporphine), norharman, chrysin, coumarin, gamma-sitosterol, *n*-hexadecanoic acid, methoxy-6-vinylphenol and (9*S*,13*R*)-12-oxo-phytodienoic acid exhibited a wide range of pharmacological potential including analgesic and anti-inflammatory effects.^{52,53,70} They have been known to inhibit 5-lipoxygenase and cyclooxygenase pathways in acetic acid induced writhing assay.^{71,72} The flavonoid metabolites present in this *A. heterophyllum* species are considered responsible for analgesic property; however, the involvement of other secondary metabolites in the plant species cannot be ruled out.

3.3.3. Anti-inflammatory Activity (Carrageenan-Induced Hind Paw Edema). The *aconitum* specie has been reported to comprise numerous bioactive metabolites which retain various pharmacological potential including analgesic, anti-inflammatory, blood pressure, antiviral, anesthetic and cardio tonic effect.^{11,16,18,20} The treatment of mice with both methanol and *n*-hexane extracts of AHS (50, 100, and 200 mg/kg) doses, before the carrageenan injection significantly ($p \leq 0.01$)

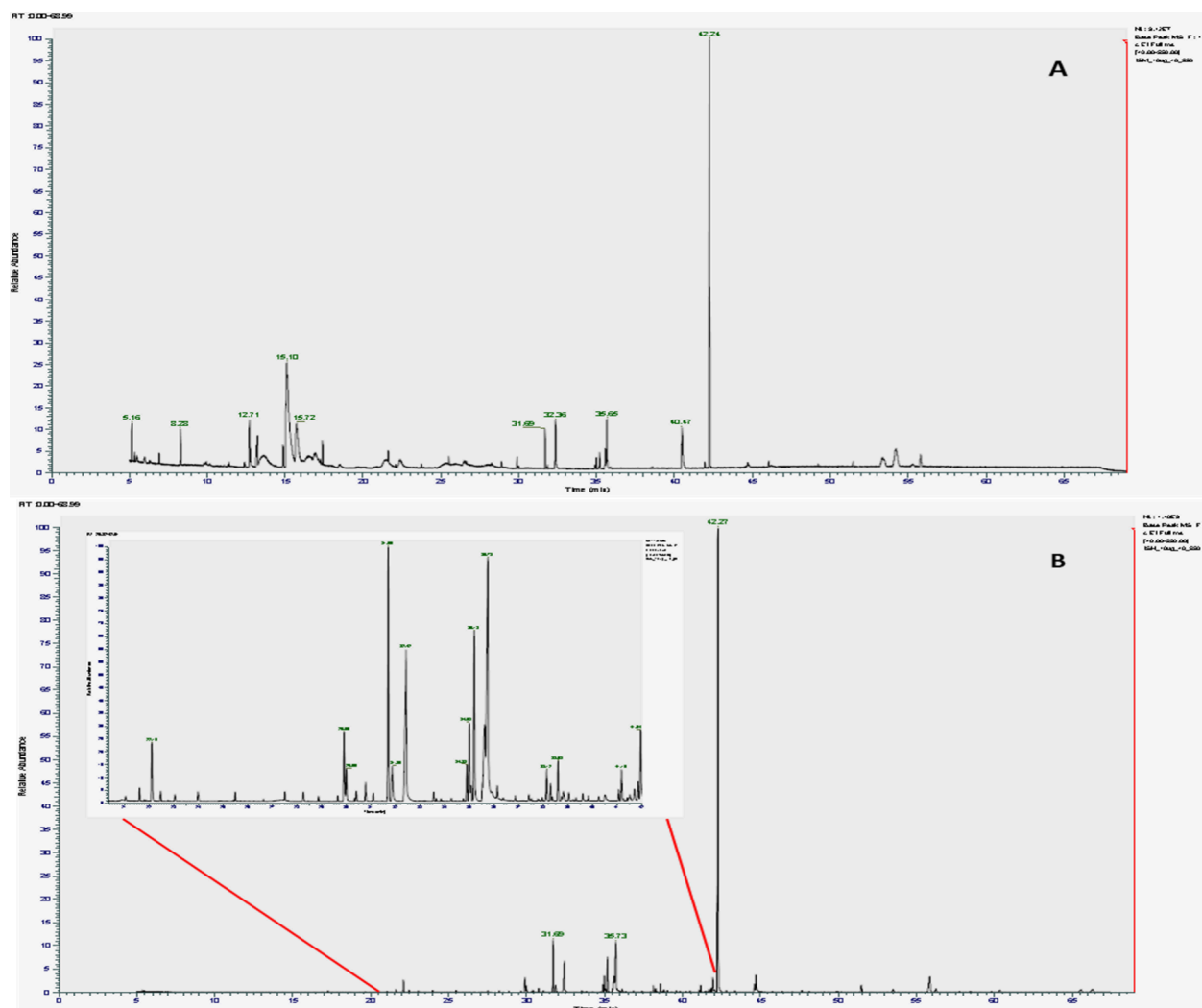


Figure 3. GC-MS phytochemical profiling of methanol (A) and *n*-hexane (B) extracts of AHS. A total ion chromatogram (TIC) was obtained via gas chromatography mass spectrometry electron ionization (GC-MS-EI) analysis.

Table 7. IC₅₀ of Both MeOH and *n*-Hexane Extract of AHS^a

Test sample	Cell lines	IC ₅₀ μg/mL		
		Methanol extract	<i>n</i> -hexane extract	Aconitine
<i>Aconitum heterophyllum</i> stem (AHS) extracts	NIH3T3	216.14	---	161.36
	MDCK	199.63	247.12	141.58
	H9c2	186.39	341.92	132.32

^aNote: (---) represents that IC₅₀ is not promising.

inhibited the edema formation in 1 to 5 h study compared to the control group.³⁶ The methanol extract at dose (200 mg/kg) significantly inhibited the formation of paw edema from 69.41 to 82.94%, and 49.07–63.97% by *n*-hexane extract. At the high dose of 200 mg/kg, no significant difference (Dunnett's-posthoc test) was noted between methanol extract and diclofenac sodium (DS), suggesting effective anti-inflammatory activity for AHS extract (Table 9 and Figure 7).

However, the MeOH extract was more potent at a time interval of 1 to 5 h compared to *n*-hexane extract. The % anti-inflammation for diclofenac sodium (DS) was recorded as 67.03% after 1 h, which increased to 98.19% after 5 h of the

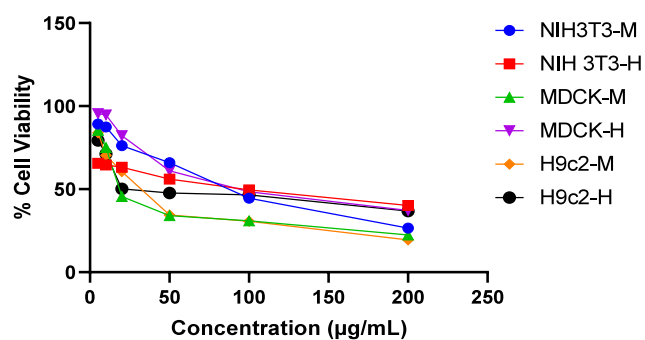


Figure 4. Percent cell viability of various cancer cell lines at different doses of methanol and *n*-hexane stem extracts of *A. heterophyllum*. Note. M: Methanol extract, H: *n*-Hexane extract. Statistically data were analyzed through one-way ANOVA followed by multiple-comparison test (Dunnett's test). Values with distinct letters are significantly different from each other ($p > 0.05$).

study. Significance was assumed as $p \leq 0.05$ (****), $p \leq 0.04$ (**), $p \leq 0.03$ (*) vs Diclofenac Sodium Table 9 and Figure 7. It was also determined from the study that the effect in the *n*-hexane extract was not significant between the doses. Furthermore, the difference between the anti-inflammatory

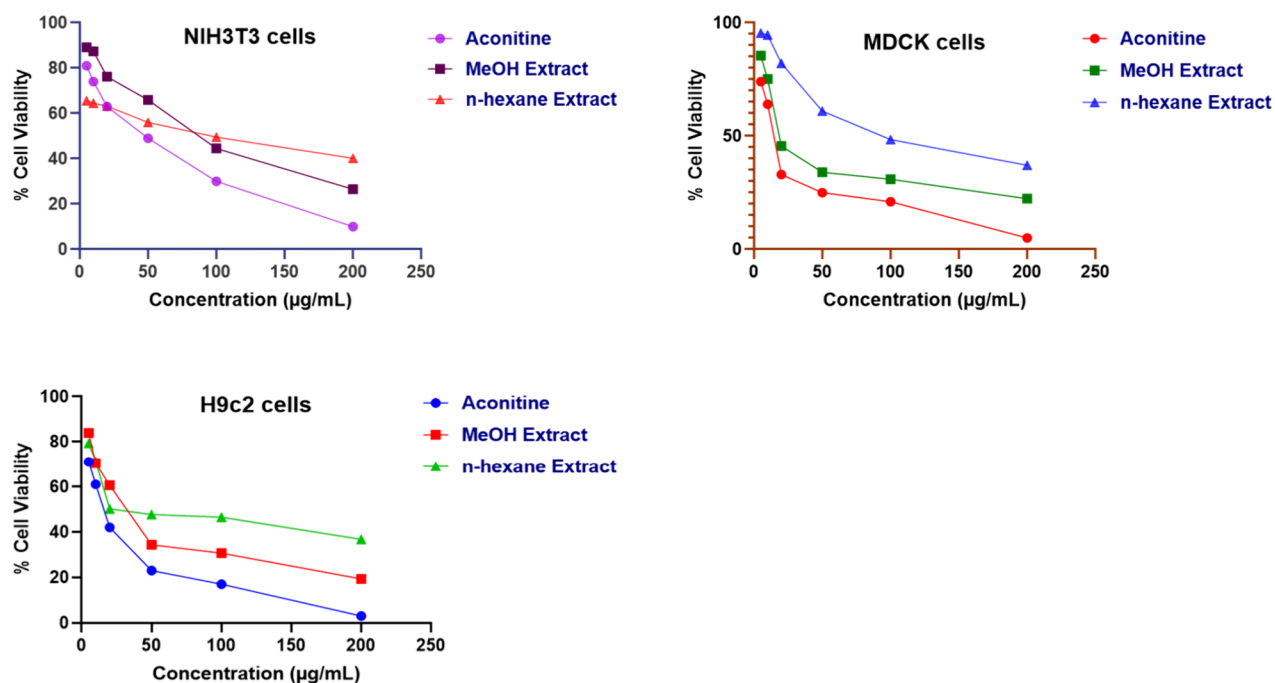


Figure 5. Comparative representation of % cell between cancer cell lines at different doses of methanol, *n*-hexane stem extracts, and Aconitine (standard drug).

Table 8. Effect of AHS Extract on Acetic Acid Induced Writhing Reflex in Mice^a

Treatment	Dose (mg/kg)	Number of writhes	Percentage Inhibition (%)
Diclofenac Sodium (DS)	10	33.54 ± 1.06	68.15
Methanol extract	50	79.41 ± 0.76 ^c	23.23
	100	63.86 ± 0.84 ^b	38.46
	200	53.25 ± 0.92 ^a	48.85
<i>n</i> -Hexane extract	50	92.35 ± 0.96 ^d	10.56
	100	84.61 ± 1.05 ^c	18.14
	200	73.67 ± 1.03 ^b	28.85

^aThe values represented above as mean ± SEM ($n = 3$) standard deviation (SD) followed by letters (a, b, c), indicating a significant difference LSD at $p \leq 0.05$. $p < 0.05$ vs control, $p < 0.05$ vs Diclofenac Sodium (DS).

effect of the methanol AHS extract and the positive control (Diclofenac sodium) was not significant at all dosages, which is a good sign for this study.

Previous studies on the metabolite songorine and analogues of *Aconitum Napellus* have reported its highly significant anti-inflammatory potential.²⁰ Similarly, in another study conducted, the nonalkaloid part of *Aconitum flavum* significantly reduced carrageenan-induced paw edema in mice.⁷³ Although important and meaningful results were obtained in our study, the decision as to which chemical class is responsible for this effect is not entirely clear because the LC-MS/MS results of both extracts consist of various secondary metabolites, such as alkaloids, phenolic, saponins, flavonoids, and sterols.

3.4. Computing the Drug-Relevant Properties of Specific Metabolites from *A. heterophyllum* AHS Extract. A total of seven key phytochemicals (metabolites, ligands), categorized as two phenolic, two flavonoids, one

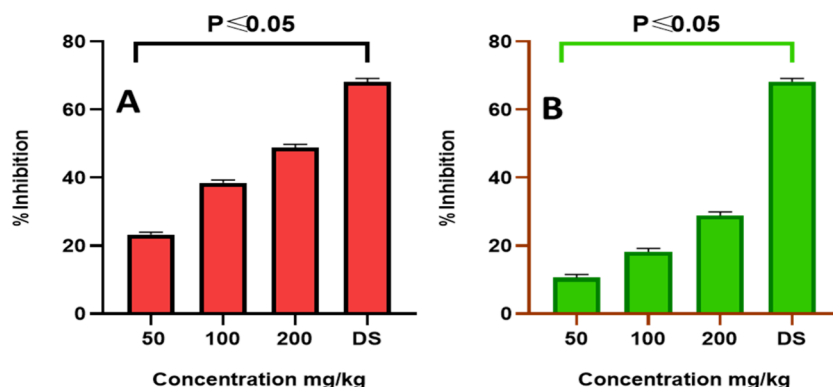


Figure 6. Antinociceptive activity of AHS (A) methanol and (B) *n*-hexane extracts (50–200 mg/kg). The percentage inhibition of writhing responses was calculated in comparison with the control with the vehicle (5% DMSO, 1% Tween80). The positive control (DS), administered at a dose of 10 mg/kg, resulted in the significant reduction of the number of writhes to 68.15%. Values with distinct letters are significantly different from each other ($p \leq 0.05$).

Table 9. Anti-inflammatory Activity of AHS Extract^a

Treatment	Dose(mg/kg)	%Inhibition after Various Time Intervals				
		1 h	2 h	3 h	4 h	5 h
DS	10	67.03 ± 1.10	79.12 ± 0.96	90.65 ± 0.94	95.60 ± 1.05	98.19 ± 1.02
Methanol extract	50	61.21** ± 0.67	66.27** ± 0.45	68.48** ± 0.95	70.30** ± 0.73	73.33** ± 0.76
	100	64.25* ± 0.56	69.04* ± 0.61	71.60* ± 1.23	75.30* ± 0.79	77.77* ± 0.73
	200	69.41 ± 0.76	73.52 ± 0.42	76.47 ± 1.05	80.58 ± 0.96	82.94 ± 0.64
<i>n</i> -Hexane extract	50	43.47**** ± 0.54	44.72**** ± 0.31	45.34**** ± 1.21	47.82**** ± 1.01	51.03**** ± 0.93
	100	44.65**** ± 0.86	45.28**** ± 0.79	47.79**** ± 1.14	49.68**** ± 1.05	55.55**** ± 0.86
	200	49.07**** ± 1.12	52.14**** ± 0.92	57.05**** ± 1.01	62.57**** ± 1.07	63.97**** ± 0.84

^aOne-way ANOVA was performed followed by posthoc (Dunnett's) test. Each value was expressed as mean ± SEM ($n = 3$). Differences from the control group Diclofenac sodium were determined by ANOVA followed by Dunnett's test. Significance was assumed as $P \leq 0.05$ (****), $P \leq 0.04$ (**), $P \leq 0.03$ (*) vs Diclofenac sodium (DS).

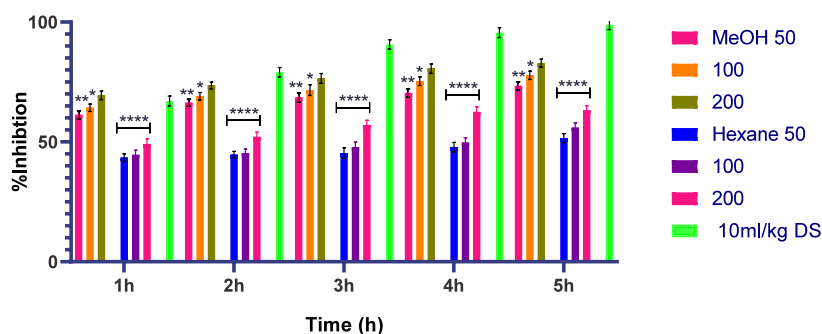


Figure 7. Anti-inflammatory activity of AHS methanol and *n*-hexane extracts (50–200 mg/kg). One-way ANOVA was conducted followed by posthoc analysis using Dunnett's test. Each value was expressed as mean ± SEM ($n = 3$). Differences from the control group, Diclofenac sodium were determined by ANOVA followed by Dunnett's test. Significance was assumed as $p \leq 0.05$ (****), $p \leq 0.04$ (**), $p \leq 0.03$ (*) versus Diclofenac sodium.

Table 10. Molecular Docking Detail of Identified Compounds (L1–L7) against Receptor COX-2 (5JVZ)^a

Ligands	Biding energy(kcal/mol)	Number of Interaction	Nature of Interaction	Interaction distance (Å ⁰)	Interacting Amino acids residues
L1	-6.1209	01	H-donor	3.87	PHEA372
L2	-7.3424	01	H-acceptor	3.12	ARG276
L3	-7.0427	02	2H-acceptor	3.55, 3.41	PHE368, PHE368
L4	-6.2950	02	2H-acceptor	3.30, 2.90	2ARG 377
L5	-6.0845	01	H-donor	2.97	GLU237
L6	-5.6613	02	H-donor, H-acceptor	3.41, 3.21	GLN 375, TRP140
L7	-8.3562	03	2 pi-H, H-donor	4.24, 3.35, 3.75	LEU146, PHE143,ASN376

^aL1= Chrysin, L2= Cohumulone, L3= Colneleate, L4= Formononetin, L5= Medicarpin, L6= Traumatina and L7= Guineesine.

Table 11. Molecular Docking Detail of Identified Compounds (L1–L7) against Receptor ADAM10 (6BDZ)^a

Ligands	Biding energy(kcal/mol)	Number of Interactions	Nature of Interaction	Interaction distance (Å ⁰)	Interacting Amino acids residues
L1	-8.0650	02	H-donor, H-acceptor	2.96,2.97	ASN308,TYR312
L2	-6.6200	01	H-acceptor	3.12	ARG276
L3	-5.5757	03	H-acceptor, 2 ionic	3.07, 3.07, 3.75	3ARG239
L4	-7.7276	03	H-donor, 2pi-H	3.35, 4.00, 3.75	CYS473, 2ARG239
L5	-5.0845	01	H-donor	2.97	GLU237
L6	-7.0454	02	H-donor, H-acceptor	3.41,3.21	GLN375, TRP140
L7	-7.2080	01	pi-cation	3.66	ARG276

^aL1= Chrysin, L2= Cohumulone, L3= Colneleate, L4= Formononetin, L5= Medicarpin, L6= Traumatina and L7= Guineesine.

alkaloid, and two other organic classes were selected from the metabolites identified in AHS by LC-MS/MS analysis. To assess their potential as drugs, using molecular docking *in silico* study. This investigation aimed to evaluate their ability to bind to receptors or other target enzymes that may mediate therapeutic activities. The preferred metabolites were analyzed for their interaction characteristics, binding energy, proximity of binding, and primary interacting amino acids related to their

binding to two enzymes: cyclooxygenase-2 (COX-2, PDB ID: 5JVZ), responsible for mediating anti-inflammatory activity, and the extracellular domain of ADAM10 (A Disintegrating and Metalloproteinase 10) (ADAM10, PDB ID: 6BDZ), which is associated with the proteolytic processing of extracellular protein domains. The selected metabolites comprise Chrysin (L1, compound CID: 5281607), Cohumulone (L2, compound CID: 196915), Colneleate (L3, compound CID: 25245832),

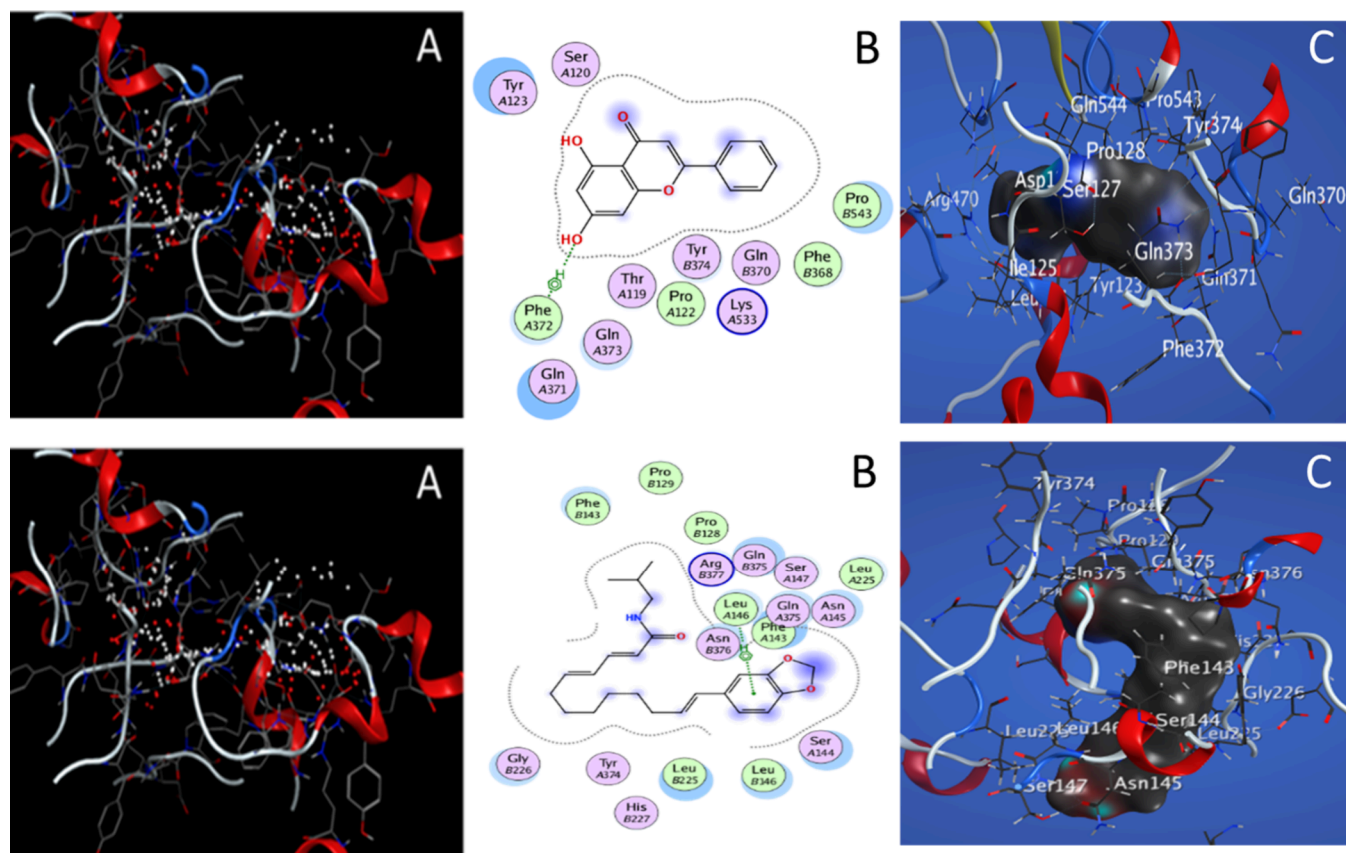


Figure 8. Molecular docked-pose of best docked phytocompounds (ligands) of *A. heterophyllum* stem against protein (COX-2, PDB-ID: 5JVZ): (A) 3D structures of 5JVZ enzyme (B) 2D interaction of L1 = Chrysin, compound CID: 5281607 and L7 = Guineesine, compound CID: 6442405 with interacting amino acids of 5JVZ; (C) 3D interactions of L1 and L7 with enzymes 5JVZ. **Note:** L = Ligands.

Formononetin (L4, compound CID: 5280378), Medicarpin (L5, compound CID: 336327), Traumatin (L6, compound CID: 5312889), and Guineesine (L7, compound CID: 6442405).

3.4.1. Molecular Docking Studies. The docking results depicted in Table 10 and Table 11 revealed that phytocompounds (L1–L7) exhibited strong and reasonable interactions with both receptors (enzymes). Among all compounds, the ligand L7 showed strong physical interaction of hydrogen-bonds 2 Pi-H and H-donors with receptor COX-2 (5JVZ) enzyme involving amino acid residues LEU146, PHE143, and ASN376 resulting in promising binding energy of -8.3562 kcalmol $^{-1}$ Figure 8. The compounds L1, L2, L3, L4, L5, and L6 displayed physical interactions (1, 1, 2, 2, 1, 2) with hydrogen-bonds (H-donor, H-acceptor, 2 H-acceptor, 2 H-acceptor, H-donor, H-donor, H-acceptor, 2 Pi-H, H-donor) by amino acid residues PHEA372, ARG276, 2PHE368, 2ARG377, GLU237, GLN375, TRP140, LEU146, PHE143, and ASN376 against receptor COX-2 (5JVZ) enzyme resulting in binding energy kcalmol $^{-1}$ (-6.1209 , -7.3424 , -7.0427 , -6.2950 , -6.0845 , and -5.6613), respectively Table 10. However, previously these ligands have shown potential for the inhibition of COX-2 (5JVZ) enzyme.^{37,71}

Likewise, compound L1 established hydrogen-bonds, H-donor and H-acceptor with receptor ADAM10 (6BDZ) resulting in the highest binding energy of -8.0650 kcalmol $^{-1}$ Figure 9. One H-bond was established among the hydrogen atom of a hydroxyl-group of L1 and carbonyl oxygen of amino acid ASN308; the second H-acceptor was generated between

the oxygen atom of the hydroxyl-group of L1 and the hydrogen atom of the hydroxyl group of amino acid TYR312 residue Table 11. The compound L4 showed a strong physical interaction of Hydrogen-bonds (H-donor, 2pi-H) with the receptor ADAM10 (6BDZ) resulting in a binding energy of -7.7276 kcalmol $^{-1}$ Figure 9. One H-bond was generated between hydrogen atom of hydroxyl group of L4 of residues CYS473; the 2 Hydrogen-bonds was between oxygen atom of hydroxyl group of L4 with amino acid residues 2ARG239. The strong physical interactions and binding affinity create L1 and L4 the most potential candidates for the inhibition of ADAM10 (6BDZ) enzyme. The compound L6 indicated their inhibitory effects of binding energies -7.0454 kcalmol $^{-1}$ and physical interaction (2) with amino acid residues GLN375 and TRP140 against ADAM10 (6BDZ) enzyme, respectively. The compound L7 showed a physical interaction of hydrogen bond (pi-cation) with interacting amino acid residue ARG276 against receptors 6BDZ resulting in binding energy of -7.2080 kcalmol $^{-1}$. Similarly, compounds L2, L3 and L5 displayed reasonable effect of binding energies with physical interactions 1,3, and 1 against ADAM10 (6BDZ) enzyme, Table 11.

The docking results discriminated that phytocompound L7 has the strongest potential for the inhibition of COX-2 (5JVZ) inflammation and L1 and L4 for ADAM10 (6BDZ) anticancer supporting enzymes. However, the other compounds also indicated reasonable inhibitory effects.^{63,74–76} Keeping in view their strong binding affinities and physical interactions, the tentatively identified phytocompounds (ligands) of AHS

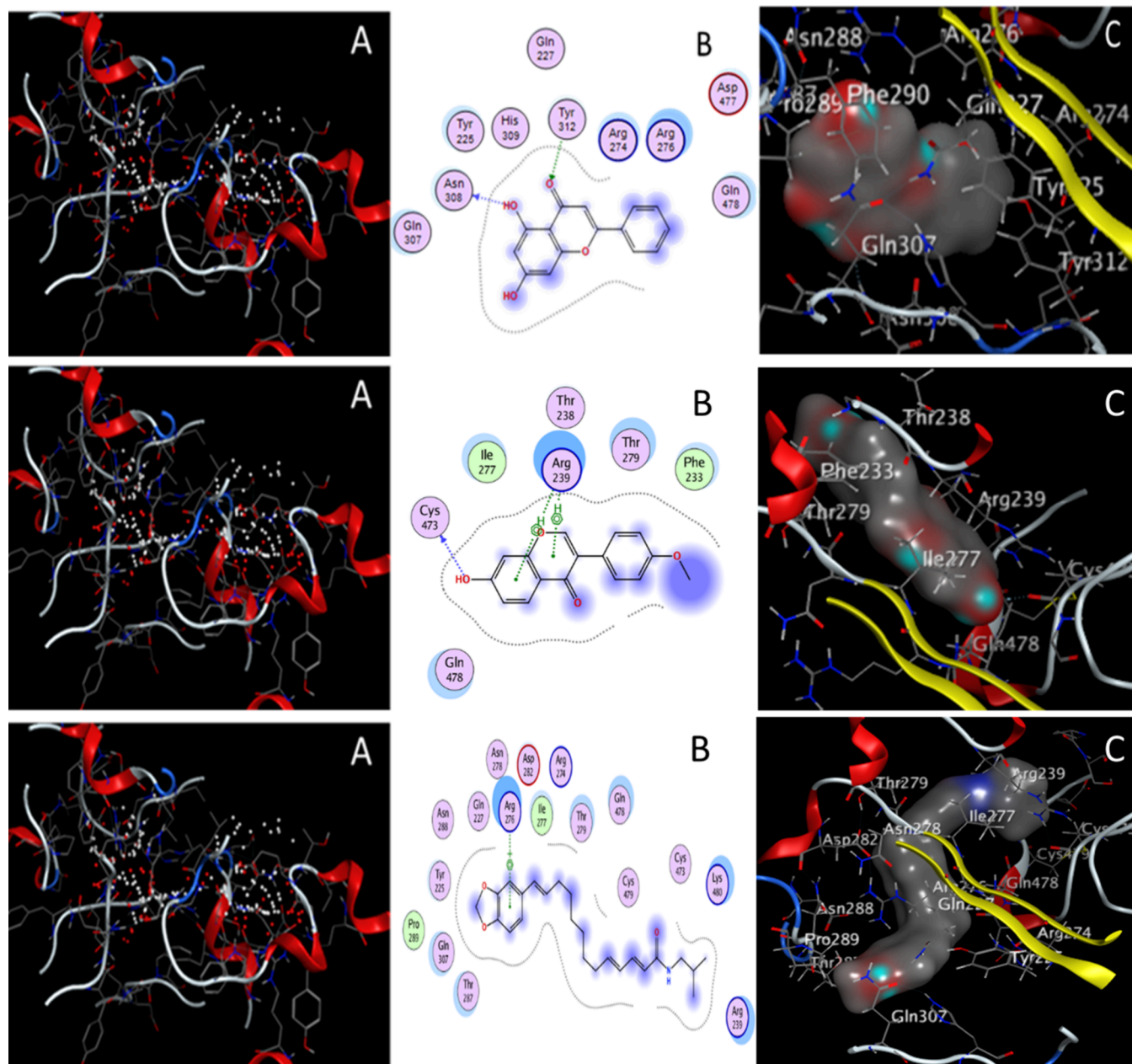


Figure 9. Molecular docked-pose of best docked phytochemicals (ligands) of *A. heterophyllum* stem against protein (ADAM10, PDB-ID: 6BDZ): (A) 3D structures of 6BDZ enzyme (B) 2D interactions of L1 = Chrysin, compound CID: 5281607, L4 = Formononetin, compound CID: 5280378 and L7 = Guineesine, compound CID: 6442405) with interacting amino acids of 6BDZ; (C) 3D interaction of L1, L4, and L7 with enzymes 6BDZ. **Note:** L = Ligands.

Table 12. Toxicity Study for the Prediction of the Compounds or/and Ligands (L1–L7)^a

Ligands	Hepatotoxicity	Carcinogenicity	Cytotoxicity	Mutagenicity	Immunotoxicity
L1	Inactive (pro 0.68)	Inactive (pro 0.62)	Inactive (pro 0.87)	Inactive (pro 0.57)	Inactive (pro 0.99)
L2	Inactive (pro 0.78)	Inactive (pro 0.61)	Inactive (pro 0.76)	Inactive (pro 0.67)	Inactive (pro 0.89)
L3	Inactive (Pro 0.63)	Inactive (pro 0.55)	Inactive (pro 0.70)	Inactive (pro 0.97)	Inactive (pro 0.74)
L4	Inactive (pro 0.73)	Active (pro 0.50)	Inactive (pro 0.88)	Inactive (pro 0.74)	Active (pro 0.54)
L5	Inactive (pro 0.80)	Active (pro 0.67)	Inactive (pro 0.89)	Active (pro 0.62)	Active (pro 0.78)
L6	Inactive (pro 0.68)	Inactive (pro 0.70)	Inactive (pro 0.76)	Inactive (pro 0.86)	Inactive (pro 0.99)
L7	Inactive (pro 0.86)	Inactive (pro 0.50)	Inactive (pro 0.71)	Inactive (pro 0.72)	Active (pro 0.99)

^aL1 = Chrysin, L2 = Cohumulone, L3 = Colneleate, L4 = Formononetin, L5 = Medicarpin, L6 = Traumatin and L7 = Guineesine. **Note.** pro = probability and L = Ligands.

strongly supported the anti-inflammatory and anticancer effects.

3.5. *In Silico* Toxicity Assessment. The compounds L1 to L7 were further utilized for predicting compound *in silico* toxicity via the ProTox-II web server (<https://tox-new.charite.de/protolxII/>).⁷⁷ Various toxicity parameters, including hepatotoxicity, immunogenicity, mutagenicity, cytotoxicity, and carcinogenicity, were assessed, as depicted in Table 12. The predictions for each compound were as follows: L1: predicted LD50:3919 mg/kg, predicted toxicity class: 5, average similarity: 82.19%, and prediction accuracy: 70.97%. L2: predicted LD50:100 mg/kg; predicted toxicity class: 3, average similarity: 66.77%; prediction accuracy: 68.07%. L3: predicted LD50:3000 mg/kg, predicted toxicity class: 5, average similarity: 59.01%, and prediction accuracy: 67.38%. L4: predicted LD50:2500 mg/kg, predicted toxicity class: 5, average similarity: 86.47%, and prediction accuracy: 70.97%. L5: predicted LD50:500 mg/kg, predicted toxicity class: 4, average similarity: 72.28%, and prediction accuracy: 69.26%. L6: predicted LD50:2610 mg/kg, predicted toxicity class: 4, average similarity: 84.11%, and prediction accuracy: 70.97%. L7: predicted LD50:760 mg/kg, predicted toxicity class: 4, average similarity: 76.71%, and prediction accuracy: 69.26%.

4. CONCLUSION

In this paper, the LC-MS/MS-orbitrap technique was used for the first time to explore the metabolite profiling of AHS extracts. A novel and previously known 44 metabolites in methanol and 74 in the *n*-hexane extracts were successfully isolated and identified. The phytochemical analysis of both extracts detected and confirmed the presence of 24 phenolics, 18 alkaloids, 10 flavonoids, 24 terpenoids, 2 coumarins, 2 lignans, and 38 other fatty acids and organic class compounds. The methanol and *n*-hexane extracts of this species showed potential biological and pharmacological activities; however, comparatively, the methanol extract showed strong potency in anticancer and anti-inflammation studies. The results of the molecular docking analysis for compounds L1, L4, and L7 identified them as active agents with promising binding energy, providing robust evidence for their potential anti-inflammatory and anticancer effects. The *in vivo* study was conducted on the randomized doses which needs to be optimized on different parameters before conducting such study in future. These results suggested that AHS has the potential to be used as a promising therapeutic for the drug discovery program. Nevertheless, further detailed investigation is needed to evaluate the therapeutic potential and the mechanism of action of these metabolites. The targeted biological assays (e.g., cell-based assays, enzyme inhibition studies, animal model for other experiments) are needed to comprehend specific mechanisms of action of the identified compounds in AHS. The findings of this study will help to understand the medicinal prominence and importance of new source compounds in this species, which will help to promote the significance and rational development of medicinal plants.

Data Processing and Statistical Analysis. Mass spectrometry data were collected in both electrospray ionization (ESI⁺ and ESI⁻ ions) modes using UPLC-MS/MS, and GC-MS in electron ionization (EI) mode. The Molecular Operating Environment (MOE) software were used for *in silico* molecular docking study. The toxicity prediction of the compounds was conducted by using ProTox-II version 3.0. Three replicates of samples were taken for each measurement,

and the reported values include the mean \pm SEM ($n = 3$) standard deviation. The results were analyzed using Dunnett's posthoc test to determine the level of significance, p values less than 0.05 considered as statistical significant. The statistical analysis was performed employed one-way ANOVA, utilizing the GraphPad Prism software 8.0 statistical package (GraphPad software Inc., version 8, Chicago, IL, USA).

■ ASSOCIATED CONTENT

Data Availability Statement

The data presented in this study are contained within the article and Supporting Information.

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsomega.3c09668>.

Additional Supporting Information, including structures of docked ligands (compounds) of AHS, along with other molecular docking figures (both 3D and 2D), as well as additional details, available in the Supporting Information (PDF)

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Notes

The authors declare no competing financial interest.

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