

Phytochemical, LC-MS Studies, and Assessment of Antioxidant, Anti-inflammatory, α -amylase inhibitory activity, and amelioration against rheumatoid-arthritis with hydroethanolic flower-extract of *Acmella paniculata* (Wall. ex DC.) : *in-silico* and *in-vitro* appraisal

Abu Md Ashif Ikbal,^[a] Avik Sarkar,^[b] Abhishek Saha,^[b] Prashant Bhardwaj,^[c] Rajib Panchadhayee,^[d] Suveena S,^[e] Ravi Adinarayan Somabattini,^[f] Nirupam Das,^[a] Satheesh Kumar Nanjappan,^[f] Banappa S Unger,^[g] Abhijit Hazra,^[f] Parasar Ghosh,^{*,[b]} Suboj Babykutty,^[h] Lutfun Nahar,^{*,[i]} Satyajit D. Sarker,^[j] Partha Palit,^{*,[a]} Debprasad Chattopadhyay^{*,[g, k]}

^[a]Department of Pharmaceutical Sciences, Drug Discovery Research Laboratory, Assam University, Silchar-788011, India

^[b]Department of Clinical Immunology and Rheumatology, Institute of Post-Graduate Medical Education & Research, Kolkata-700020, India

^[c]Department of Computer Science and Engineering, National Institute of Technology, Agartala- 799046, India

^[d]Department of Chemistry, Assam University, Silchar-788011, India.

^[e]Centre for Venom Informatics, Dept. of Computational Biology & Bioinformatics, University of Kerala, Thiruvananthapuram

^[f]Department of Natural Products, National Institute of Pharmaceutical Education and Research (NIPER), Kolkata-700054, India

^[g]ICMR-National Institute of Traditional Medicine, Nehru Nagar, Belagavi 590010, Karnataka, India.

^[h]Centre for Tumor Immunology and Microenvironment, Department of Zoology, Mar Ivanios College, Nalanchira, Thiruvananthapuram, Kerala, India

^[i]Laboratory of Growth Regulators, Palacký University and Institute of Experimental Botany,
The Czech Academy of Sciences, Šlechtitelů 27, 78371 Olomouc, Czech Republic

^[j]Centre for Natural Products Discovery, School of Pharmacy and Biomolecular Sciences,
Liverpool John Moore's University, Byrom Street, Liverpool L3 3AF, UK

^[k] School of Life Sciences, Swami Vivekananda University, Barrackpore, Kolkata, India

Short running text: Phytochemical and bioactivity studies on *Acmella paniculata* flower: .

Address for Correspondence*

Dr. Partha Palit

¹Department of Pharmaceutical Sciences, Drug Discovery Research Laboratory, Assam
University, Silchar-788011, India

Email: itspartha_p@yahoo.com

Prof. Dr. Debprasad Chattopadhyay

School of Life Sciences, Swami Vivekananda University, Barrackpore, Kolkata, India

Email: debprasadc@yahoo.co.in

Prof. Parasar Ghosh

Department of Clinical Immunology and Rheumatology, Institute of Post-Graduate Medical
Education & Research, Kolkata-700020, India

Email: drparasar@gmail.com

Prof. Lutfun Nahar

Laboratory of Growth Regulators, Palacký University and Institute of Experimental Botany,
The Czech Academy of Sciences, Šlechtitelů 27, 78371 Olomouc, Czech Republic

Email: nahar@ueb.cas.cz

Funding information

Indian Council of Medical Research - Ref. 59/03//BMS/2020

Abstract

Acmella paniculata (AP), an ethnomedicinal plant, is traditionally used for toothache, pain, inflammation, and arthritis, as edible tribal vegetables and as an antimicrobial agent. This study aimed to evaluate the protective role of AP against inflammatory pain, rheumatoid arthritis (RA), and hyperglycemia. Hydro-ethanolic flower extract of AP (HEFeAP) was prepared via maceration, followed by ultrasonication. Physicochemical characterization was performed using Thin Layer Chromatography (TLC), Liquid Chromatography-Mass Spectrometry (LC-MS), Ultra-violet Spectroscopy (UV), and Fourier Transform Infrared Spectroscopy (FTIR). Major phytochemicals were assessed against inflammatory mediators using molecular docking and Molecular Dynamics (MD) simulations. HEFeAP's antioxidant activity was assessed using 2,2-diphenyl-1-picrylhydrazyl (DPPH) (31.22 ± 0.53 $\mu\text{g/ml}$) and hydrogen peroxide (H_2O_2) scavenging (40.36 ± 0.47 $\mu\text{g/ml}$). The anti-inflammatory activity, protective role against RA, and anti-hyperglycaemic activities were evaluated via egg-albumin denaturation, MTT assay against human-fibroblast cells and α -amylase inhibition, showing IC_{50} values of 27.61, 65.75, and 85.43 $\mu\text{g/ml}$, respectively. Tumor Necrosis Factor-alpha (TNF- α), and nitric-oxide (NO) levels were significantly downregulated in LPS-stimulated PBMC-derived macrophages ($\text{IC}_{50} = 152$ and 164.56 $\mu\text{g/ml}$). Phytochemical analysis revealed coumarins, flavonoids, polyphenols, steroids, and local-anesthetic alkylamides. Active phytomolecules in HEFeAP exhibited strong and stable binding with inflammatory biomarkers. These findings suggest HEFeAP can be used as a potential candidate for treating inflammatory pain, Rheumatoid Arthritis (RA), and diabetes.

Keywords

Acmella paniculata, LC-MS, Anti-inflammatory, DPPH, Rheumatoid arthritis, α -amylase inhibition.

Introduction

Medicinal plants of the Indian subcontinent are traditionally used to manage diverse ailments^[1] including chronic conditions.^[2] The ethnic cultures of India have a long history of medicinal plant use, which plays a significant role in India's healthcare system. Ayurveda and other traditional healing systems fulfil around 70% of the medical demands of India's rural population.^[3] The medicinal plants of North-East India serve as a valuable and versatile source of raw materials for the country's herbal industry.^[4-6] A survey was conducted in tribal areas of North-Eastern India to explore potential ethnomedicinal plants for inflammation and pain-reduction properties. It was found that tribal communities mostly use *Acmella paniculata* (Wall. ex DC.) R. K. Jansen (Asteraceae), an important ethnomedicinal herb of the tropical and subtropical world including North-East India, locally known as Bonnarji, or as toothache plant or Akarkara, for the treatment of toothache.^[7,8] *Acmella paniculata* flowers, leaves, stems, and roots, used in different remedies,^[9] contain alkaloids, flavonoids, saponins, and tannins as major phytoconstituents.^[10] In the Indian system of medicine (ISM), *A. paniculata* is frequently used to reduce acute dental pain and to treat inflammation-associated ailments.^[9] We found that the local tribal communities chew floral leaf paste and put it on painful dental cavities, morning and night, to relieve dental pain, and mouth ulcers, and to ease the stammering of children. Our survey among the local tribal communities also revealed that the ethnic people use the fresh flowers and leaves of *A. paniculata* as an edible vegetable as reported earlier.^[11] Moreover, *A. paniculata* is used in snake bites,^[12] and mouth ulcers,^[13] as well as an antiarthritic,^[14] anti-inflammatory, and antioxidant ^[15,16] plant. *A. paniculata* contains a significant amount of "spilanthol", a *N*-isobutylamide, as a potential active constituent, having local anesthetic^[7] and insecticidal^[9] properties. Spilanthol [IUPAC name (2*E*,

1 6Z, 8E)-N-isobutylamide-2,6,8-decatrienamide] is bitter, may cause salivation^[17] and has several
2 bioactivities with significant industrial applications. Contemporary literature showed that despite
3 several publications on the related species, the chemical and biological activities of *A. paniculata*
4 have not been extensively studied to date. Based on the current literature and our survey among
5 North East tribes, we hypothesized that the local anesthetic property of *A. paniculata* flower head
6 (capitulum) would help manage RA-induced pain via skin-mediated delivery.^[18] A recent report
7 has revealed that in a pre-clinical model, *Acmella oleracea*, combined with *Boswellia serrata*, is
8 effective against neuropathic pain.^[19] So Antioxidant, anti-inflammatory, and antidiabetic
9 assessment are essential for preventing and treating oxidative stress, inflammation, and metabolic
10 disorders. Oxidative stress contributes to the development of inflammatory illness followed by
11 auto-immune disorders including RA as well as cardiovascular difficulties.^[20] Plant-based
12 antioxidants, such as flavonoids and polyphenols, can scavenge free radicals, reduce oxidative
13 damage, and lessen disease risk. Chronic inflammation plays a vital role in illnesses such as
14 rheumatoid arthritis, inflammatory bowel disease, and psoriasis, where excess pro-inflammatory
15 cytokines like TNF- α and IL-6 promote disease progression.^[21] Bioactive phytochemicals, such as
16 alkylamides and polyphenols, have been shown to modulate immune responses by inhibiting
17 inflammatory mediators. Antioxidants can improve glucose metabolism and prevent pancreatic β -
18 cell destruction, which leads to insulin resistance and diabetes.^[22] Phytochemicals with α -amylase
19 and α -glucosidase inhibitory properties, such as flavonoids and saponins, might delay
20 carbohydrate digestion, regulate glycemic levels, and reduce the global diabetes burden.^[23] Given
21 these factors, investigating *Acmella paniculata's* antioxidant, anti-inflammatory, and
22 antihyperglycemic properties might provide new therapeutic insights into managing these chronic
23 conditions.

Rheumatoid arthritis (RA), a chronic inflammatory autoimmune disorder, is characterized by chronic pain caused by impaired innate immunity when the immune system attacks the healthy synovial lining of joints. This immune impairment activates complement for adaptive responses against 'self'-antigens, with dysregulated cytokines, chondrocytes, and osteoclasts. The RA progresses from early, moderate, severe, to end-stage, while symptoms appear in moderate/stage-2. The autoimmune attack initiates a series of inflammatory responses, resulting in classic signs of pain, stiffness, and edema leading to swollen and malfunctioning joints with a substantial impact on the quality of life, daily activities and emotional misery. Thus, effective RA management requires a focus on reducing inflammation to relieve discomfort and avoid joint degeneration to improve quality of life.^[24–26] The treatment of RA includes nonsteroidal anti-inflammatory drugs (NSAIDs), with disease-modifying anti-rheumatic drugs (DMARDs) cyclophosphamide, hydroxyquinoline (cardiotoxic), leflunomide, methotrexate, sulfasalazine and intramuscular gold nanoparticles.^[27] However, prolonged use of these expensive drugs may cause hepato-renal dysfunction^[28,29], hypertension^[30–32], and stomach ulcers^[33–35] that harm normal systems, with poor recovery and recurrence^[30] of the symptoms. Traditionally used herbal medicines have gained popularity as an alternative treatment for chronic inflammatory disorders including diabetes, RA, etc. not only to mitigate the side effects but also to offer affordability of long-term treatments with conventional drugs.^[36,37] Polyherbal formulations are recommended for intended pharmacological action in difficult-to-treat and chronic diseases, by reducing side effects and eliciting synergistic benefits of diverse phytochemicals.^[16] Earlier reports revealed that several Ayurvedic medicines as polyherbal formulations might help manage acute and chronic inflammatory diseases, via the lab-to-fab route.^[38]

Thus, the current study aimed to explore the potential and validate the ethnic use scientifically of *A. paniculata* flower head of North-Eastern India, using *in-vitro* anti-oxidant, anti-inflammatory,

1 α -amylase inhibition, and *ex-vivo* synovial fluid-derived fibroblast cells (fd-FLS) growth
2 inhibition in rheumatoid arthritis disease model.

3 To characterize and standardize the HEFeAP, TLC, UV, FTIR, and LC-MS studies were
4 undertaken, and the major phytochemicals were detected by LC-MS chromatogram. The bioactive
5 constituents were subjected to *in-silico* docking and simulation studies against potential
6 inflammatory biomarkers, followed by *in-vitro* validation of *A. paniculata* flower-derived
7 bioactive fraction against inflammatory pain and RA. Based on anti-inflammatory activity, the
8 extract was subjected to an MTT assay on fd-FLS cells derived from RA patients to authenticate
9 the *ex-vivo* anti-RA activity. Moreover, to understand the possible mode of action, the pro-
10 inflammatory cytokines TNF- α and nitric oxide of untreated and fraction-treated cells were studied
11 to substantiate their effect on auto-immune disorder. On the other hand, the literature reveals that
12 auto-inflammatory complications make RA patients who are susceptible to developing Diabetes
13 melitus type 2 in several cases^[39,40] Thus, our study was further extended to investigate the
14 protective role of HEFeAP against diabetes type 2 using *in-vitro* α -amylase inhibition model.
15 Hence, the most likely correlation between RA and T2 diabetes in the line of inflammation will be
16 explored in the discussion part following the study result of the undertaken experiments.

17 18 **Material and Methods**

19 **Collection of Plant Material**

20 Flower heads of *Acmella paniculata* (Wall. ex DC.) R. K. Jansen **Figure 1** were collected in August
21 2021 from the adjoining tribal area of Mahadebpur [24°48'N and 92°45'E, Barak valley, Assam
22 (Herbarium Ref. No: 4415, identified by Prof. B.K. Dutta, Department of Botany, Tripura Central
23 University, Agartala). The plant samples were thoroughly washed in running tap water, followed
24 by sterile distilled water, and then shed-dried in an airy room. The dried samples were processed

for extraction to evaluate their bioactivities if any, using *in-vitro*, and *ex-vivo* models with possible modes of action.

Processing of Plant Material for Physicochemical Characterization

The dried samples were ground into powder, sieved (number 10), and kept in an air-tight container (Figure 1) to store at room temperature. Hydroalcoholic (70% ethanol in water) extract was prepared by maceration for 3 days with constant shaking at 20°C^[41] followed by ultrasonication in a probe sonicator (3110-Rivotek) for 30 min at 20°C. The extract was concentrated using a rotary vacuum evaporator followed by freeze-drying in a lyophilizer. Phytochemical screening was conducted to detect the phytochemical groups present in the hydroethanolic extract of *A. paniculata* flower heads (HEFeAP), using a standard method.^[42] The percentage of extractive yield was calculated by the formula (1):^[43]

$$\text{Percentage (\%) yield} = \frac{\text{Weight of the obtained lyophilized extract}}{\text{Weight of the dried crude flower material taken}} \times 100 \dots\dots (1)$$



Figure 1. Processing the sample of *Acmella paniculata* (A & B: Flower of *Acmella paniculata* – The fresh flowers of *Acmella paniculata* are shown, C: Dried Flowers – The flowers are dried to remove moisture, D: Grinding Process – Dried flowers being ground into a fine powder, E: Grinded Powder – The powdered form of the dried flowers is displayed, F: Lyophilized Extract – The processed lyophilized extract of *Acmella paniculata* flowers).

Phytochemical profiling and quantitative estimation of the chemical group(s) from a hydroethanolic fraction of *Acmella paniculata* flower head (HEFeAP).

Phytochemical Screening of HEFeAP

Using a colorimetric method^[44] the extract was phytochemically profiled for the major chemical classes/groups (alkaloids, anthocyanidin, coumarin, flavonoids, glycosides, phenols, steroids, tannins, terpenoids), present in the extract, by comparing the color intensity of equivalent standards. The concentration of the secondary metabolites present in the test extract was evaluated using standard phytomolecules of the corresponding phytochemical group(s) and comparing the color intensity of the standard and sample.

Determination of Total Alkaloid Content

Quantitative titration was used to determine the presence of alkaloids.^[45] Briefly, 50 mg of HEFeAP was dissolved in 10 ml of *n*-butanol, followed by adding 10 ml 0.1N HCl in a separating funnel. The solution was vigorously shaken for 3-4 min and left for layers to separate. A few drops of methyl red indicator were added to the denser layer and allowed to separate from the bottom of the separating funnel. The endpoint was reached when the solution changed from light pink to pale yellow after titrated with 0.1N NaOH. A blank containing 10 ml of 0.1N HCl and a few drops of methyl red is used as an indicator. The indicator's color was titrated with 0.1N NaOH, until it changed from light pink to pale yellow, at the endpoint, repeating the same step three times for each solvent.^[45] The following equivalency was considered while calculating the total amount of alkaloids: 1.0 ml 0.1N HCl $\equiv$ 0.01629 g of alkaloid.

Estimation of Total Phenolic Compounds. The colorimetric Folin-Ciocalteu (FC) method has been utilized to quantify the total phenolics of HEFeAP^[46,47] with minor modifications, using tannic acid (1.0 mg/ml in 70% ethanol) as a standard stock solution. Briefly, the stock solution was diluted 10 times to get five different concentrations (0.1, 0.2, 0.3, 0.4, and 0.5 mg/ml) in separate test tubes. The FC reagent was added to each tube after each standard solution was diluted with double-distilled water in a volume of 3 ml. The reaction mixture was left at room temperature for 3 min and then added with 2 ml of 35% (w/v) sodium carbonate. The tubes were left at room temperature for 45 min in the dark. A calibrated UV-Vis spectrophotometer was used to measure the absorbance of the reaction mixture at 650 nm for constructing a calibration curve. After removing 200 μ l of the sample (in duplicate), the color reverted to its original state. The calibration curve was utilized to estimate the total phenolic content in the extract by comparing the absorbance values with tannic acid, which served as the positive control.^[48]

Determination of Total Tannin Content

UV-VIS spectrophotometer was used to estimate the total tannin content^[49,50] of the HEFeAP, using tannic acid as the reference standard. For the optimization of the wavelength, 25 µg/ml of tannic acid, prepared in methanol (as solvent), was scanned between 200-1000 nm to get the $\lambda_{\max}$, followed by optimization at 457 nm. The same wavelength was used to get the absorbance of the sample and control. A calibration curve of the standard sample, plotted by absorbance vs concentrations, was prepared to determine the HEFeAP concentration, and the total yield of tannins present in the sample was calculated and expressed in mg/g.

Phytochemical Profiling and Physicochemical Characterization of HEFeAP

Phytochemical profiling and physicochemical characterization of HEFeAP were achieved using TLC, UV, FTIR, and LC-MS. Phytochemical characterization and separation of chemical groups of secondary metabolites present in HEFeAP were carried out by TLC on silica gel with a fluorescent indicator at 254 nm on aluminum sheets (layer thickness 0.2 mm; 10×20 cm) using toluene: ethyl acetate = 8:2; ethanol: *n*-hexane = 3:7 and *n*-hexane: ethyl acetate = 2:1 as eluting mobile phase in v/v.^[51] Separated bands were optimized by detecting under a UV chamber at 366 nm and results were expressed as R_f of the respective bands. Physicochemical characterization was used to standardize the test extract (HEFeAP), diluted in ethanol in quartz cuvettes of 1 cm thickness (d), using a double-beam UV-Vis Spectrophotometer (Hitachi-U-2910) by measuring the absorbance at a wavelength between 190 to 800 nm, to get $\lambda_{\max}$ of the test extract. The FT-IR spectrometric study of HEFeAP was performed using a Bruker Alpha (II) Spectrometer (Germany) with FT-IR via the KBr pellet technique. Before the examination, the materials were ground with KBr pellets, and a disc was made by combining a 2% HEFeAP sample with 100 mg of KBr to determine the functional groups of the test sample, compared to the standard compounds. Further, the phytochemical analysis of HEFeAP was conducted by an Agilent UHPLC-Q-TOF-

MS (6545B) system connected to a Dual AJS-ESI source and operated with a Mass-Hunter Workstation. The separation of HEFeAP was accomplished in the mass range of 50-3200 by an Agilent Eclipse Plus C₁₈ column (dimensions 2.1 x 100 mm, particle size 1.8 µm), and the oven temperature was maintained at 40°C. The mobile phase consisted of a mixture of 0.1% formic acid (Solvent A) and acetonitrile (Solvent B) using the gradient mobile phase as follows: (i) 0-2 min, B 35%, from 2-10 min the proportion of solvent B increases from 35-60%; from (ii) 10-15 min the proportion of solvent B maintained to 60%; and from (iii) 15-35 min the proportion of solvent B increased from 60-85%; and from (iv) 35-45 min, the proportion of solvent B was increased 85-92%; and maintained for next 5 min at 92%; and from (v) 50-57 min, the proportion of solvent B was decreased from 92-35%; and maintained for 3 min. The total run time was 60 min, with a flow rate of 0.3 ml/min, and an injection volume of 5µl. The Dual AJS-ESI source was configured to operate in positive ionization mode. The instrument settings are: the gas temperature was set at 320°C, flow rate 9 L/min, the nebulizer pressure is 35 pounds per square inch gauge (psig), and the sheath gas temperature was 350°C with a flow rate of 11 and zero collision energy. The Scan Source Parameters are configured as Vcap set to 5300 V, Nozzle Voltage set to 1000 V, Fragmentor set to 175, Skimmer1 set to 65, and Octopole RF Peak set to 750 V.

***In-vitro* Experiments**

Antioxidant Activity Using DPPH Free-radical Scavenging Assay

The 2,2-diphenyl-1-picryl-hydrazyl (DPPH) assay was used to evaluate the *in-vitro* antioxidant (free-radical-scavenging) activity of HEFeAP following a modified method. Ascorbic acid prepared in 0.1% DMSO at a concentration of 1 mg/ml serves as a positive control. This stock solution was divided into different quantities (10, 20, 40, 80, 100 µg/ml) and a same volume of DPPH in ethanol (0.1 mM) was added. After this, the mixture was incubated for 30 min in total

darkness at ambient temperature. The absorbance was assessed at 517 nm after 30 min, against an ethanol blank. The control solution contained 0.1 mM of DPPH. The percentage (%) inhibition by ascorbic acid was estimated using the following equation (2).^[52,53]

$$\% \text{ of DPPH scavenging effect} = [\text{ControlOD} - \text{SampleOD}] / \text{ControlOD} \times 100 \dots\dots(2)$$

The above procedure was strictly followed for the determination of the percentage of free-radical-scavenging potential of HEFeAP at graded concentrations (10, 20, 40, 80, 100 µg/ml).

Hydrogen Peroxide (H₂O₂) Scavenging Assay

H₂O₂ scavenging activity was determined using the procedure by Keser et al., (2012) with some modifications. H₂O₂(40 mM) and test extract solution(s) of 1000 µg/ml of HEFeAP were prepared in phosphate buffer (pH 7.4). From this, 40, 80, 160, 320, and 400 µl were put into separate test tubes and added to hydrogen peroxide (40 mM) solution to make a volume of 4 ml, to achieve the final concentrations of 10, 20, 40, 80 and 100 µg/ml, respectively in triplicate sets. The mixture was allowed to incubate for 15 min at room temperature in the dark. The absorbance was measured at 230 nm, and the percentage inhibition was determined using the equation (3).^[54]

$$\text{Hydrogen peroxide scavenging effect} = \frac{\text{ControlOD} - \text{SampleOD}}{\text{ControlOD}} \times 100 \dots\dots(3)$$

Protein denaturation Assay

Aspirin prepared in 0.1% DMSO at a concentration of 1 mg/ml serves as a positive control. and different concentrations of up to 2 ml using 0.1% DMSO were prepared from this stock solution, at the final concentrations of 0, 5, 25, 50, 100, 200 µg/ml. Each sample was treated with 1.5 ml of phosphate-buffered saline (PBS) and 0.4 ml of fresh egg albumin. After incubation for 15 min at 37 °C in a BOD incubator, each reaction mixture was heated for 5 min at 70 °C. After cooling, the absorbance was measured at 660 nm using 0.1% DMSO as vehicle control. Similarly, for HEFeAP, duplicate measurements were recorded for each concentration. Without the sample or test extract,

the untreated set served as a control.^[55] The percentage (%) inhibition of protein denaturation was determined^[56,57] by the following formula (4):

$$\text{Percentage (\% inhibition)} = 100 \times (V_t/V_c - 1) \dots\dots\dots(4);$$

where V_t is the absorbance of the test sample; and V_c is the absorbance of the control. A dose-response curve was plotted to calculate the HEFeAP concentration for 50% inhibition (IC_{50}) by plotting the percentage of inhibition compared to the control against the treatment concentration ranging from 5-200 $\mu\text{g/ml}$.^[58]

Antihyperglycemic activity using α -amylase

Preparation of 3,5-dinitro Salicylic Acid (DNSA) Reagent:

About 30 ml of 96 mM 3,5-dinitro-salicylic acid in 2M NaOH was prepared. From this solution, 20 ml was taken out and mixed with 5.31M sodium potassium tartrate in 8 ml 2M NaOH. The volume was adjusted to 40 ml with distilled water in an amber-colored bottle.

Preparation of α -Amylase Enzyme Solution:

Ten (10) mg of α -amylase was dissolved in 100 ml of 20 mM sodium phosphate buffer, at pH 6.9 adjusted by using a 6.7 mM NaCl solution.

Preparation of Starch Solution (Potato Starch):

About 0.5% (w/v) potato starch was dissolved in 15 ml of distilled water, followed by boiling for 15 min. A 1.0 mg/ml metformin standard solution was prepared using methanol as a standard α -amylase Enzyme inhibitor^[59,60]. Then five different concentrations in 5 test tubes were prepared from the stock solution, with resultant concentrations of 5,10,20,50, and 100 $\mu\text{g/ml}$. Finally, the content of each test tube was mixed with 0.5 ml α -amylase solution and incubated for 30 min at $25^\circ\text{C} \pm 2^\circ\text{C}$. Following this, 0.5 ml of the respective standard mixtures were mixed with 0.5 ml of potato starch and 3 mins incubation at room temperature. Subsequently, DNSA reagent (0.5ml) was added, followed by the incubation for 15 min at 85°C . After cooling, the volume was made

up to 5 ml using distilled water, and the absorbance was checked at 540 nm.^[61] A blank was prepared using DNSA reagent and distilled water, while a control was prepared without the sample/drug solution, following the same procedure. The percent inhibition of α -amylase was calculated using the formula (5):

$$\text{Percentage (\%) Inhibition of } \alpha - \text{amylase} = \frac{\text{ControlOD} - \text{SampleOD}}{\text{ControlOD}} \times 100 \dots (5)$$

MTT Assay on Fibroblast Cells Derived from Knee Joint Synovial Fluid of RA patient The synovial fluid was drawn from RA patients, and the sample was centrifuged (10 x 2000 rpm) up to 15 min. Following the Helsinki Declaration^[62] the study was conducted with the approval of the Institutional Human Ethics Committee of Post Graduate Medical Education & Research (IPGMER) and SSKM Hospital, Kolkata (IPGME&R/IEC/2023/980; dated 7/10/2023). The resultant pellet was resuspended in 15% fetal bovine serum (FBS)-containing Dulbecco's Modified Eagle Medium (DMEM, Sigma). After cultivation, fluid-derived synovial fibroblast cells (fd-FLS) were examined by flow cytometry to determine their phenotypes.^[63] The MTT assay was used to assess the viability of fd-FLS.^[64] Briefly, the cells (5×10^4) were seeded in 96-well plates overnight and then treated with HEFeAP at graded dosages of 10, 50, 100, 200, and 500 $\mu\text{g/ml}$, dissolved in cell-culture DMSO for 48 h. A microplate reader was used to record the absorbance of the treated and untreated synovial fibroblast cell pellet suspension, solubilized in 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide solution (MTT). Electron microscopy of untreated controls and HEFeAP-treated fd-FLS cells were captured to analyze the cell morphology.

Determination of Pro-inflammatory Cytokines (TNF- α and NO) from Untreated and HEFeAP-treated Supernatants of Peripheral Blood Mononuclear Cells

To evaluate the anti-inflammatory potential of HEFeAP, the peripheral blood mononuclear cells (PBMCs) were separated from the Buffy coats of venous blood as gift samples drawn from healthy volunteers who attended a blood donation program at a Registered approved Blood Bank (no special ethics permission or approval for this study is needed to use this material of human origin). The PBMCs were separated by Ficoll-Paque gradient centrifugation, following the manufacturer's (Sigma, Aldrich) protocols three 20 ml centrifuge tubes with 4 ml of Ficoll-Paque gradient Donor blood (heparinized, 25 ml) were carefully placed on the Ficoll-Paque gradient (12 ml/tube) and diluted 1:1 in PBS (0.01 M) and tubes were centrifuged at 1020 x g for 20 min. After removal of the cell interface layer, the blood sample was centrifuged (at 640x g followed by 470x g), and washed twice in PBS for 10 min. Before counting, the sample was resuspended in full-strength RPMI 1640 containing Glutamax, penicillin (50 U/ml), streptomycin (50 µg/ml), and 10 mM HEPES. The PBMCs (2×10^6) were then suspended in 1.0 ml of complete medium with colony-stimulating factor-1 (CSF-1) for five days. Macrophages matured on day 6 were used in the following experiments: The macrophages were exposed to 20 ng/ml of lipopolysaccharide (*E. coli* 055: B5, Sigma) for 24h to release the pro-inflammatory cytokines. Following LPS exposure HEFeAP was added at 50-500 µg/ml, and incubated for 48 h. After incubation, the cells were separated by centrifugation for 10 min at 1500 rpm. TNF-α was measured by the ELISA kit (BD Pharma) following the manufacturer's instruction; while NO was determined by Griess reagent.^[65]
[66][67,68][21,69]

***In-silico* Studies**

Binding Site Prediction

The Discovery Studio package anticipated the binding location based on the receptor's internal shape.^[70] Initially, a grid was used to map receptor molecules, such as Lox-5 and phospholipase-2, as potential indicators of inflammation. The atoms of the receptor that are close to the grid points

were considered and found to be unsatisfactory as locations for the binding of ligand atoms. Two methods were considered for determining a binding location: one was the rubber algorithm, used to determine locations based on the receptor's shape, and the other was the volume, a well-known ligand that had been previously positioned at a receptor active site had engaged likewise ascertaining the second method. Here, an eraser algorithm was used to eliminate any grid points not in contact with the receptor. A flood-filling method investigated connecting sections with linked and unoccupied grid points near the receptor. CDOCKER, an application of a CHARMM-based docking program that follows a precise receptor protocol, was used to perform the docking. Using a variety of random seeds and high-temperature molecular dynamics, a set of ligand conformations was created that accounted for the appropriate number of low-energy orientations. A simulation of annealing molecular dynamics was run on each orientation. Using non-softened potential, a final minimization of the ligand in the rigid receptor was carried out. Both the interaction energy alone and the CHARMM energy (interaction energy plus ligand strain) were computed for each final position. The sites with the greatest values (more negative, facilitating binding) had the lowest CHARMM energy scores. To produce more accurate data for ligand-protein complexes with greater affinities (less than 12 kcal/mol), refinement docking studies were conducted.^[71,72] The tests were carried out 100 times with the complete version of the Discovery Studio 4.1 software for each ligand-macromolecular protein target.

Molecular Docking-interaction study

To learn more about their preferences for binding at the predicted active site of LOX-5 and phospholipase A2, which is believed to be a key inflammatory biomarker protein, spilanthol and (*E*)-*N*-isobutylundeca-2-en-8,10-diamide, the two active constituents of the bioactive fraction of *A. paniculata* flower, were subjected to molecular docking studies. To create the test ligands, the 3D structures of spilanthol and (*E*)-*N*-isobutylundeca-2-en-8,10-diyamide were downloaded from

the PubChem database **Table 3**. Using the given Momanye-Rone electrostatic charge and the CHARMM force field, the structure's energy was decreased. This structure was chosen as the first conformation for the docking process. Two significant inflammatory signaling target proteins from the homo-sapiens species-phospholipase A2 and LOX-5-had their X-ray crystal structures, retrieved and saved in the Protein Data Bank (PDB; <http://www.rcsb.org/pdb>). To get the protein ready for docking, the heteroatoms (i.e., non-receptor atoms like water and ions) and conformers from the crystal structure were taken out. Invalid residues were also corrected. Momanye Rone charges and the CHARMM force field were applied to the macromolecular structure Using Discovery Studio 4.1.^[58]

Molecular Dynamic Simulation

The best top-scored receptor-ligand complexes obtained after docking were evaluated to investigate molecular dynamics simulation using GROMACS 2021 version, with CHARMM 27 force field and TIP3P water model with the aid of dodecahedron box with periodic boundary conditions. The ligand topology was created with the SWISSPARAM online server. Sodium ions were added to PDB ID: 2IUJ (lipoxygenase) and Chlorine ions were added to PDB ID: IDB4 phospholipase A2 human enzyme proteins for neutralization at 0.1M concentration. The simulation started at 300 K and 1 bar pressure and ran for 100 ns. The particle mesh Ewald method allowed us to locate the long-range electrostatic interactions.^[73] In a simulation that combines molecular mechanics with a generalized model for solvent effects, approximate results were obtained. NVT dynamics, which maintain constants for material quantity (N), volume (V), and temperature (T), have been developed. The system underwent energy reduction during MD, which was finished using the steepest descent method at a threshold of 10 kcal/mol (nsteps 50000). Particle Mesh Ewald (PME) was used to calculate electrostatic interaction, and the linear constraint solver (LINCS) to estimate the van der Waals interaction algorithm.^[74] The standard temperature was

maintained at 300K using a modified Berendsen thermostat and the Parrinello-Rahman coupling technique. Finally, each compound underwent a 100 ns-long simulation of molecular dynamics. Additionally, QT Grace was used to perform the MD trajectory analysis to look into the Radius of Gyration (Rg), Root Mean Square Deviation (RMSD), Fluctuations (RMSF), and Hydrogen Bond.

Statistical Analysis

Statistical analysis was carried out using Graph Pad Prism version 5 and IBM SPSS software. A one-way analysis of variance (ANOVA), Tukey's multiple comparison analysis, and unpaired student *t-test* were undertaken to determine the statistical differences between the means of various groups. The treated, untreated, and positive control groups were regarded as statistically significant at $P < 0.05$, where applicable.

Results and Discussion

Physical Properties of HEFeAP

Physically the HEFeAP sample was dark green solid powder and is soluble in water, ethanol, methanol, acetone, and DMSO. The solubility of the phytoconstituents of HEFeAP in the above solvents suggested the presence of polar and nonpolar phytoconstituents. **Percentage Yield**

The percentage yield of HEFeAP was calculated to be 1.5% (w/w), by the formula referred to (1).

Qualitative Screening of Secondary Metabolites of *Acmella paniculata*

Qualitative phytochemical screening of HEFeAP, presented in **Table 1**, revealed the presence of alkaloids, alkyl amides, anthocyanidin, phenols, steroids, tannins, and terpenoids.

Table 1. Qualitative testing of phytochemical groups presents in HEFeAP

Secondary metabolites	HEFeAP
Alkaloids	+++
Anthocyanidin	++

Coumarin	+
Flavonoids	+
Glycosides	+
Phenols	+++
Saponin	++
Steroids	+++
Tannins	++
Terpenoid	++

+, low concentration; ++, moderate concentration; +++, high concentration; -, absent, based on the comparative phytochemical test with standard phytochemicals, using the same concentrations.

Quantitative Estimation of Phytochemical Groups and Metabolites in HEFeAP

Quantitative estimation of phytochemical groups in HEFeAP revealed the presence of alkaloids, phenolics, and tannins at 325.8, 240.07, and 117.68 mg/g of the sample, respectively. This estimation suggested that alkaloids were present in higher concentrations than tannin and phenolics. **Table 2.** The LC-MS fingerprinting (**Figure 4** and **Supplementary files**) showed the presence of pungent alkyl-amides viz. 94.2% spilanthol, 79.21% (2*E*,7*Z*,9*E*)-*N*-isobutylundeca-2,7,9-trienamide, 88.59% (*E*)-*N*-isobutylundeca-2-en-8,10-diynamide, 94.46% 2*E*-*N*-(2-methylbutyl)-2-undecene-8,10-diynamide, and 92.24% *N*-isobutyl-(2*E*,4*Z*,8*Z*,10*E*)-dodecatetraenamide by score analysis, indicating that the hydroalcoholic extract of *A. paniculata* flower head could have an anti-inflammatory and analgesic effect. These results prompted us to evaluate the anti-inflammatory activity relevant to RA effects of the test extract via *in-silico*, *in-vitro*, and *ex-vivo* methods.

Table 2. Quantitative estimation of three major phytochemical groups of HEFeAP. Values are represented as Mean $\pm$ SD of three independent experiment (n=3)

Total Alkaloids ^[a] (Mean ± SDs)	Total Tannins ^[a] (Mean ± SDs)	Total Phenolic compounds ^[a] (Mean ± SDs)
325.8 ± 0.89	117.68 ± 0.92	240.07 ± 0.42

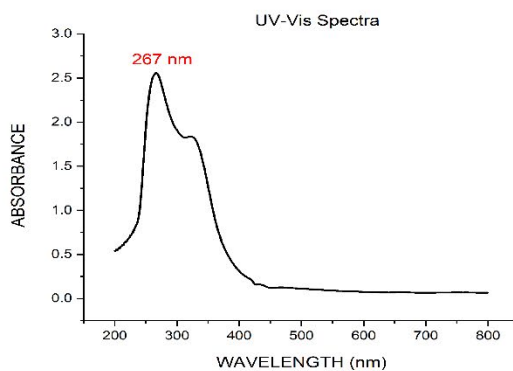
[a] mg/g of dry extract

UV-VIS Spectrophotometric analysis

The UV-absorption spectra of hydroalcoholic extract of HEFeAP via photometric scan using wavelength vs absorbance is presented in **Figure 2a**. UV-Vis spectroscopy at wavelengths between 190 and 800 nm was used to confirm the absorption spectra of HEFeAP. The extract had a distinctive peak in the absorption spectra at 267 nm. **Figure. 2b** is documented as λ_{max} .



a



b

Figure 2. UV-Vis spectra analysis of HEFeAP – [a]. UV observation of HEFeAP (by UV chamber) and [b]. UV-Vis absorption spectra of HEFeAP (λ_{max} at 267 nm).

The absorption spectrum of **267 nm** λ_{max} signifies that the compounds present in the test sample have conjugated double bonds, indicating the similarity with the standard compound, spilanthol.

TLC Characterization

TLC characterization of HEFeAP was carried out in three optimized solvent systems (toluene: ethyl acetate = 8:2; ethanol: *n*-hexane = 3:7 and *n*-hexane: ethyl acetate = 2:1), revealing 4 bands, 3 bands, and 4 bands, respectively **Figure 3**. The R_f was calculated for each band and 0.50 R_f was matched with the R_f of spilanthol, as per the literature, in the same solvent system of *n*-hexane: ethyl acetate = 2:1, as reported earlier.^[75,76] This infers the possible presence of the bioactive compound spilanthol in HEFeAP, as validated by the LC-MS profile. At 366 nm UV light, four bands with R_f 0.24, 0.33, 0.40, and 0.50 were observed. While, at the optimized solvent system of toluene: ethyl acetate (8:2) the HEFeAP showed 4 bands under UV light, with R_f 0.16, 0.50, 0.55, and 0.60. Three bands were detected on the TLC plate after spraying with iodine spray with the R_f values of 0.10, 0.44 and 0.58; the mobile phase was ethanol: *n*-hexane 3:7. Again four bands were visible under UV light with R_f 0.24, 0.33, 0.40, 0.50 at 366 nm in the optimized solvent system of *n*-hexane: ethyl acetate = 2:1 in the chromatogram.

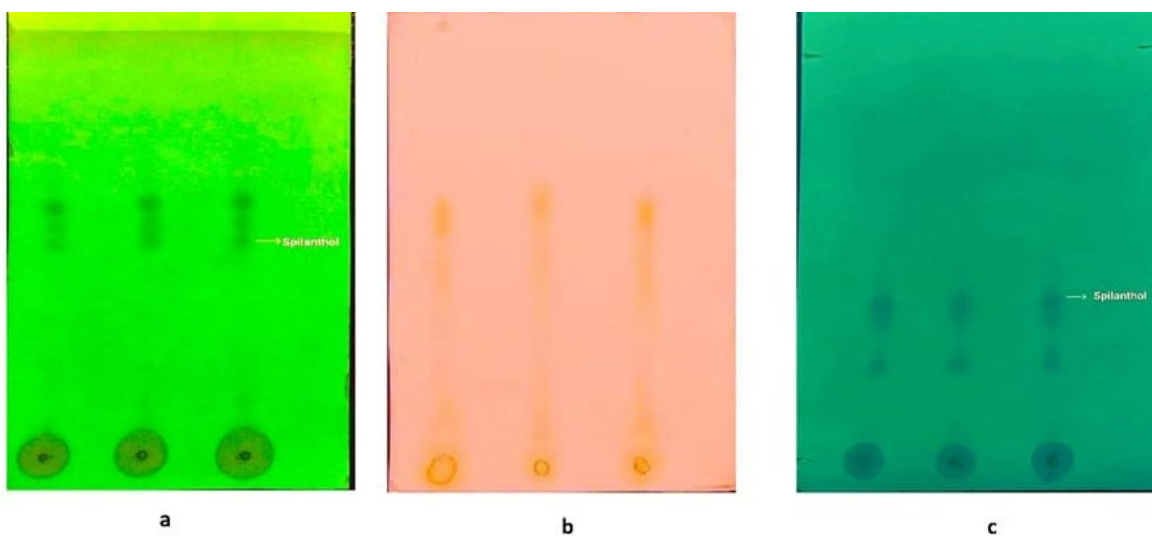


Figure 3. TLC Chromatogram of HEFeAP. [a]: TLC of HEFeAP; [b]: Iodine spray TLC of HEFeAP; and [c]: TLC of HEFeAP. TLC of HEFeAP with three optimized solvent system (Toluene: Ethyl acetate = 8:2; Ethanol: n-Hexane = 3:7 and n-Hexane: Ethyl acetate = 2:1) presented 4 bands (Figure 3a), 3 bands (Figure 3b), and 4 bands (Figure 3c), respectively].^[75,76]

FT-IR Spectroscopic analysis

The FT-IR was carried out to determine the nature and presence of functional groups in HEFeAP. In FT-IR spectra, a total of six different peaks were detected. The HEFeAP bioactive fraction A1 **Figure 4** reveals the presence of stigmasterol and β -amyrin, as found in the LC-MS profile. The major peaks at 1054 cm^{-1} could be due to C-C, C-O, and C-N single bond stretching, the peak at 1399 cm^{-1} may be due to -O-H bending, the peak at 1595 cm^{-1} is due to aromatic C=C skeletal stretching; while the peak at 2849 cm^{-1} and 2925 cm^{-1} could be due to aromatic C-H stretching and the peak at 3285 cm^{-1} could be ascribed to O-H stretching of hydroxyl (-OH) group.

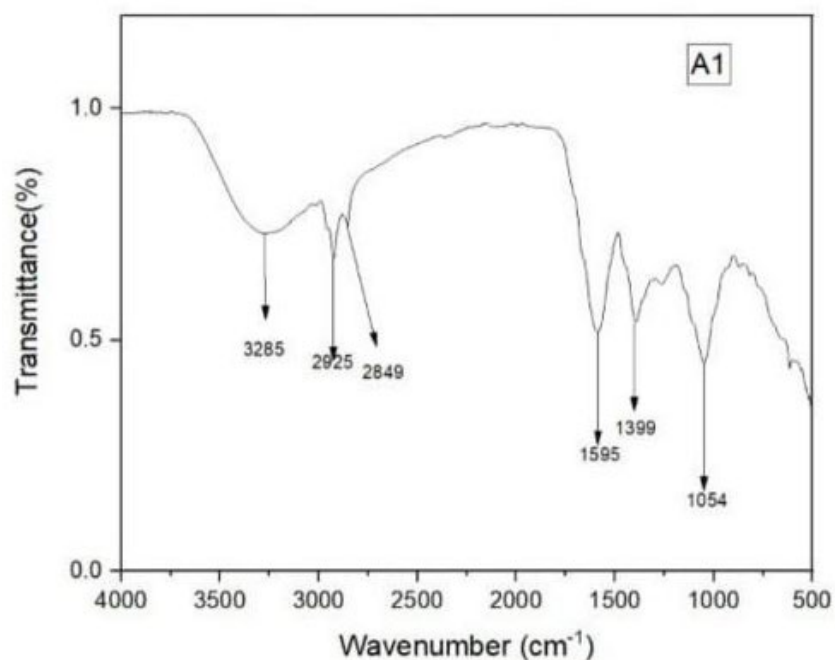
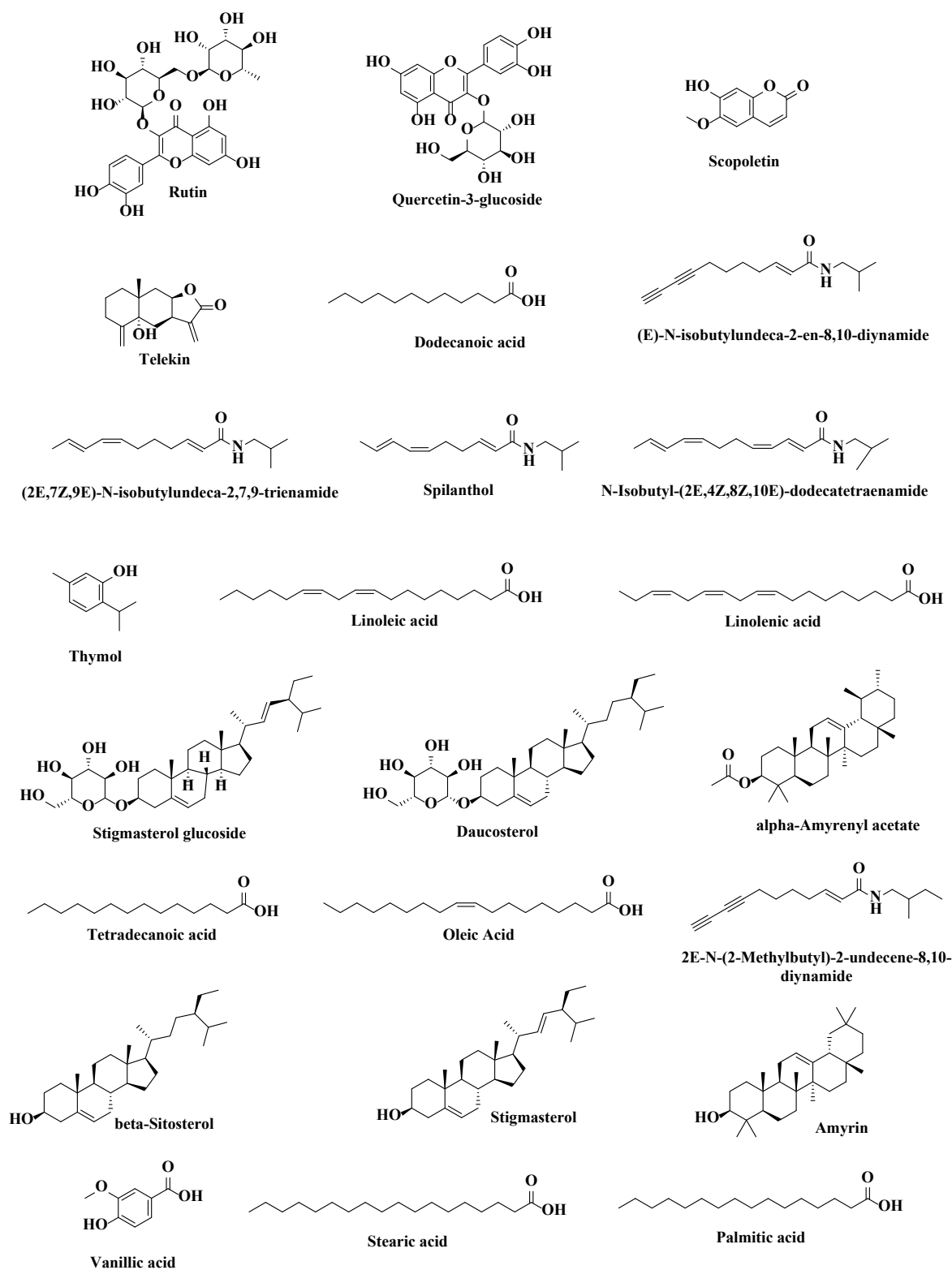


Figure 4 FT-IR spectra of HEFeAP

Determination of active phytoconstituents by liquid chromatography-mass spectrometry (LC-MS)

The phytochemicals present in the HEFeAP were identified with database matching from the LC-MS library, based on their molecular formula/weight, and relative number of compounds, using LC-MS interpretation by score value. A total of 24 compounds were detected, of which 13 major documented compounds from bioactive reference phytochemical library were (i) spilanthol, (ii) (2*E*,7*Z*,9*E*)-*N*-isobutylundeca-2,7,9-trienamide, (iii) (*E*)-*N*-isobutylundeca-2-en-8,10-diynamide, (iv) 2*E*-*N*-(2-methylbutyl)-2-undecene-8,10-diynamide, (v) *N*-isobutyl-(2*E*,4*Z*,8*Z*,10*E*)-dodecatetraenamide, (vi) β -sitosterol, (vii) stigmasterol, (viii) tetradecanoic acid, (ix) oleic acid, (x) dodecanoic acid, (xi) amyirin, (xii) vanillic acid, and (xiii) scopoletin. However, 24 phytochemicals were found in LC-MS separation, followed by fragmentation. The validated phyto-compounds as per LC-MS study were presented in **Figure 5** and **Table 3**. These compounds were characterized by their respective retention time in the LC-MS chromatogram via comparison with reference standards having similar chromatographic parameters.



1

2 **Figure 5. LC-MS of HEFeAP. [5a].** Structures of 24 compounds listed in table 3, detected by LC-

3 MS studies.

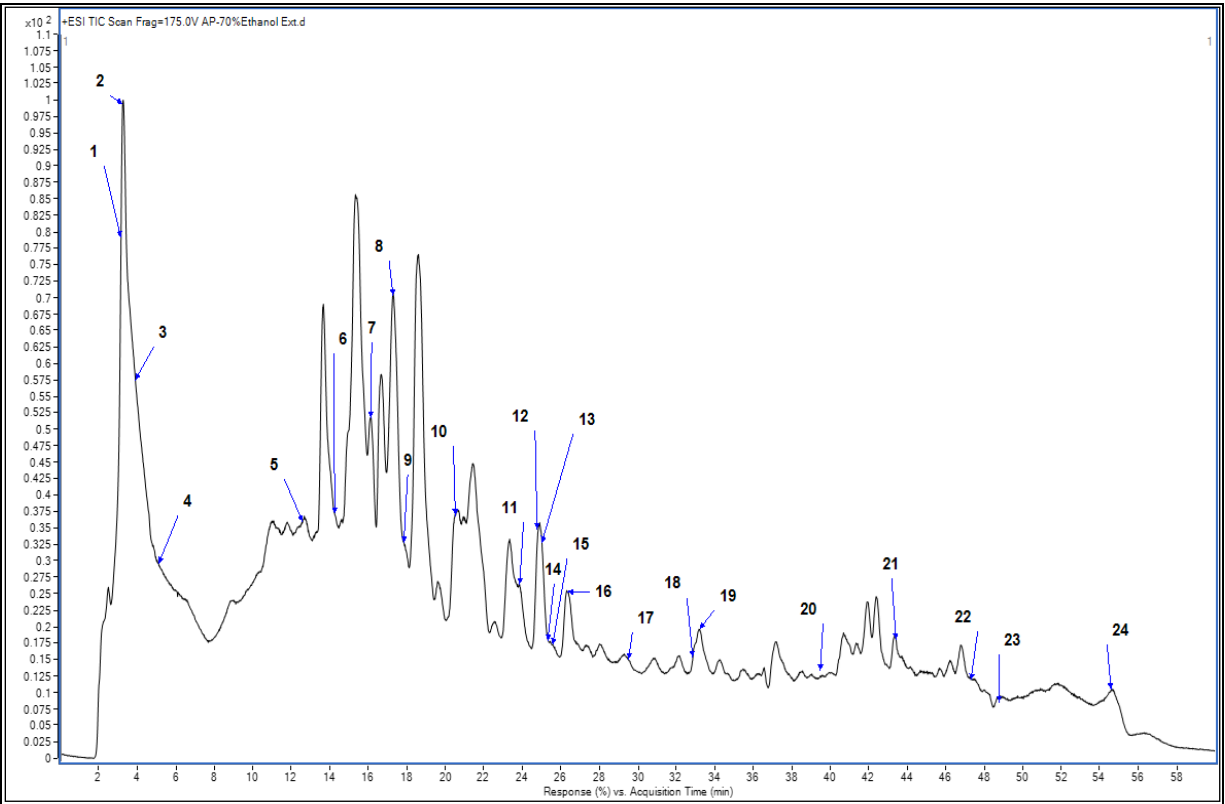


Figure 5. LC-MS of HEFeAP. (5b) Total Ion Chromatogram of 24 active-phytocompounds present in HEFeAP

Table 3. List of 24 Compounds detected

Compound no.	Name	Formula	Mass	^{a)} (Min)	Width	Height	Area	[M+H] ⁺ ^[b]	Error ^[c]	Score
1	Rutin	C ₂₇ H ₃₀ O ₁₆	610.1519	3.144	0.254	59075	1189526	611.1585	-2.4	63.72
2	Quercetin 3-glucoside	C ₂₁ H ₂₀ O ₁₂	464.0948	3.227	0.297	77862	1811173	465.101	-1.41	65.91
3	Scopoletin	C ₁₀ H ₈ O ₄	192.0412	3.892	0.618	27968	957228	193.0487	-5.56	58.29
4	Telekin	C ₁₅ H ₂₀ O ₃	248.139	5.105	0.995	21920	1015584	249.1462	-9.01	42.6
5	Dodecanoic acid	C ₁₂ H ₂₄ O ₂	200.1831	12.601	0.625	1473	2541	201.1904	27.37	2.21
6	(<i>E</i>)- <i>N</i> -isobutylundeca-2-en-8,10-diynamide	C ₁₅ H ₂₁ NO	231.1613	14.296	0.223	1291431	19712581	232.1683	-4.34	88.59
7	Spilanthol	C ₁₄ H ₂₃ NO	221.1771	16.373	0.311	5037265	103917325	222.1843	-3.94	94.02
8	(2 <i>E</i> ,7 <i>Z</i> ,9 <i>E</i>)- <i>N</i> -isobutylundeca-2,7,9-trienamide	C ₁₅ H ₂₅ NO	235.1923	17.42	0.562	17095426	616164111	236.1995	-5.67	79.21
9	<i>N</i> -Isobutyl-(2 <i>E</i> ,4 <i>Z</i> ,8 <i>Z</i> ,10 <i>E</i>)-dodecatetraenamide	C ₁₆ H ₂₅ NO	247.1925	17.786	0.363	340402	6151474	248.1999	-4.46	92.24
10	Linolenic acid	C ₁₈ H ₃₀ O ₂	278.2232	20.811	0.319	628515	10973108	279.2305	-5.1	92.66
11	Thymol	C ₁₀ H ₁₄ O	150.1042	23.836	0.416	72835	2053680	151.1116	-1.71	84.61
12	α-Amyrenyl acetate	C ₃₂ H ₅₂ O ₂	468.3807	24.85	0.105	7587	29935	469.3885	-34.13	29.98
13	Daucosterol	C ₃₅ H ₆₀ O ₆	576.4492	24.949	0.355	28346	608072	577.4586	17.73	10.87
14	Linoleic acid	C ₁₈ H ₃₂ O ₂	280.2382	25.032	0.564	22361	684324	281.2455	-7.18	80.15
15	Stigmasterol glucoside	C ₃₅ H ₅₈ O ₆	574.4443	25.182	0.016	27362	25394	575.4521	36.52	48.86
16	Oleic acid	C ₁₈ H ₃₄ O ₂	282.2543	26.262	0.393	254381	7125921	283.2616	-5.43	92

17	Tetradecanoic acid	C ₁₄ H ₂₈ O ₂	228.2075	29.52	8.599	1980	18847	229.2148	-6.21	66.57
18	Vanillic acid	C ₈ H ₈ O ₄	168.0417	33.143	28.984	2316	22563	169.049	-3.43	45.83
19	2 <i>E</i> - <i>N</i> -(2-methylbutyl)-2-undecene-8,10-diynamide	C ₁₆ H ₂₃ NO	245.1769	33.276	0.356	664524	15206340	246.1841	-4.44	94.46
20	β-Sitosterol	C ₂₉ H ₅₀ O	414.3763	39.708	6.686	3530	45635	415.384	-23.84	7.14
21	Stearic acid	C ₁₈ H ₃₆ O ₂	284.2702	43.164	16.777	5296	100400	285.278	-4.56	82.04
22	Stigmasterol	C ₂₉ H ₄₈ O	412.3666	47.12	0.792	7898	239800	413.3739	-9.39	70.55
23	Amyrin	C ₃₀ H ₅₀ O	426.3859	48.582	8.626	3966	57137	427.3932	-0.56	72.71
24	Palmitic acid	C ₁₆ H ₃₂ O ₂	256.2498	55.995	0.595	23775	42359	257.2567	37.27	34.35

[a] RT: Retention time. [b] *m/z*: mass to charge ratio. [c] ppm: parts per million.

***In-vitro* Assays**

Antioxidant Activity by DPPH

The *in-vitro* antioxidant activity at five different concentrations revealed the potent free-radical scavenging potential of HEFeAP, compared with the standard drug ascorbic acid **Figure 6**. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay is a widely accepted method for assessing the free-radical scavenging activity and our results revealed that HEFeAP possesses dose-dependent antioxidant activity. The percentage inhibition of DPPH-radicals was directly proportional to HEFeAP concentrations **Figure 6**. The IC₅₀ of HEFeAP (31.22 ± 0.53 µg/ml) indicated the concentration at which the extract scavenged 50% of DPPH radicals, calculated by linear regression analysis. Ascorbic acid (IC₅₀ 22.22 ± 0.75 µg/ml), a well-known antioxidant, is used as the reference standard, to compare and validate the antioxidant potential of HEFeAP. The results confirmed significant antioxidant activity, indicated by IC₅₀, compared to untreated controls. The observed antioxidant activity of HEFeAP thus, suggested the presence of bioactive antioxidant compounds in the test extract, while the LC-MS profile indicated the presence of vanillic acid, scopoletin, and phytochemicals of alkyl amide family with well-known free-radical-scavenging potential. As the results were comparable to ascorbic acid, the strong antioxidant ability of HEFeAP could be established.

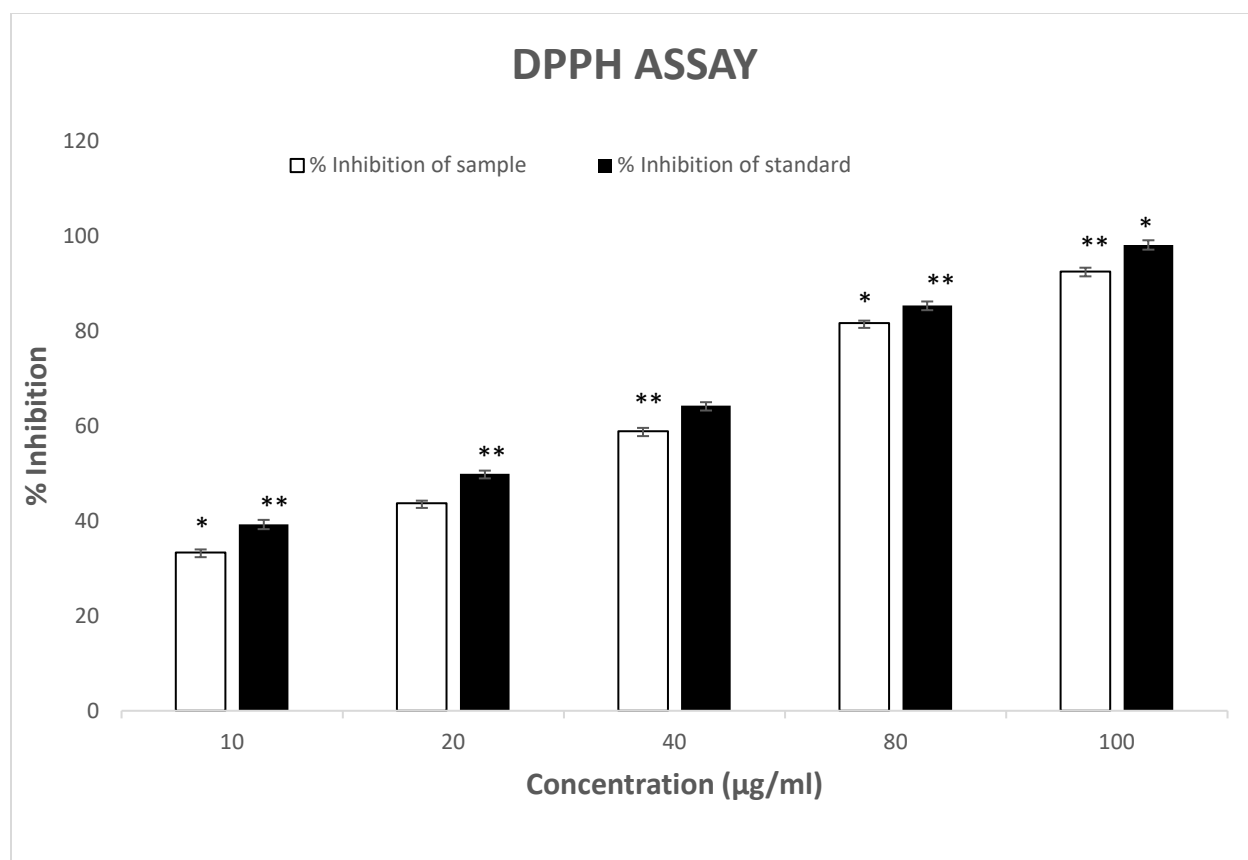


Figure 6. *In-vitro* antioxidant activity of HEFeAP. Bar-graph showing % inhibition of oxidation by HEFeAP at different concentrations, in triplicate (n=3), using ascorbic acid as standard. Values are expressed as Mean $\pm$ SD (n=3). Statistical significance was determined using One-Way ANOVA followed by Tukey's post hoc test (**P < 0.001 & *P < 0.01).

H₂O₂ Radical Scavenging assay

H₂O₂ radical scavenging is a widely known method, and our findings showed that HEFeAP possesses dose-dependent scavenging action. *In-vitro* H₂O₂ radical-scavenging activity of HEFeAP, at five different concentrations, demonstrated a higher free-radical scavenging potential than ascorbic acid **Figure 7** and the % inhibition of H₂O₂ radicals was related to HEFeAP

concentrations. The IC₅₀ of HEFeAP (40.36 ± 0.47 µg/ml), the concentration at which the extract scavenged 50% of H₂O₂ radicals, was estimated by linear regression analysis, using Ascorbic acid (IC₅₀ 4.1 ± 0.97 µg/ml) as a reference standard.^[77] The results demonstrated substantial antioxidant activity (IC₅₀) of HEFeAP, compared to untreated controls, suggesting the extract had bioactive antioxidant compounds, while the LC-MS profile revealed the presence of vanillic acid, scopoletin, and phytochemicals of the alkyl amide family with well-known free radical scavenging properties.

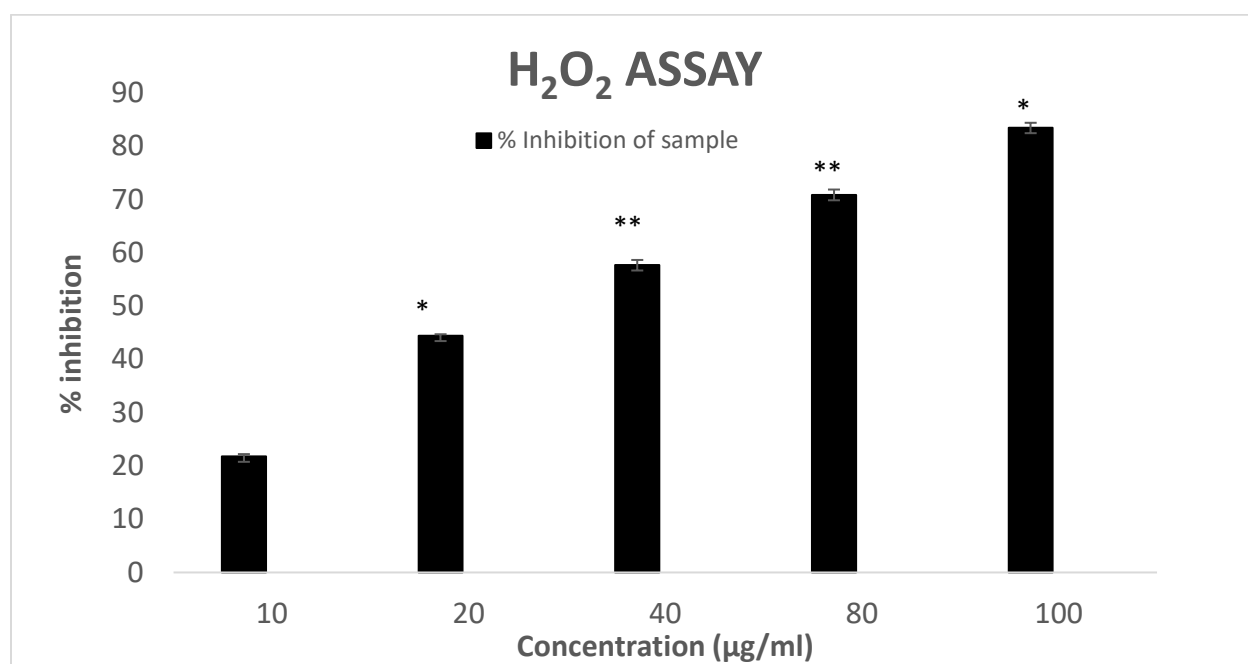


Figure 7 *In-vitro* H₂O₂ radical scavenging assay of HEFeAP. Bar-graph showing % inhibition of oxidation by HEFeAP at different concentrations, Values are expressed as Mean $\pm$ SD (n=3). Statistical significance was determined using One-Way ANOVA followed by Tukey's post hoc test (**P < 0.001 & *P < 0.01).

Protein Denaturation assay

- 1
- 2
- 3
- 4
- 5
- 6
- 7
- 8
- 9
- 10
- 11
- 12
- 13
- 14
- 15
- 16

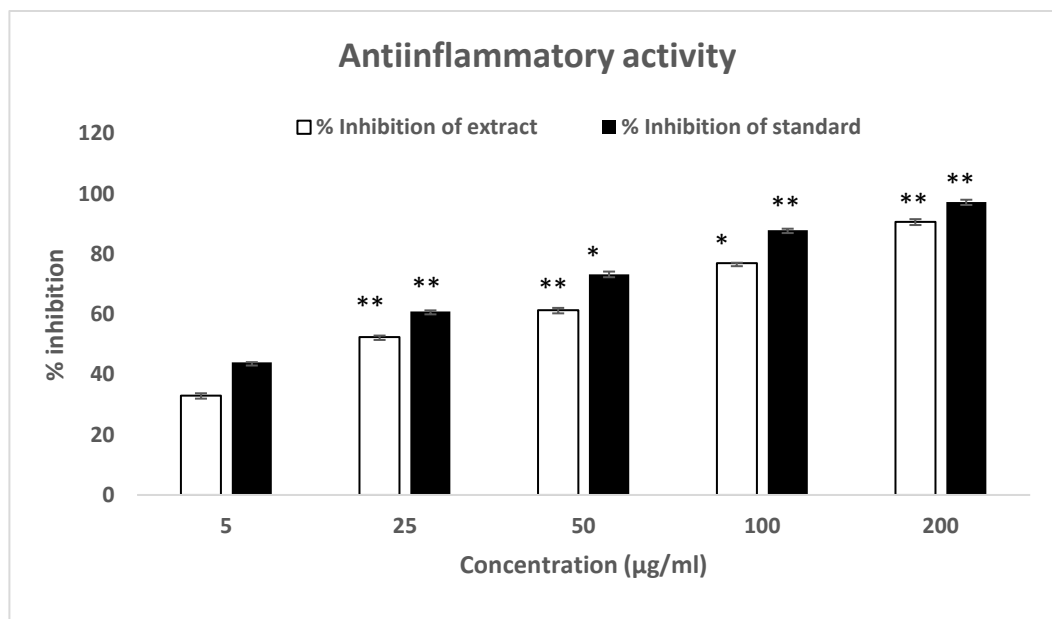


Figure 8 *In-vitro* anti-inflammatory activity of HEFeAP. Bar graph showing % inhibition of inflammation by HEFeAP at different concentrations, in triplicate (n=3), using aspirin as standard drug. Results are presented as Mean $\pm$ SD (n=3). Statistical significance was determined using One-Way ANOVA followed by Tukey's post hoc test (**P < 0.001 & *P < 0.01).

The result of the *in-vitro* protein denaturation assay exhibited potent anti-inflammatory activity of HEFeAP, compared to the standard drug aspirin, in the egg albumin denaturation model with an IC₅₀ of 27.61 µg/ml **Figure 8**. Results suggest that HEFeAP can be developed as a strong herbal healer against degeneration of joint cartilage during rheumatoid arthritis.^[64,65]

Antihyperglycemic Activity using α -amylase

The hypoglycaemic effects of HEFeAP were evaluated through α -amylase assay, using metformin as the standard drug. This *in-vitro* assay showed that HEFeAP could exert a concentration-dependent inhibition of α -amylase activity, as higher concentrations of the test agent resulted in

increased inhibition from **2.73** to **58.42%**, a proportional rise with concentration **Figure 9**. The IC_{50} , calculated as **$85.43 \pm 0.52 \mu\text{g/ml}$** , indicated the hypoglycaemic potential of HEFeAP across different concentrations. The efficacy, compared to metformin, exhibited an IC_{50} of **$72.96 \pm 0.45 \mu\text{g/ml}$** **Table 4**. These findings suggest that HEFeAP could effectively regulate carbohydrate digestion and absorption, which might add to diabetes management.

Table 4. Percent (%) Inhibition of α -amylase, as an anti-hyperglycaemic assay of HEFeAP using Metformin as standard drug. Values are Mean $\pm$ SD (n=3).

Concentration ($\mu\text{g/ml}$)	% Inhibition by HEFeAP $\pm$ SEM			% Inhibition by standard drug $\pm$ SEM		
5	2.73	$\pm$	0.58	6.2	$\pm$	0.42
10	6.1	$\pm$	1.02	9.71	$\pm$	0.98
20	12.94	$\pm$	0.85	16.13	$\pm$	0.26
50	29.21	$\pm$	0.46	36.31	$\pm$	0.74
100	58.42	$\pm$	0.25	66.82	$\pm$	1.05
	$IC_{50} = 85.43 \pm 0.52 \mu\text{g/ml}$			$IC_{50} = 72.96 \pm 0.45 \mu\text{g/ml}$		

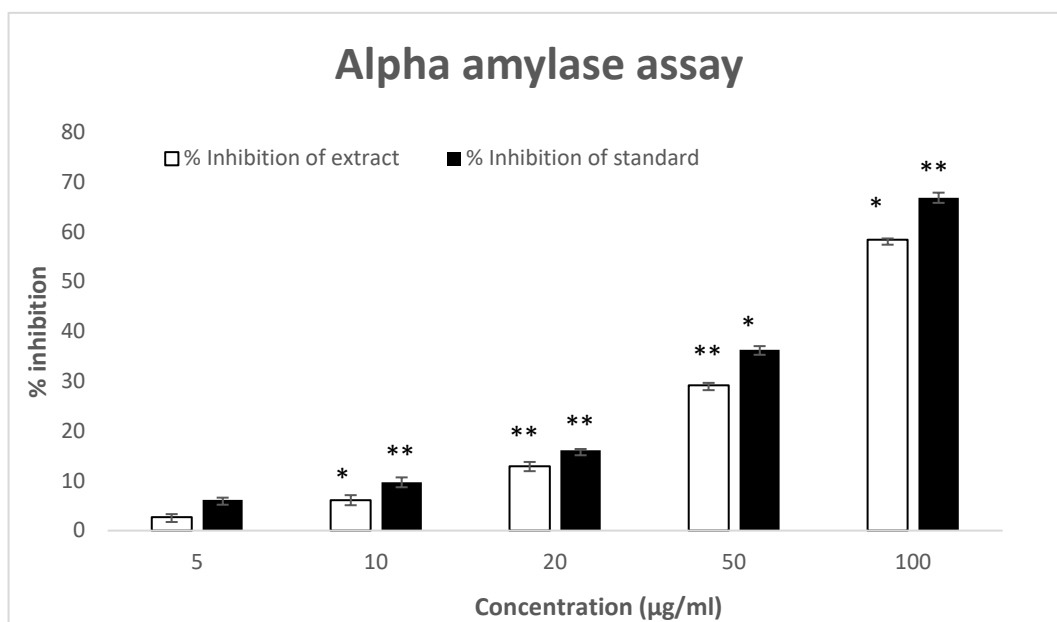
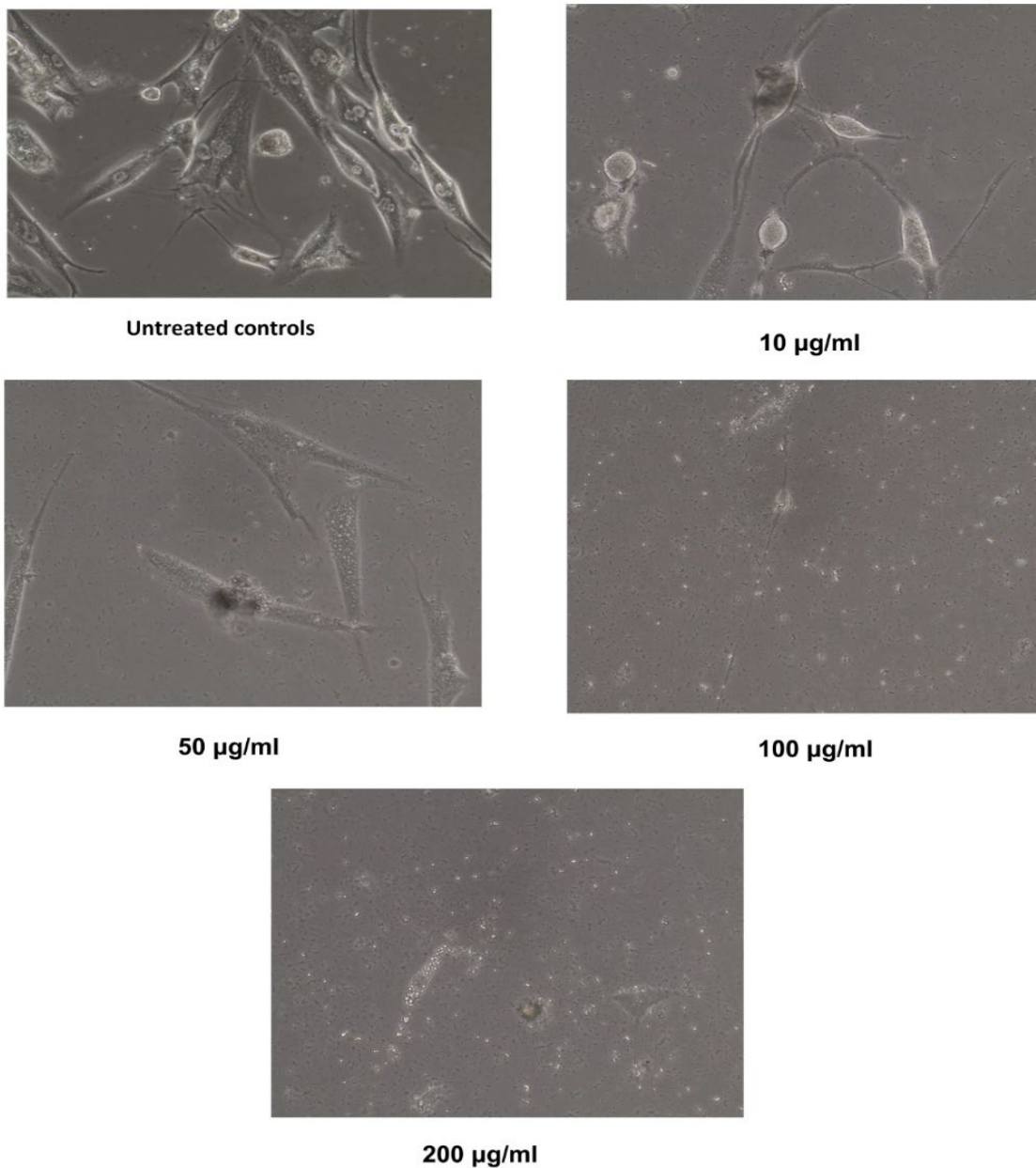


Figure 9. Percentage inhibition of α -amylase activity by HEFeAP. Data are shown as Mean $\pm$ SD (n=3). Statistical significance was determined using One-Way ANOVA followed by Tukey's post hoc test (**P < 0.001 & *P < 0.01).



1
2
3 **Figure 10a MTT assay on synovial fluid-derived fibroblast cells. 10a.** Growth-inhibition of
4 RA-promoting fd-FLS cell's viability treated with HEFeAP and observed under Electron
5 Microscope. Microscopical observation shows that upon exposure of HEFeAP at 200 µg/ml most

1 of the fd-FLS cells were lost their viability. Similarly, 50 and 100 µg/ml concentrations induced
2 growth inhibition of fd-FLS cells were significant, too.

3 **Effect of HEFeAP on the Viability of Synovial Fluid-derived Fibroblast Cells (fd-FLS)**

4 The cell viability assay of HEFeAP was conducted on Fd-FLS of RA patients at 50-500 µg/ml for
5 48 h. The results revealed that the growth of the disease-promoting fd-FLS cell(s) was significantly
6 inhibited with the HEFeAP **Figure 10a**. However, the cell death was not significant below 50
7 µg/ml, as 72.35% of fd-FLS cells were killed at 500 µg/ml in 48 h of treatment. The IC₅₀ of
8 HEFeAP was 65.75 µg/ml on exposure to fd-FLS cells for 48h treatment. Results also revealed
9 that 69.19 % of fd-FLS cells lost their viability when exposed to the test extract HEFeAP at 200
10 µg/ml, as shown in **Figure 10b**. Interestingly, HEFeAP-treated fd-FLS cells showed membrane
11 shrinkage, loss of motility, and inhibition of viability when the doses were gradually increased.
12 The HEFeAP arrested most of the fd-FLS cell's viability at 200 µg/ml.

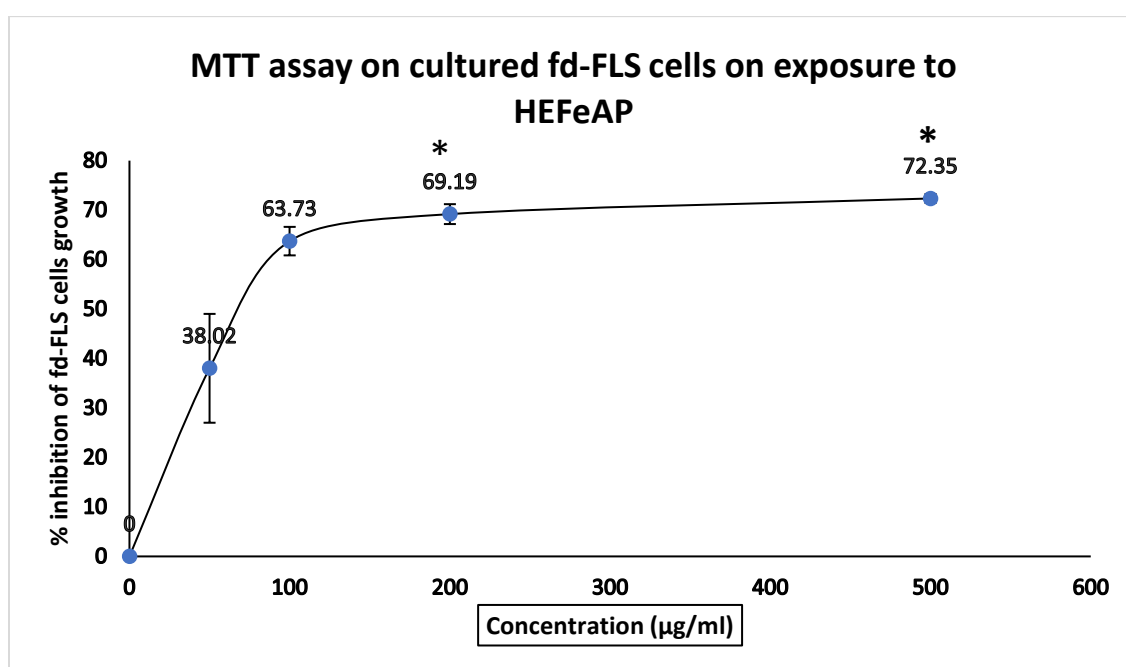


Figure 10b MTT Assay on Synovial fluid-derived fibroblast cells. Effect of HEFeAP on the viability of synovial fluid-derived fibroblast cells (fd-FLS) in the dose-dependent response curve showing significant inhibition of the growth of inflammatory fibroblast cells, may lead to suppression of inflammatory mediators towards attenuation of RA complications ^[78]. One way

ANOVA was carried out between dose conc vs % of cell growth inhibition. We found a positive correlation ($P < 0.05$ *). Total experimental P value among the different groups.

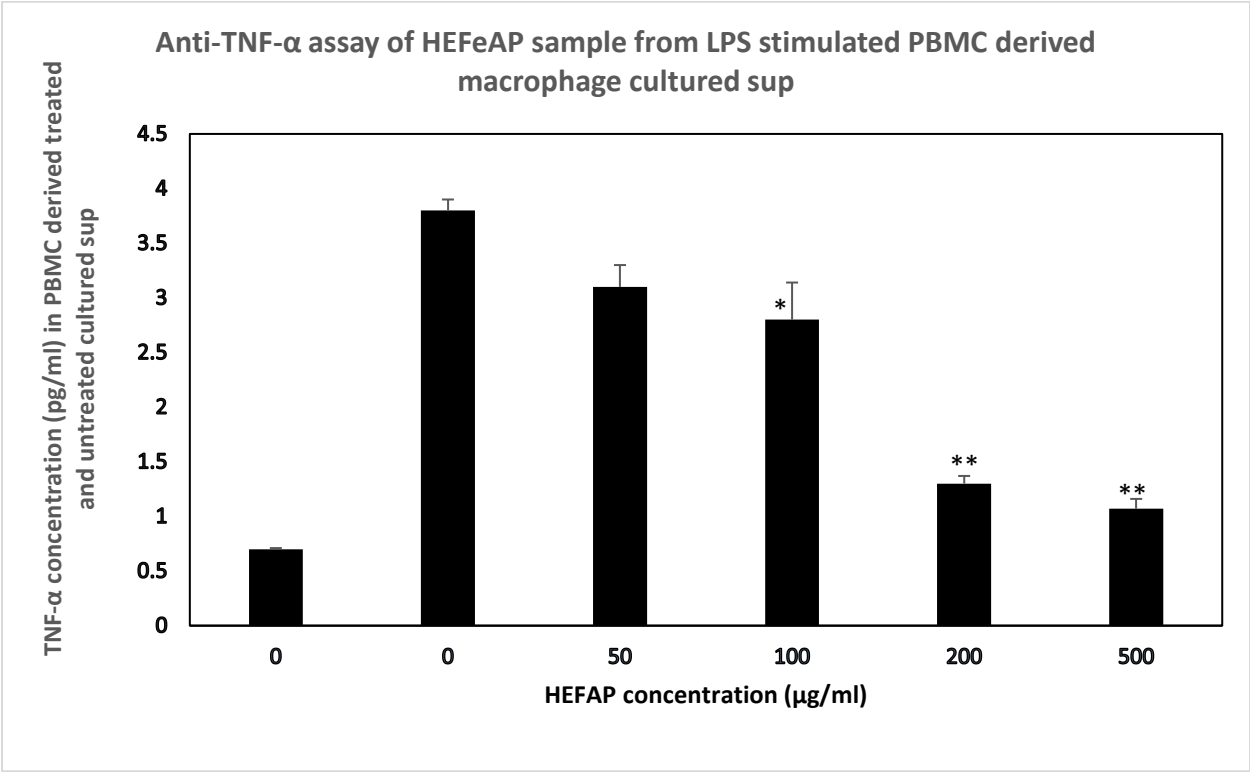
Downregulation of TNF- α and NO in HEFeAP-treated Macrophage

The culture supernatants of human PBMC-derived macrophages, treated with LPS, release a considerable amount of TNF- α **Figure 11a**. The treatment with HEFeAP dose-dependently reduced the TNF- α levels to 18.43%, 26.32% ($P < 0.001$), 65.79% ($P < 0.0001$) and 71.84% ($P < 0.0001$) at 50, 100, 200 and 500 $\mu\text{g/ml}$, respectively. TNF- α is a crucial pro-inflammatory cytokine associated with chronic inflammatory disorders of auto-immune complications including rheumatoid arthritis and metabolic issues. RA disease progression stage while joint synovial fluid derived fibroblast cell's growth upregulates, then a huge amount of TNF- α liberates from the growing fibroblast cells.^[66] TNF- α , a pro-inflammatory upstream mediator, triggers downstream signaling cascades, producing other cytokines such as IL-6 and IL-1 β and RA-specific inflammatory IL-9 and IL-17A cytokines.^[67,68] So, TNF- α reduction may attenuate acute and chronic inflammatory complications and decline associated disease progression, making it an essential biomarker for evaluating medical treatments of inflammatory diseases including rheumatoid arthritis.^[21,69] TNF- α monitoring was emphasized to assess HEFeAP efficiency in reducing inflammatory prognosis in RA complications. Similarly, the elevated inflammatory mediator NO concentration in LPS-stimulated macrophages was significantly downregulated in graded doses (50, 100, 200, 500 $\mu\text{g/ml}$) when pretreated with HEFeAP, providing 16.67, 31.42, 60.24, and 56.41% protection **Figure 11b**. Moreover, cells treated with HEFeAP led to 1.2, 1.46, 2.52, and 1.39 -fold reduction of NO production, compared to untreated controls.

1

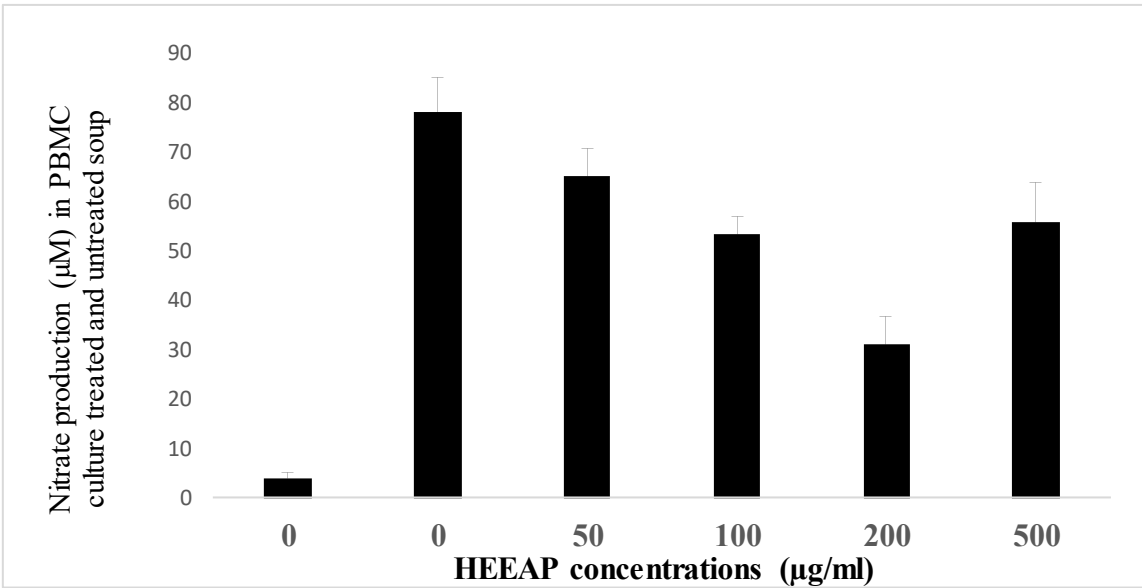
2

3



4

Figure 11a *In-vitro* determination of pro-inflammatory cytokine TNF- α using HEFeAP in



LPS-stimulated human PBMC-derived Macrophages. Results are expressed as mean $\pm$ S.D. (n=3) of three independent experiments. The first bar column indicates without treatment and LPS stimulation. Second bar column from the left indicates LPS stimulated without treatment of HEFeAP sample. Third to sixth bar column indicate the LPS stimulated as well as HEFeAP treated sample. *P < 0.001 and **P < 0.0001 were considered as significant, analysed by unpaired student *t* test, compared to LPS stimulated untreated control group [second bar column].

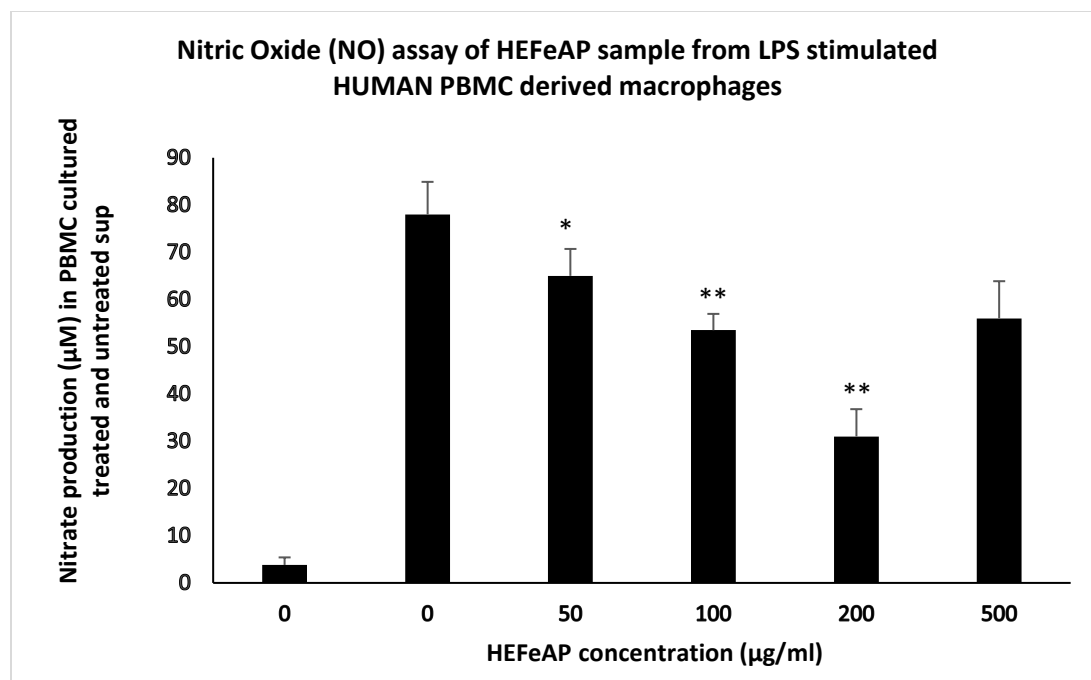


Figure 11b *In-vitro* determination of inflammatory mediator NO using HEFeAP in LPS-stimulated human PBMC-derived Macrophages. Results are expressed as mean $\pm$ S.D. (n=3) of three independent experiments. The first bar column indicates without treatment and LPS stimulation. The second bar column from the left indicates LPS stimulated without treatment of HEFeAP sample. The third to sixth bar column indicates the LPS stimulated as well as HEFeAP treated sample. * $P < 0.05$ and ** $P < 0.001$ were considered as significant analyzed by unpaired student *t-test*, compared to LPS stimulated untreated control group [second bar column].

Table 5. Physicochemical properties and Lipinski filter analysis using Swiss ADME software of 24 LC-MS detected compounds from HEFeAP.^[79]

NA = not available

Name of compounds	Molecular Formula	Molecular weight [a]	No. of H-bond acceptors	No. of H-bond donors	No. of heavy atoms	No. of rotatable bonds	Molar refractivity	Log P (o/w)	Lipinski violation	Skin permeation [b]
Spilanthol	C ₁₄ H ₂₃ NO	221.34	1	1	16	8	70.99	3.39	No	-5.12
(2 <i>E</i> ,7 <i>Z</i> ,9 <i>E</i>)- <i>N</i> -Isobutylundeca-2,7,9-trienamide	C ₁₅ H ₂₅ NO	235.37	1	1	17	9	75.80	3.53	No	-4.82
(<i>E</i>)- <i>N</i> -Isobutylundeca-2-en-8,10-diynamide	C ₁₅ H ₂₁ NO	231.33	1	1	17	8	73.07	3.39	No	-5.13
2 <i>E</i> - <i>N</i> -(2-Methyl butyl)-2-undecene-8,10-diynamide	C ₁₆ H ₂₃ NO	245.36	1	1	18	9	77.88	3.69	No	-4.96
<i>N</i> -Isobutyl-(2 <i>E</i> ,4 <i>Z</i> , 8 <i>Z</i> ,10 <i>E</i>)-dodecatetra enamide	C ₁₆ H ₂₅ NO	247.38	1	1	18	9	80.13	3.78	No	NA
β-Sitosterol	C ₂₉ H ₅₀ O	414.71	1	1	30	6	133.23	5.05	Yes	-2.20
Stigmasterol	C ₂₉ H ₄₈ O	412.69	1	1	30	5	132.75	5.04	Yes	-2.74
Tetradecanoic acid	C ₁₄ H ₂₈ O ₂	228.37	2	1	16	12	71.18	3.32	No	-3.35
Oleic Acid	C ₁₈ H ₃₄ O ₂	282.46	2	1	20	15	89.94	4.01	No	-2.60
Dodecanoic acid	C ₁₂ H ₂₄ O ₂	200.32	2	1	14	10	61.57	2.70	No	-4.54
Amyrin	C ₃₀ H ₅₀ O	426.72	1	1	31	0	135.14	4.80	No	-2.51

Vanillic acid	C ₈ H ₈ O ₄	168. 15	4	2	12	2	41.92	1.40	No	-6.31
Scopoletin	C ₁₀ H ₈ O ₄	192. 17	4	1	14	1	51.00	1.86	No	-6.39
Rutin	C ₂₇ H ₃₀ O ₁₆	610. 52	16	10	43	6	141.38	1.58	Yes	-10.26
Quercetin 3-glucoside	C ₂₁ H ₂₀ O ₁₂	464. 38	12	8	33	5	110.16	1.69	Yes	-8.88
Telekin	C ₁₅ H ₂₀ O ₃	248. 32	3	1	18	0	69.15	2.27	No	-6.31
Linolenic acid	C ₁₈ H ₃₀ O ₂	278. 43	2	1	20	13	88.99	5.66	No	Not Available
Thymol	C ₁₀ H ₁₄ O	150. 22	1	1	11	1	48.01	2.32	No	-4.87
Alpha Amyrenyl acetate	C ₃₂ H ₅₂ O ₂	468. 75	2	0	34	2	144.88	5.17	No	-2.36
Daucosterol	C ₃₅ H ₆₀ O ₆	576. 85	6	4	41	9	165.61	4.63	No	-4.32
Linoleic acid	C ₁₈ H ₃₂ O ₂	280. 45	2	1	20	14	89.46	5.88	No	NA
Stigmasterol glucoside	C ₃₅ H ₅₈ O ₆	574. 83	6	4	41	8	165.14	5.24	No	-4.86

Stearic acid	C ₁₈ H ₃₆ O ₂	284.48	2	1	20	16	90.41	4.30	No	-2.19
Palmitic acid	C ₁₆ H ₃₂ O ₂	256.42	2	1	18	14	80.80	3.85	No	-2.77

1

2 [a] g/mol. [b] cm/s

3 **Physicochemical Properties of the Bioactive Compounds of HEFeAP**

4 *In-silico* drug-like property studies, evaluated through Swiss ADME software online,^[79] revealed
5 that most of the active phyto-compounds follow the Lipinski rule of 5, suggesting that it hold good
6 promise for oral absorption and bioavailability of HEFeAP as an oral drug candidate (**Table 5**).
7 Skin permeability of most compounds including local anesthetics viz. spilanthal, (*E*)-*N*-
8 isobutylundeca-2-en-8,10-diyamide, vanillic acid, and scopoletin were found to be significant
9 with a higher rate of penetration across the skin layer stratum corneum. The results suggested that
10 HEFeAP could help to manage RA and associated inflammatory conditions, including pain,
11 through skin-mediated topical herbal nanogel formulation.

12

13 ***In-silico* Studies**

14 **Molecular docking studies of spilanthal and (*E*)-*N*-isobutylundeca-2-en-8,10-diyamide**

15 We have tested two main bioactive compounds of HEFeAPs on two inflammatory marker proteins,
16 LOX-5 and phospholipase A2, as these proteins are found in the Pub-Chem and PDB libraries,
17 respectively. The enzymes LOX-5, and phospholipase-A2 make leukotrienes and prostaglandins,
18 which are known to cause inflammation. Molecular docking analysis may provide useful
19 information on the inflammation inhibitors found in HEFeAP. The molecular docking studies of

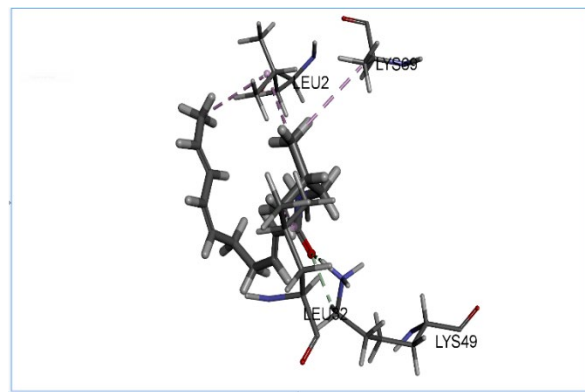
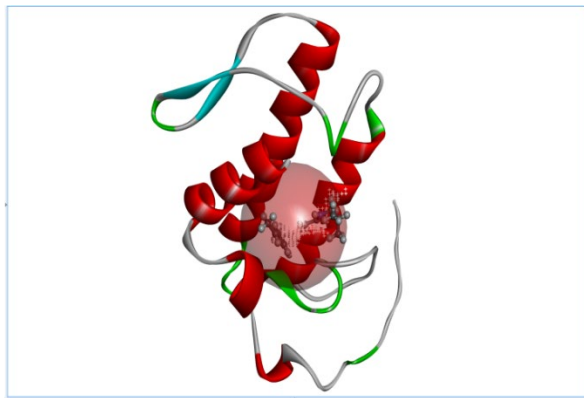
spilanthol and (*E*)-*N*-isobutylundeca-2-en-8,10-diyamide, the two most active phytoconstituents inHEFeAP, were shown in **Table 6** and **Figures 12a, 12b, 12c, and 12d**. The Molecular Docking was conducted in the molecular binding site generated by the Discovery studio platform. The top 10 binding poses were obtained after the molecular docking. The best docking pose with the Docking energy score has been presented in table 6. The RMSD between the best reported binding pose and the other 9 binding poses ranged from 0.66 to 6.3 Å. The RMSD value of the best docking score on the best optimized pose for each ligand against the target inflammatory protein was less than 0.66 Å , have been presented in the Table 6. Lower RMSD value of the best docked pose indicate the best interaction between ligand and target protein with improved binding energy as depicted in Table 6.

Table 6. Energy and interaction energy calculations of spilanthol and (*E*)-*N*-isobutylundeca-2-en-8,10-dynamide when docked with LOX-5 and Phospholipase-2.

Protein	PDB	Ligand	CDOCKER Energy kcal/mol (ΔG)	CDOCKER Interaction Energy kcal/mol (ΔG)
LOX-5	2IUJ	Spilanthol	-2.12125	29.1652
LOX-5	2IUJ	(<i>E</i>)- <i>N</i> -isobutylundeca-2-en-8,10-dynamide-	20.0638	36.5921
Phospholipase 2	3T0R	Spilanthol	10.2062	34.8296

Phospholipase 2	3T0R	(E)-N-isobutylundeca-2-en-8,10-diynamide-	24.9079	30.022
-----------------	------	---	---------	--------

1



2

3

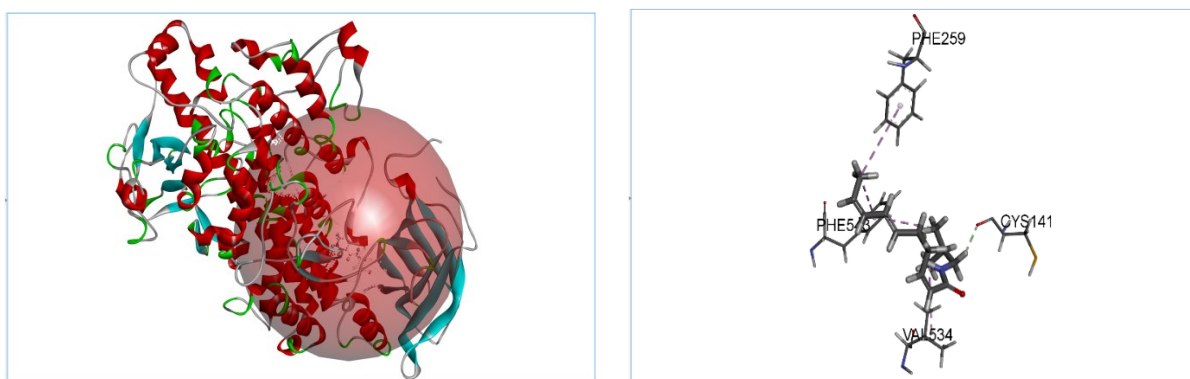
Name	Distance	category	Type	From	From Chemistry	To	To Chemistry
A:LYS49:HZ1 - 5353001:O1	2.73024	Hydrogen Bond	Conventional Hydrogen Bond	A:LYS49:HZ1	H-Donor	5353001:O1	H-Acceptor
A:LYS49:HE1 - 5353001:O1	2.41426	Hydrogen Bond	Carbon Hydrogen Bond	A:LYS49:HE1	H-Donor	5353001:O1	H-Acceptor
A:LYS69 - 5353001:C5	4.43176	Hydrophobic	Alkyl	A:LYS69	Alkyl	5353001:C5	Alkyl
5353001:C5 - A:LEU2	4.8521	Hydrophobic	Alkyl	5353001:C5	Alkyl	A:LEU2	Alkyl

5353001:C6 - A:LEU32	4.75246	Hydrophobic	Alkyl	5353001:C6	Alkyl	A:LEU32	Alkyl
5353001:C16 - A:LEU2	4.33069	Hydrophobic	Alkyl	5353001:C16	Alkyl	A:LEU2	Alkyl

1

2

Figure 12a Interaction of spilanthol and phospholipase-2



3

4

Name	Distance	category	Type	From	From Chemistry	To	To Chemistry
5353001:H18 - A:CYS141:O	2.44544	Hydrogen Bond	Carbon Hydrogen Bond	5353001:H18	H-Donor	A:CYS141:O	H- Acceptor
5353001:C5 - A:VAL534	4.40355	Hydrophobic	Alkyl	5353001:C5	Alkyl	A:VAL534	Alkyl
A:PHE259 - 5353001:C16	5.06813	Hydrophobic	Pi-Alkyl	A:PHE259	Pi-Orbitals	5353001:C16	Alkyl

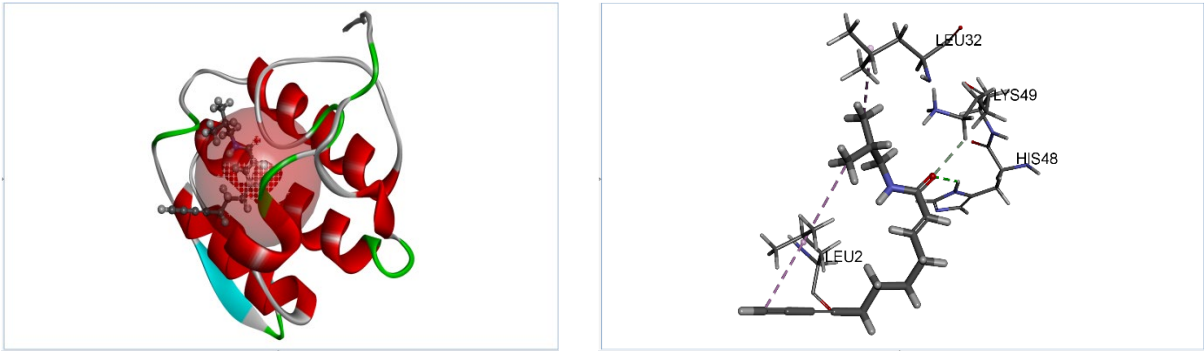
A:PHE543 - 5353001:C6	4.66897	Hydrophobic Pi-Alkyl		A:PHE543	Pi-Orbitals	5353001:C6	Alkyl
A:PHE543 - 5353001:C16	4.27836	Hydrophobic Pi-Alkyl		A:PHE543	Pi-Orbitals	5353001:C16	Alkyl

1

2

Figure 12b Interaction of spilanthol and LOX-5

3



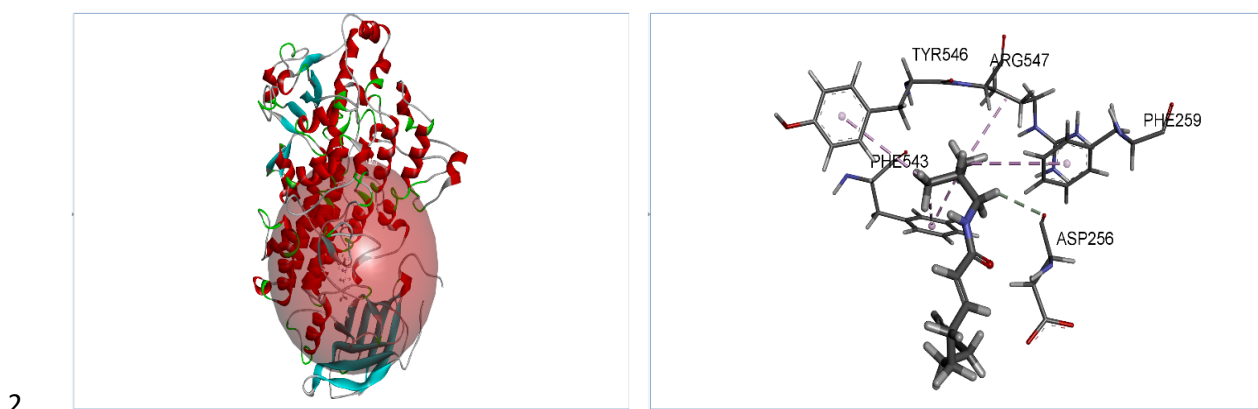
4

5

Name	Distance	category	Type	From	From Chemistry	To	To Chemistry
A:HIS48:HD1 - 5368482:O1	2.3735	Hydrogen Bond	Conventional Hydrogen Bond	A:HIS48:HD1	H-Donor	5368482:O1	H-Acceptor
A:LYS49:HE1 - 5368482:O1	2.64982	Hydrogen Bond	Carbon Hydrogen Bond	A:LYS49:HE1	H-Donor	5368482:O1	H-Acceptor

5368482:C8 - A:LEU32	5.182	Hydrophobic	Alkyl	5368482:C8	Alkyl	A:LEU32	Alkyl
5368482:C9 - A:LEU2	4.93246	Hydrophobic	Alkyl	5368482:C9	Alkyl	A:LEU2	Alkyl
5368482:C17 - A:LEU2	4.90061	Hydrophobic	Alkyl	5368482:C17	Alkyl	A:LEU2	Alkyl

1 **Figure 12c** Interaction of (*E*)-*N*-isobutylundeca-2-en-8,10-diynamide and phospholipase-2



3

Name	Distance	category	Type	From	From Chemistry	To	To Chemistry
5368482:H20 - A:ASP256:O	2.86454	Hydrogen Bond	Carbon Hydrogen Bond	5368482:H20	H-Donor	A:ASP256:O	H- Acceptor
5368482:C8 - A:ARG547	4.03186	Hydrophobic	Alkyl	5368482:C8	Alkyl	A:ARG547	Alkyl

A:PHE259 - 5368482:C8	5.28619	Hydrophobic	Pi-Alkyl	A:PHE259	Pi-Orbitals	5368482:C8	Alkyl
A:PHE543 - 5368482:C8	3.89374	Hydrophobic	Pi-Alkyl	A:PHE543	Pi-Orbitals	5368482:C8	Alkyl
A:PHE543 - 5368482:C9	4.11331	Hydrophobic	Pi-Alkyl	A:PHE543	Pi-Orbitals	5368482:C9	Alkyl
A:TYR546 - 5368482:C9	5.17516	Hydrophobic	Pi-Alkyl	A:TYR546	Pi-Orbitals	5368482:C9	Alkyl

Figure 12d Interaction of (*E*)-*N*-isobutylundeca-2-en-8,10-diynamide and LOX-5

Figure 12. Molecular docking of HEFeAP. **12a.** Interaction of spilanthol and phospholipase-2; **12b.** Interaction of spilanthol and LOX-5; **12c.** Interaction of (*E*)-*N*-isobutylundeca-2-en-8,10-diynamide and phospholipase-2; **12d.** Interaction of (*E*)-*N*-isobutylundeca-2-en-8,10-diynamide and LOX-5

Molecular Dynamic Simulation

Structural Deviation and Compactness

In the case of 2UIJ, the Root Mean Square Deviation (RMSD) of 2UIJ – E_N_iso-complex and 2UIJ – spilanthol complex was 0.275 nm and 0.22 nm, respectively. **Figure 13** (a) indicated that RMSD for the 2UIJ – E_N_iso-complex was increasing at the initial phase up to ~25 ns and started to decrease till 50 ns, then followed by a uniform appearance towards the end of the simulation. In the case of 2UIJ – Spilanthol small crust and troughs in RMSD can be seen scattered up to 50ns and then appeared to be uniformly distributed with an RMSD of 0.22 nm till 100 ns simulation. The stability of each amino acid in the protein was further confirmed by the Root Mean Square Fluctuation (RMSF) plot. The residue fluctuations at the 20th -30th residues of 2UIJ – E_N_iso-

complex were below the range of 1 nm and that of ~60th residue shows 0.6 nm, whereas in 2UIJ – Spilanthol complex 20th -30th residues had fluctuation below 0.6 nm. **Figure 13(b)** reveals that both complex's residues follow the above-stated phenomenon and all the remaining amino acids exhibit low RMSF (>0.37 nm) values, suggesting the stability of the protein backbone after binding the compounds. The H-bonds play as a critical role in determining the stability of the complex. Here, both complexes established an average of two H-bonds consistently observed after 100 ns simulation. By considering these two compounds with 2UIJ, the spilanthol bound complex showed higher stability than E_N_iso.

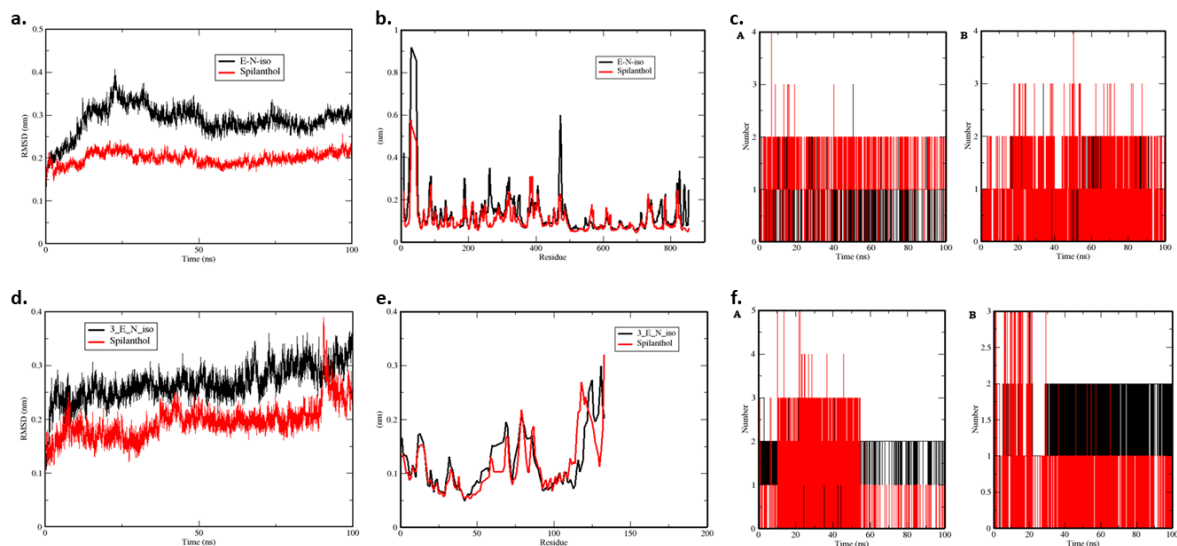


Figure 13. MD Simulation-Structural Deviation and Compactness

Discussion

In the traditional system of medicines, usually, a combination of bioactive secondary metabolites is thought to be responsible for therapeutic effects. It is known that plants produce an unmatched variety of phytochemicals that have endless potential for developing molecules having “drug-likeness”. Traditional and contemporary literature reported that *A. paniculata* flower heads (APF)

1 are used as a dental analgesic, while the present survey documented that in addition to its use as a
2 dental analgesic, the flower head has other uses including as local food. Thus, our study focuses
3 on the bioactive potential with qualitative and quantitative estimation and physicochemical
4 characterization of major bioactive secondary metabolites of hydroalcoholic extract of *Acmella*
5 *paniculata* flower (HEFeAP) to check and validate the phytotherapeutic value logically. The
6 phytochemical screening revealed the presence of alkaloids, flavonoids, phenols, steroids, and
7 tannins, while quantitative estimation, showed varied quantities of alkaloids, phenols, and tannins
8 as major secondary metabolites, compared to the standard phytomolecules. HEFeAP (1 µg/ml)
9 revealed λ_{max} as 267 nm, which might be due to the conjugated double bonds having similarity
10 with the structure of spilanthal, scopoletin, vanillic acids and other alkyl diamides. In the FTIR,
11 altogether there were six peaks showing the presence of functional groups similar to the active
12 phyto-compound, Spilanthol. The TLC with three optimized solvent systems (toluene: ethyl
13 acetate 8:2, ethanol: *n*-hexane 3:7, and *n*-hexane: ethyl acetate 2:1) reveals 4, 3 and 4 bands,
14 respectively. The R_f for each band matched with 0.50; similar to the R_f of Spilanthol,^[75,76] a
15 potential standard compound in the test extract having anti-inflammatory and local anesthetic
16 activities. The LC-MS data confirmed the presence of 24 compounds in the HEFeAP with
17 distinctive molecular formula and mass of each compound, indicating the presence of Spilanthol
18 and 23 other compounds. LC-MS results suggested the presence of several potential anti-oxidant,
19 anti-inflammatory phytomolecules including coumarin, flavonoids, polyphenols, terpenoids,
20 steroids, glycosides, and local anesthetics, for which the test extract HEFeAP showed remarkable
21 antioxidant, anti-inflammatory activities, followed by the growth inhibition of cultured synovial
22 fluid derived fibroblast cells and antidiabetic potential. These *in-vitro* studies were carried out
23 following the encouraging results of molecular docking, and molecular dynamic simulation studies

1 with the potential reference phytochemicals present in HEFeAP. Further, a literature search
2 revealed that the major compound Spilanthol downregulates the expression of i-NOS, and the
3 production of nitric oxide (NO) and inhibits inflammatory transcription factors.^[80] Here, the
4 presence of spilanthol in or test extract HEFeAP was confirmed by LC-MS, while the present
5 bioactivity study revealed *in-vitro* antioxidant and anti-inflammatory activities along with the
6 suppression of inflammatory mediators TNF- α and NO. These results are consistent with the
7 literature report. Thus, the flower extract of *A. paniculata* may serve as a good source of anti-
8 inflammatory lead.

9 (2*E*,7*Z*,9*E*)-*N*-Isobutylundeca-2,7,9 trienamide, present in the test extract, is known to down-
10 regulate the release of proinflammatory molecules from macrophages and inhibit influenza A and
11 the lipopolysaccharide of bacteria.^[81] It also acts on cannabinoid receptors and is used for treating
12 inflammation.^[82] The third compound (*E*)-*N*-isobutylundeca-2-ene-8,10 diynamide in the current
13 LC-MS study is known to inhibit NO, interleukin (IL)-8, IL-2 and COX-dependent prostaglandin,
14 with upregulation of phagocytic activity, and alveolar macrophage index as a prominent anti-
15 inflammatory agent.^[83] The next compound, identified by LC-MS, *N*-isobutyl-(2*E*,4*Z*,8*Z*,10*E*)-
16 dodecatetraenamide was reported to inhibit iNOS and COX expressing in LPS-induced
17 macrophage.^[84] The next three compounds, in LC-MS chromatogram, ephedrine's, glycyrrhizic
18 acid, and 6 gingerol are reported here for the first time in this plant *A. paniculata*, which have
19 proven anti-inflammatory potential.^[85,86] The β -sitosterol present in our test extract is known to
20 reduce arthritis by inhibiting inflammatory mediators, as well as a vascular and intracellular
21 adhesion molecule that expresses in TNF- α stimulated human aortic endothelial cells, along with
22 the inflammatory cell infiltration, IL-4, and IgE level, as a potential anti-inflammatory agent.^[87]
23 On the other hand, the stigmasterol present in the extract was reported to reduce leukocyte

1 infiltration and oedema and may bind with glucocorticoid receptors; while the genes involved in
2 the turnover of cartilage-like MMP-3, with antiarthritic action.^[88] The seventh compound
3 tetradecanoic acid is also known to have anti-inflammatory activity that enhances IL-10 production
4 in LPS-induced macrophages.^[89] The oleic acid, on the other hand, reduces IL-6, and TNF- α , but
5 enhances IL-10, inhibits NF κ B activation, and induces pro-inflammatory cytokines and
6 chemokines; along with upregulation of FFAR1, FFAR2, but down-regulate COX-2, and IL-12 as
7 an anti-inflammatory agent.^[90] Amyrin present in the extract, is known to reduce PGE by inhibiting
8 COX due to its anti-inflammatory potential. The inflammatory mediators released due to sub-
9 cellular degranulation inhibit inflammation by activation of NF κ B and cAMP response element-
10 binding protein.^[91] Dodecanoic acid inhibits IL-6, and CXCL8 of THP-1 cells by inhibiting
11 activated NF κ B and MAP kinase phosphorylation.^[92] Another important compound scopoletin
12 reduces NO synthase and inhibits the over-production of PGE2 and TNF- α along with the synthesis
13 of eicosanoid, peroxidation, and proinflammatory cytokine production.^[93] Vanillic acid, the 12th
14 compound in the LC-MS profile is known to reduce TNF, IL-6, and the production of
15 proinflammatory cytokines as an anti-inflammatory agent.^[94] Rutin on the other hand acts as an
16 anti-inflammatory agent by downregulating TNF- α , IL-6, COX-2, and IL-1 β , and by blocking
17 associated pathways NF κ B, MAPK, and JAK/STAT.^[95] While quercetin 3-glucoside, present in
18 the test extract, may act as an anti-inflammatory agent by inhibiting various inflammatory
19 mediators such as IKK, NF κ B, STAT1, iNOS, NO, TNF α , MAPKs, Akt, Src, JAK-1, and Tyk2;
20 along with the nuclear translocation of NF- κ B p65 leading to the reduced expression of key pro-
21 inflammatory cytokines IL-1 β , TNF- α , and IL-6 as anti-inflammatory agent.^[96] Telekin and its
22 derivatives are known as anti-cancer agents, with improved cytotoxicity against HepG-2 and A549,
23 with selective cytotoxicity against A549 cells, as potential anticancer molecules.^[97] The well-

known linoleic acid combined with TNF may serve as a potential anti-inflammatory agent by reducing NF- κ B activation and have a modulatory role in inflammation.^[98] Alpha-linolenic acid (ALA), is an anti-inflammatory polyunsaturated fatty acid that reduces the production of pro-inflammatory cytokines in activated macrophages and decreases the production of arachidonic acid (AA) derived oxylipins, suggesting a unique mechanism of inflammation modulation.^[99] Thymol, present in many plants including thyme, oregano, and tangerine peel displays anti-inflammatory effects by reducing the production of TNF- α and IL-6 and inhibiting the expression of COX-2 and iNOS in LPS-stimulated mouse mammary epithelial cells along with blocking NF- κ B and MAPK signaling pathways in mastitis model.^[100] The compound daucosterol exhibits anti-inflammatory activity by reducing NO production in LPS-induced rat peritoneal macrophages and RAW 264.7 cells, expression of COX-2, iNOS, as well as TNF- α and IL-6 along with inhibition of heat-induced protein denaturation and mouse ear oedema, as an anti-inflammatory agent.^[101] Palmitic acid, prevalent in obesity, can trigger inflammation by activating toll-like receptors (TLR), and induce LPS-mediated TLR4 activation. Its metabolic derivatives enhance inflammatory signaling, promote NF- κ B activation and inflammasome NLRP3, and lead to increased proinflammatory cytokines TNF- α , IL-1 β , and IL-6, contributing to insulin resistance rather than exerting anti-inflammatory effects.^[102]

We have used molecular docking study to validate our *in-vitro* results using inflammatory mediator proteins LOX-5 and phospholipase A2, the enzymes responsible for the production of leukotrienes and prostaglandins to initiate the progression of RA.^[103] Our docking results revealed the downregulation of inflammation by binding LOX-5 and Phospholipase A2 with the active-compounds, spilanthol and (*E*)-*N*-isobutylundeca-2-en-8,10-diyamide. The MD simulation was also used to validate the binding stability with target proteins. Impressive simulation suggests a

stable complex with the active phytomolecule in HEFeAP. To authenticate the *in-silico* findings, we have conducted *in-vitro* antioxidant activity of HEFeAP by DPPH assay using ascorbic acid as a standard drug. Significant IC₅₀ (IC₅₀ 31.22 ± 0.53 µg/ml), compared to ascorbic acid (IC₅₀ 22.22 ± 0.75 µg/ml) elicited the potential free radical scavenging power of HEFeAP. Radical scavenging activity was also validated by using H₂O₂ showing the IC₅₀ of HEFeAP (40.36 ± 0.47 µg/ml) compared to IC₅₀ of the standard Ascorbic acid 4.1 ± 0.97 µg/ml^[77] indicating radical-scavenging activity of HEFeAP. Next, the anti-inflammatory activity was evaluated by egg-albumin assay using aspirin as a standard drug. Test extract at IC₅₀ 27.61 ± 0.82 µg/ml and standard drug (IC₅₀ 15.88 ± 0.52 µg/ml) revealed the potential anti-inflammatory activity of HEFeAP. Further, *in-vitro* anti-hyperglycemic potential by α-amylase assay revealed that HEFeAP may possess good protection (IC₅₀ 85.43 ± 0.52 µg/ml) compared to standard drug metformin (IC₅₀ 72.96 ± 0.45 µg/ml). The cell viability assay on fd-FLS cells conducted to check the cytotoxic effects of HEFeAP revealed that the flowers of *A. paniculata* are safe as a dental analgesic in traditional medicine, and have diverse phytochemicals with anti-inflammatory and analgesic activities to manage inflammatory dysregulation including RA. We have studied the 24 bioactive compounds of HEFeAP, obtained from LC-MS, using Swiss ADME software (**Table 5**) for their *in-silico* pharmacokinetics parameters and pre-formulation studies. Our data suggest that most of the compounds are biologically active and follow the Lipinski Rule having high log *P* value, indicating that they are lipophilic. Further, the skin permeation values obtained from the software also support the observations. So, future research on nano-gel formulation of HEFeAP for therapeutic use may elicit better efficacy. These results suggest further *in-vivo* and clinical studies for better insight into *A. paniculata* for mitigation of ailments related to inflammatory pain and auto-immune disorders.

Conclusions

1 This study explored the bioactivity and mechanistic insights of *A. paniculata* flower, traditionally
2 used as a dental pain reliever, coupled with anti-inflammatory potential including a protective role
3 against RA and α -amylase inhibition relevant to diabetes using *ex-vivo* and *in-vitro* studies. The
4 investigation included extraction, physicochemical standardization, phytochemical profiling, *in-*
5 *vitro* antioxidant, anti-inflammatory followed by *ex-vivo* fd-FLS cells growth inhibition in RA and
6 α -amylase inhibition relevant to hypoglycaemic activities, molecular docking, and molecular
7 dynamics simulation. Our results authorize the traditional uses of *A. paniculata*, demonstrating its
8 anti-inflammatory activity against inflammatory models. The presence of various bioactive
9 compounds, confirmed by FTIR, TLC, LC-MS, suggests the potential pharmacological activities
10 of the flower extract. The presence of local anesthetic alkyl-amide compounds such as spilanthol,
11 (2*E*,7*Z*,9*E*)-*N*-isobutylundeca-2,7,9-trienamide, (*E*)-*N*-isobutylundeca-2-ene-8,10-dynamide, and
12 flavonoids, tri-terpenoids and steroids may be attributed for the noteworthy display of anti-
13 inflammatory effect through various mechanisms including the downregulation of inflammatory
14 markers and arresting proinflammatory molecules. Significant inhibition of fd-FLS cells treated
15 by bioactive *A.a paniculata* may alleviate the RA disease progression arresting the expression of
16 specific inflammatory cytokine of IL-17a signalling^[104]. The *in-vitro* studies demonstrated its
17 significant antioxidant and anti-inflammatory activities and it may support its significant potential
18 as an anti-inflammatory phytopharmaceutical development. Further, a molecular docking study
19 with LOX-5 and phospholipase A2, key enzymes involved in inflammation, may authenticate the
20 inhibitory activities of phyto-compounds present in HEFeAP against inflammatory pain. For the
21 first time, the current study unfolded the significant growth inhibitory role of bioactive *Acmella*
22 *paniculata* flower extract against RA diseases progressing fd-FLS cells. It also disclosed the α -
23 amylase inhibitory activity that may suggest the prevention of hyperglycaemic syndrome in RA

patients. The present results may emphasize the need for future *in-vivo* preclinical and clinical studies on the therapeutic potential of *A. paniculata*. Overall, this research shares valuable insights into the bio-medicinal activity of HEFeAP, its chemistry, and molecular mechanism, paving the way for further exploration and development of novel anti-inflammatory agents for the management of inflammatory disorders followed by industrial scale-up for phytopharmaceutical polyherbal formulations development using HEFeAP as an active medicament.

Acknowledgments

The authors thank the Indian Institute of Technology-Kharagpur and CSIR-Indian Institute of Chemical Biology, Kolkata for the analytical experiments. The authors immensely thank the Indian Council of Medical Research (Ref. 59/03//BMS/2020) for financially supporting the research studies. Lutfun Nahar gratefully acknowledges the financial support of the European Regional Development Fund - Project ENOCH (No. CZ.02.1.01/0.0/0.0/16_019/0000868) and the Czech Agency Grants - Project 23-05474S and Project 23-05389S.

Declarations

Ethics Approval: Human ethical clearance was taken with prior approval (IPGME&R/IEC/2023/980; dated 7/10/2023)

Consent to Participate Not applicable.

Consent for Publication: Not applicable.

Author Contribution: AMAI—Writing an original draft, the methodology adopted, review, and, interpretation.

ABS-execution of few experiments.

AH- supervision of LC-MS and interpretation

AS—execution of two experiments and its analysis,

BSU-reviewing, few analyses.

DC- overall supervision, editing, guiding

LN-review and editing

ND-formal analysis of spectra of FTIR

PB-molecular docking

PG-clinical study analysis and supervision
PP=conceptualization, designing, execution, supervision, analysis, editing, reviewing
RP- FTIR study
RS- LC-MS study
SB-formal analysis and review
SDS-overall review and interpretation.
SKN-formal analysis and review of LC-MS
SS- molecular dynamics study

The results were evaluated by all authors and the final version of the manuscript was approved.

Conflict of Interest: The authors declare no competing interests

Funding: The research study was sponsored by The Indian Council of Medical Research funded Research project (Ref. 59/03//BMS/2020).

Data and materials: Data and materials will be provided upon request

References

- [1] M. Eddouks, D. Chattopadhyay, V. De Feo, W. C. Cho, *Evidence-Based Complementary and Alternative Medicine* **2012**, 2012, 1–2.
- [2] R. Rupani, A. Chavez, *Clin Dermatol* **2018**, 36, 306–309.
- [3] S. Chandra, K. Patwardhan, *J Ayurveda Integr Med* **2018**, 9, 143–150.
- [4] H. Tag, P. Kalita, P. Dwivedi, A. K. Das, N. D. Namsa, *J Ethnopharmacol* **2012**, 141, 786–795.
- [5] S. S. Ningthoujam, A. Das Talukdar, K. S. Potsangbam, M. D. Choudhury, *J Ethnopharmacol* **2013**, 147, 136–147.
- [6] R. Gogoi, R. Loying, N. Sarma, S. Munda, S. Kumar Pandey, M. Lal, *Ind Crops Prod* **2018**, 125, 131–139.
- [7] S. Dubey, S. Maity, M. Singh, S. A. Saraf, S. Saha, *Adv Pharmacol Sci* **2013**, 2013, 1–9.
- [8] R. Panmei, P. R. Gajurel, B. Singh, *J Ethnopharmacol* **2019**, 235, 164–182.
- [9] T. G. G. Uthpala, S. B. Navaratne, *Food Reviews International* **2021**, 37, 399–414.
- [10] Y. Bibi, *Med Discoveries* **2023**, 2, DOI 10.52768/2993-1142/1036.
- [11] S. Konsam, B. Thongam, A. K. Handique, *J Ethnobiol Ethnomed* **2016**, 12, 11.
- [12] C. P. Kala, *J Ethnobiol Ethnomed* **2005**, 1, 11.
- [13] J. Paulraj, R. Govindarajan, P. Palpu, *Adv Pharmacol Sci* **2013**, 2013, 1–22.
- [14] S. Paul, S. Sarkar, T. Dutta, S. Bhattacharjee, *J Intercult Ethnopharmacol* **2016**, 5, 257.

- 1 [15] R. Abdul Rahim, P. A. Jayusman, N. Muhammad, N. Mohamed, V. Lim, N. H. Ahmad, S. Mohamad,
2 Z. A. Abdul Hamid, F. Ahmad, N. Mokhtar, A. N. Shuid, I. N. Mohamed, *Int J Environ Res Public*
3 *Health* **2021**, *18*, 3532.
- 4 [16] B. Gorain, V. Karmakar, B. Sarkar, M. Dwivedi, J. T. L. Leong, J. H. Toh, E. Seah, K. Y. Ling, K. Y. Chen,
5 H. Choudhury, M. Pandey, *Int J Biol Macromol* **2023**, *253*, 126623.
- 6 [17] P. J. MacMahon, S. J. Eustace, E. C. Kavanagh, *Radiology* **2009**, *252*, 647–661.
- 7 [18] S. Priya, G. Singhvi, *Biomedicine & Pharmacotherapy* **2022**, *155*, 113717.
- 8 [19] S. Boccella, C. Mattia, M. Perrone, A. M. Morace, E. Karabacak, F. Guida, S. Maione, L. Luongo,
9 *Phytotherapy Research* **2024**, *38*, 1731–1734.
- 10 [20] B. Halliwell, *Nutr Rev* **2012**, *70*, 257–265.
- 11 [21] A. K. Garg, B. B. Aggarwal, *Mol Immunol* **2002**, *39*, 509–517.
- 12 [22] J. L. Evans, I. D. Goldfine, B. A. Maddux, G. M. Grodsky, *Endocr Rev* **2002**, *23*, 599–622.
- 13 [23] R. Tundis, M. R. Loizzo, F. Menichini, *Mini-Reviews in Medicinal Chemistry* **2010**, *10*, 315–331.
- 14 [24] F. Salaffi, G. Giacobazzi, M. Di Carlo, *Pain Res Manag* **2018**, *2018*, 1–14.
- 15 [25] A. Zhang, Y. C. Lee, *Curr Osteoporos Rep* **2018**, *16*, 603–610.
- 16 [26] S. Safiri, A. A. Kolahi, D. Hoy, E. Smith, D. Bettampadi, M. A. Mansournia, A. Almasi-Hashiani, A.
17 Ashrafi-Asgarabad, M. Moradi-Lakeh, M. Qorbani, G. Collins, A. D. Woolf, L. March, M. Cross, *Ann*
18 *Rheum Dis* **2019**, *78*, 1463–1471.
- 19 [27] S. Laeeq, T. Reghefaoui, H. Zahoor, J. H. Yook, M. Rizwan, N. ul ain Shahid, N. Naguit, R. Jakkoju, L.
20 Mohammed, *Musculoskeletal Care* **2022**, *20*, 471–486.
- 21 [28] A. A. Aljohani, Y. S. Alqarni, M. N. Alrashidi, M. H. Aljuhani, S. A. Shehata, M. K. El-Kherbetawy, K.
22 Prabahar, R. Alshaman, A. Alattar, A. M. N. Helaly, H. Ateyya, E. A. Ismail, S. A. Zaitone, *Toxics*
23 **2022**, *10*, 274.
- 24 [29] M. H. Schiff, A. Whelton, *Semin Arthritis Rheum* **2000**, *30*, 196–208.
- 25 [30] S. Singh, N. Tiwary, N. Sharma, T. Behl, A. Antil, Md. Anwer, S. Ramniwas, M. Sachdeva, G.
26 Elossaily, M. Gulati, S. Ohja, *Pharmaceuticals* **2024**, *17*, 248.
- 27 [31] H. Li, R. Gou, J. Liao, Y. Wang, R. Qu, Q. Tang, J. Gan, L. Zou, S. Shi, *Acta Materia Medica* **2023**, *2*,
28 DOI 10.15212/AMM-2022-0039.
- 29 [32] J. W. Liew, M. M. Ward, J. D. Reveille, M. Weisman, M. A. Brown, M. Lee, M. Rahbar, S. R.
30 Heckbert, L. S. Gensler, *Arthritis Care Res (Hoboken)* **2020**, *72*, 1645–1652.
- 31 [33] F. W. D. Tai, M. E. McAlindon, *Clinical Medicine* **2021**, *21*, 131–134.
- 32 [34] S. Tsujimoto, S. Mokuda, K. Matoba, A. Yamada, K. Jouyama, Y. Murata, Y. Ozaki, T. Ito, S. Nomura,
33 Y. Okuda, *PLoS One* **2018**, *13*, e0200023.

- 1 [35] M. Drina, *Aust Prescr* **2017**, *40*, 91–93.
- 2 [36] M. Akram, M. Daniyal, S. Sultana, A. Owais, N. Akhtar, R. Zahid, F. Said, A. Bouyahya, E.
3 Ponomarev, M. Ali Shariat, M. Thiruvengadam, *Clinica Chimica Acta* **2021**, *512*, 142–155.
- 4 [37] B. Wang, L. Wu, J. Chen, L. Dong, C. Chen, Z. Wen, J. Hu, I. Fleming, D. W. Wang, *Signal Transduct*
5 *Target Ther* **2021**, *6*, 94.
- 6 [38] L. Deb, S. Laishram, N. Khumukcham, D. Ningthoukhongjam, S. S. Nameirakpam, A. Dey, D. S.
7 Moirangthem, N. C. Talukdar, T. R. Ningthoukhongjam, *J Ethnopharmacol* **2015**, *169*, 387–400.
- 8 [39] J. Okais, F. Fayad, R. Baddoura, O. A. Tabesh, K. Aouad, M. Ghoubar, N. Sunna, E. Mysler, *Open*
9 *Rheumatol J* **2022**, *16*, DOI 10.2174/18743129-v16-e221205-2022-13.
- 10 [40] S. Georgin-Lavialle, A. Fayand, F. Rodrigues, C. Bachmeyer, L. Savey, G. Grateau, *Presse Med* **2019**,
11 *48*, e25–e48.
- 12 [41] L. Jamir, S. K. Ghosh, H. R. Bhat, S. K. Chaudhary, A. Shakya, *South African Journal of Botany* **2023**,
13 *156*, 223–233.
- 14 [42] M. U. Farooq, M. W. Mumtaz, H. Mukhtar, U. Rashid, M. T. Akhtar, S. A. Raza, M. Nadeem, *Sci Rep*
15 **2020**, *10*, 3530.
- 16 [43] T. Lefebvre, E. Destandau, E. Lesellier, *J Chromatogr A* **2021**, *1635*, 461770.
- 17 [44] E. Gligorić, R. Igić, B. Teofilović, N. Grujić-Letić, *Int J Mol Sci* **2023**, *24*, 11848.
- 18 [45] S. Gupta, R. Acharya, R. V. Gamit, V. J. Shukla, *Journal of Indian System of Medicine* **2021**, *9*, 171–
19 174.
- 20 [46] A. E. El-Hadary, M. Taha, *Food Sci Nutr* **2020**, *8*, 1798–1811.
- 21 [47] F. Siano, E. Vasca, G. Picariello, *Food Chem* **2022**, *370*, 130990.
- 22 [48] N. Siddiqui, A. Rauf, A. Latif, Z. Mahmood, *J Taibah Univ Med Sci* **2017**, *12*, 360–363.
- 23 [49] J. Loum, R. Byamukama, P. A. G. Wanyama, *Chemistry Africa* **2020**, *3*, 449–455.
- 24 [50] T. K. Patle, K. Shrivastava, R. Kurrey, S. Upadhyay, R. Jangde, R. Chauhan, *Spectrochim Acta A Mol*
25 *Biomol Spectrosc* **2020**, *242*, 118717.
- 26 [51] H. Wagner, S. Bladt, *Plant Drug Analysis*, Springer Berlin Heidelberg, Berlin, Heidelberg, **1996**.
- 27 [52] K. Sowndhararajan, S. C. Kang, *Saudi J Biol Sci* **2013**, *20*, 319–325.
- 28 [53] M. J. Rodrigues, A. Soszynski, A. Martins, A. P. Rauter, N. R. Neng, J. M. F. Nogueira, J. Varela, L.
29 Barreira, L. Custódio, *Ind Crops Prod* **2015**, *77*, 315–322.
- 30 [54] S. Keser, S. Celik, S. Turkoglu, Ö. Yilmaz, I. Turkoglu, *Hydrogen Peroxide Radical Scavenging and*
31 *Total Antioxidant Activity of Hawthorn*, **2012**.
- 32 [55] I. Ramli, S. Zerizer, L. Gali, F. Z. Sakhri, Z. Kabouche, D. Usai, L. A. Sechi, *The Journal of Infection in*
33 *Developing Countries* **2022**, *16*, 1064–1074.

- 1 [56] P. Palit, S. C. Mandal, B. Bhunia, *Int Immunopharmacol* **2016**, *41*, 106–115.
- 2 [57] P. Palit, D. Mukherjee, P. Mahanta, M. Shadab, N. Ali, S. Roychoudhury, Md. Asad, S. C. Mandal,
3 *Inflammopharmacology* **2018**, *26*, 235–250.
- 4 [58] S. Misra, A. M. A. Ikbal, D. Bhattacharjee, M. Hore, S. Mishra, S. Karmakar, A. Ghosh, R. Srinivas, A.
5 Das, S. Agarwal, K. Das Saha, P. Bhardwaj, I. B. Ubhadia, P. Ghosh, S. De, O. N. Tiwari, D.
6 Chattopadhyay, P. Palit, *J Food Biochem* **2022**, *46*, DOI 10.1111/jfbc.14487.
- 7 [59] A. A. Bhat, N. Tandon, I. Singh, *Heliyon* **2024**, *10*, e39444.
- 8 [60] K. A. Akinyede, H. A. Oyewusi, G. D. Hughes, O. E. Ekpo, O. O. Oguntibeju, *Molecules* **2021**, *27*,
9 155.
- 10 [61] A. Jain, R. Kumar, P. Mothsra, A. K. Sharma, A. K. Singh, Y. Kumar, *Curr Top Med Chem* **2024**, *24*,
11 1829–1855.
- 12 [62] *JAMA* **2013**, *310*, 2191.
- 13 [63] M. Neidhart, C. A. Seemayer, K. M. Hummel, B. A. Michel, R. E. Gay, S. Gay, *Arthritis Rheum* **2003**,
14 *48*, 1873–1880.
- 15 [64] T. Mosmann, *J Immunol Methods* **1983**, *65*, 55–63.
- 16 [65] P. Manna, M. Sinha, P. C. Sil, *Toxicology* **2009**, *257*, 53–63.
- 17 [66] E. Neumann, S. Lefèvre, B. Zimmermann, S. Gay, U. Müller-Ladner, *Trends Mol Med* **2010**, *16*,
18 458–468.
- 19 [67] R. K. Chauhan, P. K. Sharma, S. Srivastava, *Curr Drug Res Rev* **2021**, *13*, 130–139.
- 20 [68] A. Alghasham, Z. Rasheed, *Autoimmunity* **2014**, *47*, 77–94.
- 21 [69] M. Feldmann, S. R. N. Maini, *Immunol Rev* **2008**, *223*, 7–19.
- 22 [70] K. L. B. dos Santos, J. N. Cruz, L. B. Silva, R. S. Ramos, M. F. A. Neto, C. C. Lobato, S. S. B. Ota, F. H.
23 A. Leite, R. S. Borges, C. H. T. P. da Silva, J. M. Campos, C. B. R. Santos, *Molecules* **2020**, *25*, 1245.
- 24 [71] J. J. Sahayarayan, K. S. Rajan, R. Vidhyavathi, M. Nachiappan, D. Prabhu, S. Alfarraj, S. Arokiyaraj,
25 A. N. Daniel, *Saudi J Biol Sci* **2021**, *28*, 400–407.
- 26 [72] Y. Xiao, R. J. Woods, *J Chem Theory Comput* **2023**, *19*, 5503–5515.
- 27 [73] J. P. Pederson, J. G. McDaniel, *J Chem Phys* **2022**, *156*, DOI 10.1063/5.0087386.
- 28 [74] L. López-Villellas, C. C. K. Mikkelsen, J. J. Galano-Frutos, S. Marco-Sola, J. Alastruey-Benedé, P.
29 Ibáñez, M. Moretó, J. Sancho, P. García-Risueño, **2022**, DOI 10.1101/2022.09.28.509839.
- 30 [75] A. M. A. Dias, P. Santos, I. J. Seabra, R. N. C. Júnior, M. E. M. Braga, H. C. de Sousa, *J Supercrit*
31 *Fluids* **2012**, *61*, 62–70.
- 32 [76] J. V. Franca, M. S. R. Queiroz, B. P. do Amaral, N. K. Simas, N. C. B. da Silva, I. C. R. Leal, *Food*
33 *Research International* **2016**, *89*, 781–789.

- 1 [77] L. Pierre Luhata, T. Usuki, *Bioorg Med Chem Lett* **2022**, 59, 128528.
- 2 [78] S. Samarpita, R. Ganesan, M. Rasool, *Toxicol Appl Pharmacol* **2020**, 391, 114917.
- 3 [79] A. Daina, O. Michielin, V. Zoete, *Sci Rep* **2017**, 7, 42717.
- 4 [80] E. Bakondi, S. B. Singh, Z. Hajnády, M. Nagy-Pénzes, Z. Regdon, K. Kovács, C. Hegedűs, T. Madácsy,
5 J. Maléth, P. Hegyi, M. Á. Demény, T. Nagy, S. Kéki, É. Szabó, L. Virág, *Int J Mol Sci* **2019**, 20, 4308.
- 6 [81] J. da S. Moura, E. de M. Gemaque, C. E. Bahule, L. H. da S. Martins, R. C. Chisté, A. S. Lopes,
7 *Sustainability* **2023**, 15, 15231.
- 8 [82] H. Liao, J. Ye, L. Gao, Y. Liu, *Biomedicine & Pharmacotherapy* **2021**, 133, 110917.
- 9 [83] J. N. Sharma, A. Al-Omran, S. S. Parvathy, *Inflammopharmacology* **2007**, 15, 252–259.
- 10 [84] H. Liu, C. Wang, *J Ethnopharmacol* **2022**, 282, 114642.
- 11 [85] S. Dwivedi, E. J. Testa, J. M. Modest, Z. Ibrahim, J. A. Gil, *R I Med J (2013)* **2020**, 103, 32–36.
- 12 [86] W. Wang, W. Gu, C. He, T. Zhang, Y. Shen, Y. Pu, *J Ethnopharmacol* **2022**, 291, 115085.
- 13 [87] A. Lukasz, C. Hillgruber, H. Oberleithner, K. Kusche-Vihrog, H. Pavenstädt, A. Rovas, B. Hesse, T.
14 Goerge, P. Kümpers, *Cardiovasc Res* **2017**, 113, 671–680.
- 15 [88] M. C. Recio, R. M. Giner, L. Uriburu, S. Máñez, M. Cerdá, J. R. De La Fuente, J. L. Ríos, *Life Sci* **2000**,
16 66, 2509–2518.
- 17 [89] P. C. Calder, *Eur J Pharmacol* **2011**, 668, S50–S58.
- 18 [90] A. Muñoz, M. Costa, *Oxid Med Cell Longev* **2013**, 2013, 1–11.
- 19 [91] N. N. Okoye, D. L. Ajaghaku, H. N. Okeke, E. E. Ilodigwe, C. S. Nworu, F. B. C. Okoye, *Pharm Biol*
20 **2014**, 52, 1478–1486.
- 21 [92] X. Shi, J. Guo, S. Saravelos, X. Huang, E. Xia, L. Feng, T.-C. Li, *Hum Fertil* **2023**, 26, 742–756.
- 22 [93] R. Virmani, K. Pathak, in *Herbal Bioactive-Based Drug Delivery Systems*, Elsevier, **2022**, pp. 63–92.
- 23 [94] P. Stanely Mainzen Prince, S. Rajakumar, K. Dhanasekar, *Eur J Pharmacol* **2011**, 668, 233–240.
- 24 [95] N. Muvhulawa, P. V. Dlodla, K. Ziqubu, S. X. H. Mthembu, F. Mthiyane, B. B. Nkambule, S. E.
25 Mazibuko-Mbeje, *Pharmacol Res* **2022**, 178, 106163.
- 26 [96] H. J. Ahn, H. J. You, M. S. Park, Z. Li, D. Choe, T. V. Johnston, S. Ku, G. E. Ji, *RSC Adv* **2020**, 10, 5339–
27 5350.
- 28 [97] X. Wang, X. Zhang, B. Zheng, N. Hu, W. Xie, K. Row, *Nat Prod Res* **2015**, 29, 756–763.
- 29 [98] V. M. Young, M. Toborek, F. Yang, C. J. McClain, B. Hennig, *Metabolism* **1998**, 47, 566–572.
- 30 [99] S. D. Pauls, L. A. Rodway, T. Winter, C. G. Taylor, P. Zahradka, H. M. Aukema, *J Nutr Biochem* **2018**,
31 57, 121–129.

- 1 [100] D. Liang, F. Li, Y. Fu, Y. Cao, X. Song, T. Wang, W. Wang, M. Guo, E. Zhou, D. Li, Z. Yang, N. Zhang,
2 *Inflammation* **2014**, 37, 214–222.
- 3 [101] N. El Omari, I. Jaouadi, M. Lahyaoui, T. Benali, D. Taha, S. Bakrim, N. El Menyiy, F. El Kamari, G.
4 Zengin, S. P. Bangar, J. M. Lorenzo, M. Gallo, D. Montesano, A. Bouyahya, *Applied Sciences* **2022**,
5 12, 5779.
- 6 [102] J. Korbecki, K. Bajdak-Rusinek, *Inflammation Research* **2019**, 68, 915–932.
- 7 [103] Y. Wang, S. Chen, K. Du, C. Liang, S. Wang, E. Owusu Boadi, J. Li, X. Pang, J. He, Y. Chang, *J*
8 *Ethnopharmacol* **2021**, 279, 114368.
- 9 [104] S. Samarpita, R. Ganesan, M. Rasool, *Toxicol Appl Pharmacol* **2020**, 391, 114917.

10

11