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Extraction of bioactive compounds from *Limonia acidissima* to explore their therapeutic potential

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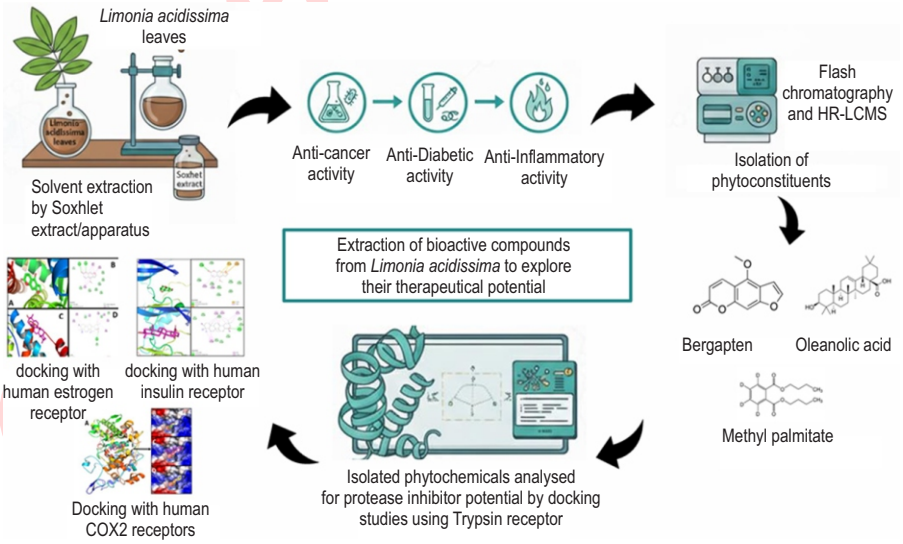
Abstract

Aim: This study evaluates the bioactivity and molecular mechanisms of *Limonia acidissima* (wood apple) leaf extracts across multiple therapeutic targets.

Methodology: Solvent extracts of *L. acidissima* (ethanol 70%, ethyl acetate 70%, and methanol 70%) were assessed for bioactivity screening, antimicrobial profiling, phytochemical characterization, and molecular docking analysis.

Results: Ethyl acetate extract exhibited higher protease inhibition (67.45±1.88%, $p<0.001$) and broad-spectrum antimicrobial activity. Ethanol extract showed high anti-diabetic potential (75.22±1.26% α-amylase inhibition, statistically comparable to acarbose, $p>0.05$) with significant dose-dependent anticancer activity against MCF-7 breast cancer cells (linear slope = 0.311% μl^{-1}, $R^2 = 0.976$, $p < 0.001$). Methanol extract showed the highest antioxidant activity (78.15±0.99%, higher than ascorbic acid control by 16%, $p<0.01$). Flash chromatography identified seven chemical entities, of which three—bergapten, methyl palmitate, and oleanolic acid—were selected for molecular docking analysis. Molecular docking revealed oleanolic acid as a promising compound with strong binding affinities across therapeutic targets: trypsin (-8.61 kcal mol^{-1}), estrogen receptor (-6.80 kcal mol^{-1}), and insulin receptor (-6.99 kcal mol^{-1}). Methyl palmitate showed the strongest COX-2 affinity (-8.07 kcal mol^{-1}) with 15 distinct interaction points.



Interpretation: The findings suggest the reactivity and stability of extracted bioactive compounds as a protease inhibitor and their potential to develop effective insecticidal agents as well as therapeutic candidate. Exploring the role of protease inhibitors as a biocontrol agent can be an effective way to replace hazardous chemical pesticides producing harmful effect on the environment.

Key words: Ethnopharmacology, *Limonia acidissima*, Molecular docking, Multi-target therapeutics, Protease inhibitors

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Introduction

In 1972, the wound-inducible protease inhibitors (PIs) revolutionized the science of plant-insect interactions, which function by blocking the digestive enzymes in the guts of herbivorous insects (Green and Ryan, 1972). Plant PIs inhibit the action of insect gut proteases, serve as natural agents to control insect populations, and are critical plant defence mechanisms that are effective against phytophagous insects and microorganisms (Napoleão *et al.,* 2019). In response to an attack of the proteinases produced by phytopathogenic bacteria, plants manufactured inhibitory polypeptides can reduce the enzyme activity (Ryan, 1989). This is evident from tomato plants infected with *Phytophthora infestans* which exhibited an increase in the levels of trypsin and chymotrypsin inhibitors. These inhibitors play an important role in providing resistance to plant PIs (Woloshuk *et al.,* 1991). Later, a type of PI that resists the infection by *Phytophthora infestans* zoospores was found in potato tubers belonging to the family of soybean Kunitz-inhibitors (Valueva *et al.,* 1998). Such plant-derived PIs may be either small molecular weight proteins or certain bioactive compounds that interact with proteases to suppress their activity. These inhibitors are used by plants to regulate a variety of physiological functions and to defend themselves against diseases and pest insects.

PIs are an integral defence mechanism against many herbivores and exhibit bactericidal and fungicidal effects on a variety of pathogenic organisms (Polya, 2003, Kim *et al.,* 2009; Kuhar *et al.,* 2013). As a result, these plant-based PIs can be efficiently used as target molecules for drug development for effective disease treatment (Barbole *et al.,* 2022). Animal studies and cell line experiments have revealed promising results of such plant-based therapeutics in treating a wide variety of health problems like arthritis, pancreatitis, hepatitis, cancer, AIDS, thrombosis, cardiovascular, and autoimmune diseases. Generally, plant therapeutic compounds in the form of inhibitor molecules are non-nutritional chemical substances that delay or inhibit the catalytic action of enzymes involved in gastric, colorectal, breast, and lung cancer (Hellinger and Gruber, 2019; Mosolov and Valueva, 2005).

Fruit extracts of *Limonia acidissima* have been reported to possess therapeutic properties enriched with phytoconstituents like saponins, flavonoids, tannins, alkaloids and glycosides (Thakur *et al.,* 2020; Saima *et al.,* 2000). Extracts from unripe fruits are often used as astringents, tonics, antiscorbutics, and alexiformics. Pharmacological activities of *L. acidissima* have been documented. It has been demonstrated to have antitumour, antimicrobial, antidiabetic, antipyretic, anti-inflammatory, analgesic, antioxidant, hepatoprotective, antimutagenic and antimalarial activities (Thakur *et al.,* 2020; Saima *et al.,* 2000). These extracts also act as an effective insecticides and natural mosquito repellents, thereby serving as inexpensive, effective and environmentally sustainable solutions which will also reduce the use of synthetic pesticides (Chellappandian *et al.,* 2022; Qotrinnada *et al.,* 2024). In view of the above, this study was conducted to explore the therapeutic potential of bioactive compounds, particularly trypsin, derived from *L. acidissima* and evaluate their potential to inhibit protease activity. Further, tests were conducted to investigate the efficacy of these phytoconstituents as therapeutics for anti-cancer, anti-diabetic, anti-inflammatory, anti-oxidant and antimicrobial activities.

Materials and Methods

Collection of plant material: Fresh leaves of *Limonia acidissima* were collected from Shrushti Rop Vatika, Ravet, Pune. *Limonia acidissima* commonly known as elephant apple or Indian wood apple has Taxonomy ID: 159053. It has heterotypic synonym: *Feronia limonia* (L.) Swingle. It is a fruit yielding tree that belongs to family Rutaceae. It is found in India, Sri Lanka, Pakistan, Indo-China, java and Malesia.

Selection of solvent systems and trypsin compatibility assessment: Trypsin was used as a protease. Six solvent systems, *i.e.,* butane (70%, 100%), diethyl ether (70%), ethanol (70%), ethyl acetate (70%, 100%), formaldehyde (70%), and methanol (70%), were used to analyze the compatibility of trypsin with respect to solvents. Standard bovine trypsin (1 mg ml^{-1}) was added to each solvent system separately and incubated at 37°C for 30 min to determine the stability of enzyme activity.

Extraction of bioactive compounds bythe Soxhlet method: The plant leaves (approx. 12 g) were washed, dried, and crushed using an appropriate solvent to form a fine paste. Further extraction was carried out using a Soxhlet apparatus, with 120 mL of each organic solvent (ethanol 70%, ethyl acetate 70%, and methanol 70%). The extraction was carried out at 60-65°C for 6 hr at a constant extraction rate of 4-6 cycles per hour. The hot extract was cooled to remove volatile organic compounds by vapor permeation and was preserved at 4°C. The concentrated extracts were stored at 4°C in opaque amber bottles until further analysis. The extract was further analyzed for protease inhibition activity using a standard inhibition assay, following the original protocol as described by Rasul (2018). Based upon different polarity, and compatibility of bioactive compounds, ethanol and methanol being polar solvents while ethyl acetate being moderately polar solvent were used for the extraction process. Butane, a highly nonpolar solvent, diethyl ether with highly flammable and volatile nature and formaldehyde with chemically reactive nature were not suitable for extraction of components.

Estimation of inhibitory potential against trypsin: All the three solvent extracts were prepared from *L. acidissima* leaves using ethanol, ethyl acetate and methanol by soxhlet method. All the three extracts were further assessed to evaluate the inhibitory potential against bovine pancreatic trypsin (T1426-100MG, Sigma-Aldrich), by Folin-Ciocalteu colorimetric assay (Lowry *et al.,* 1951). The percentage inhibition was calculated by the formula:

Percentage inhibition = [(Absorbance of_control–Absorbance of sample) / Absorbance of control] × 100

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Biochemical characterization of phytoconstituents: The plant extract, prepared following the Soxhlet extraction method, was further evaluated for the following therapeutic activities:

Anticancer activity assay (MTT Assay): Human breast cancer cells (1×10^4 cells ml^{-1}) (MCF-7, obtained from NCCS, Pune) were incubated in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin antibiotic solution for 24 hr at 37°C in a 5% CO_2 atmosphere. Thereafter, MCF7 cells (100 µl) and heat-stable plant extracts (10, 40, 100 µl) were poured into 96-well microplates. 5-Fluorouracil (55-FU) was used as a standard anticancer drug. Control wells were also prepared using DMSO and an untreated cell line. All the samples were incubated in a CO_2 incubator (Thermo Scientific BB150) with 5% CO_2 for 24 hr at 37°C. Controls were monitored to analyze the percentage of live cells. The medium was completely removed, and cells were incubated along with 20 µl of MTT reagent (5mg ml^{-1} PBS) for 4hr at 37°C in a CO_2 incubator. After the formation of formazan crystal and the complete removal of the medium, 200 µl of DMSO was added to the wells and incubated at 37°C. Optical density was read at 550 nm using an ELISA plate reader (Thermo Fisher Scientific, India). Cell viability was determined following the methods of Mosmann (Mosmann, 1983).

Determination of anti-inflammatory and antioxidant activities: Anti-inflammatory activity was determined as per the protocol by Mizushima and Kobayashi (1968). The anti-inflammatory activity was evaluated by egg albumin-induced denaturation assay. A reaction mixture was prepared using 0.4 ml of egg albumin, 5.6 ml of PBS (pH 6.4), and 100 µl plant extract (1 mg ml^{-1}) and incubated at 37°C for 15 min, followed by heating at 70°C for 5 min. A control was prepared with distilled water, and diclofenac sodium (0.5 mM) served as the standard sample. The absorbance was read at 660 nm. The anti-inflammatory activity (% inhibition of protein denaturation) was calculated as indicated above. For antioxidant activity, a reaction mixture was prepared with solvent extracts and 2,2-diphenyl-1-picrylhydrazyl (DPPH), and the mixture was incubated for 30 min in dark (Brand-Williams *et al.*, 1995). The absorbance was read at 490 nm on an ELISA plate reader. L-Ascorbic acid (1 mg ml^{-1}) was used as a control.

Antibacterial and antifungal activities: Four bacterial strains—Bacillus subtilis (MTCC 121), *Staphylococcus aureus* (MTCC 96), *Salmonella typhi* (MTCC 98), and *Shigella* spp. (MTCC 3385)—were obtained from the Institute of Microbial Technology (IMTCC), Chandigarh. A 100 µl bacterial inoculum was spread onto the nutrient agar medium. Plant extracts prepared in DMSO by the Soxhlet method were added to the prepared wells and incubated at 37°C for 24 hr (Luque de Castro and Garcia-Ayuso, 1998) and (Parente *et al.*, 1995). Streptomycin and DMSO (100%) were used as positive and negative controls. The zone of inhibition (mm) was measured. Similarly, for antifungal activity, two fungal strains—*Candida albicans* (MTCC 227) and *Aspergillus niger* (MTCC 282), procured from the Institute of Microbial Technology (IMTCC), Chandigarh, were used for antifungal assay. 100 µl of fungal inoculum was spread evenly on sterile Sabouraud dextrose agar (Hi-Media, Mumbai) plates. Wells were created, and 100 µl of plant extracts was added with further incubation at 37°C for 24 hr. Fluconazole and DMSO (100%) served as positive and negative controls. The zones of inhibition were measured (Denkova *et al.*, 2017).

Anti-diabetic activity assay: The anti-diabetic potential was determined by assessing the α-amylase inhibition activity. A reaction mixture with plant extract, fungal diastase enzyme (pH 6.9, 0.2 U m l^{-1}), and 1% starch solution was incubated for 10 min at 25°C. After incubation, the reaction was stopped by adding 3,5-dinitrosalicylic acid (DNSA) reagent, followed by heating at 100°C for 5 min (Bernfeld, 2017). The absorbance was recorded at 540 nm at room temperature. Acarbose served as a positive control. The percentage inhibition of α-amylase was calculated as:

$$\text{Inhibition (\%)} = [(\text{Absorbance\_Control} - \text{Absorbance\_Sample}) / \text{Absorbance\_Control}] \times 100$$

Flash chromatography and phytochemical isolation: On the basis of preliminary screening that showed superior biological activity, the ethanol extract was purified using flash chromatography, where a Combifash companion system (Septech Marketing Pvt. Ltd., India) equipped with a FLASH silica gel column (24 g, 40-60 µm) was used. Gradient elution was performed using hexane and ethyl acetate (100:0 to 0:100) at a flow rate of 20 ml min^{-1}.

High-resolution liquid chromatography-mass spectrometry analysis: HR-LCMS was performed on a Q-Exactive Plus Biopharma mass spectrometer (Thermo Scientific, USA) interfaced with a Hypersil Gold C18 column (3 µm, 100 × 2.1 mm). The mobile phase consisted of solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile), with a gradient program: 5-95% B over 30 min at a flow rate of 0.3 ml min-1. The mass spectrometer was run on a positive electrospray ionization (ESI+) mode with the following parameters: spray voltage 3.8 kV, capillary temperature 320°C, and mass range 100-1000 m/z. Compounds were identified as per their retention times, mass-to-charge ratios (m/z), and molecular formulas.

Molecular docking studies: Isolated phytochemicals from *L. acidissima* were analyzed for protease inhibitor potential by performing docking studies using trypsin receptor. To investigate the binding pattern of trypsin receptor with Bergapten, Methylpalmatate and Oleanolic acid, a molecular docking was performed using a AutoDock4 4.2.6 (Morris *et al.,* 1992) and target receptors: human trypsin (PDB ID: 1TRN), oestrogen receptor α (PDB ID: 1ERR), human insulin receptor tyrosine kinase domain (PDB ID: 1IRK), and cyclooxygenase-2 (COX-2, PDB ID: 1CX2). The atomic coordinates of Bergapten, Methyl palmatate and Oleanolic acid were produced using the Discovery Studio Visualizer (BIOVIA, 2016). Grid boxes of 60×60×60 were built around the active sites of trypsin. The docking conformations were generated using LGA, clustered using RMSD with a cutoff of

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4 Å, and selected based on binding interactions. Stable docked conformations of Bergapten, Methylpalmatate, and Oleanolic acid and their images were accessed by Discovery Studio visualizer (BIOVIA, 2016) and PyMol (DeLano, 2002).

Molecular docking with human oestrogen and insulin receptor: The binding modes of Bergapten and Oleanolic acid with the estrogen receptor were determined by performing molecular docking using Auto Dock 4. The anti-cancer activity of the above-mentioned phytocompounds was evaluated using the breast cancer cell line MCF-7. The atomic coordinates of Bergapten and Oleanolic acid were generated by Discovery Studio Visualizer (BIOVIA, 2016). Grid boxes were built around the active sites of the estrogen receptor. Docking was performed using LGA with 100 conformations. Anti-diabetic potential of Bergapten and Oleanolic acid was analyzed by molecular docking with human insulin receptors (1IRK.pdb) using Auto Dock 4. Insulin receptor is an important molecular target for the development of anti-diabetic drugs. The atomic coordinates of Bergapten and Oleanolicacid were generated with Discovery Studio Visualizer (BIOVIA, 2016). Grid boxes were built around the active sites of the insulin receptor. Receptor proteins are considered rigid, and Bergapten and Oleanolic acid are considered flexible molecules. Hundred conformations were clustered, and docking interactions were assessed with Discovery Studio Visualizer.

Molecular docking with human COX-2 receptors: Anti-inflammatory properties of Bergapten, Oleanolic-acid and Methyl palmitate were examined by performing molecular docking with the COX-2 receptor. The atomic coordinates of Bergapten, Oleanolic acid and Methyl palmitate were generated. The COX-2 enzymes are targets of non-steroidal anti-inflammatory drugs (Vane, 1971; Xie *et al.,* 1991).

Statistical analyses: All the experiments were conducted in triplicate. Data were expressed as mean ± S.D. Statistical analyses were carried out using One-way ANOVA to compare the bioactivity and zone of inhibition data across three solvent extraction systems. Pairwise comparisons were performed using Tukey's Honest Significant Difference (HSD) test to determine which groups differed significantly. Significance was set at α= 0.05. Statistical analyses were carried out in Microsoft Excel.

Results and Discussion

Protease inhibitors, being part of an important natural plant defence mechanism, protect plants from damage by bacteria and insects; thus, they are natural insecticides. Aqueous and alcoholic extracts of *Limonia acidissima* are also reported to have traditional medicinal properties and are widely used in Ayurveda for treating several ailments (Thakur *et al.,* 2020). *L. acidissima* belongs to the monotypic genus *Limonia,* family *Rutaceae*. They are widely found in India, and the use of extracts is a cost-effective, environmentally sustainable agent that can be used as insecticides and therapeutic agents (Wakchoure *et al.,* 2023). Solvent selection is an important factor in determining the phytochemical yield and bioactivity profile of herbal extracts. Different solvents exhibit differential affinities for bioactive compounds depending on their polarity. The use of multiple extraction solvents enables differential extraction of both polar and intermediate-polarity phytochemicals, yielding a more complete phytochemical profile and identifying the optimal solvent system for bioactivity recovery. Differential therapeutic potential of each extraction solvent is summarized in Table 1.

The ethyl acetate leaf extract of *L. acidissima* showed significantly higher protease inhibition (67.45%, $p < 0.001$) than methanol (45.12%) and ethanol (35.13%) extracts. This correlates with established literature that solvent polarity influences the selective extraction of compounds with aromatic rings and hydrophobic moieties. Plant-derived protease inhibitors also function as a natural insect defence mechanism, rendering *L. acidissima* as a potential biocontrol agent for sustainable pest management without synthetic insecticides (Singh *et al.*, 2020). Table 1 shows that the ethyl acetate extract achieved moderate anticancer activity against MCF-7 cells (26.08% inhibition at 80 µl). This was slightly higher than ethanol (22.89%) and significantly higher than methanol extract(4.60%). These differences were statistically significant ($p < 0.001$), thus indicating that solvent-dependent extraction strongly influences the anticancer potency of *L. acidissima* leaf constituents. Additional dose-response analysis was carried out, which showed

Table 1: Comprehensive bioactivity screening profile of *L. acidissima* leaf extracts

Bioactivity Assay	Positive Control	Ethanol Extract	Ethyl Acetate Extract	Methanol Extract	p-value
Protease inhibition (%)	—	35.13 ± 2.30^{a}	$67.45 \pm 1.88^{b***}$	45.12 ± 1.50^{c}	<0.001
Anticancer activity MCF-7 (% inhibition @ 80 µl-1)	5-FU: 43.42±1.00	22.89 ± 1.36^{a}	26.08 ± 0.86^{b}	4.60 ± 0.50^{c}	<0.001
Anti-diabetic α-Amylase Inhibition (%)	Acarbose: 77.79±1.08	75.22 ± 1.26^{a}	$42.37 \pm 1.16^{b***}$	69.72 ± 0.81^{c}	<0.001
Anti-inflammatory Activity (%)	Diclofenac: 34.08±0.59	26.19 ± 1.23^{a}	23.88 ± 1.34^{a}	8.50 ± 0.46^{b}	<0.001
Antioxidant DPPH Activity (%)	Ascorbic acid: 62.33±1.53	13.97 ± 0.69^{a}	34.84 ± 1.52^{b}	$78.15 \pm 0.99^{c**}$	<0.001

Data represent mean ± S.D. (n=3). Different superscript letters (^{a}, ^{b}, ^{c}) indicate statistically significant differences ($p < 0.05$) by One-way ANOVA and Tukey HSD post-hoc test. *** $p < 0.001$, ** $p < 0.01$, NS = Not Significant. 5-FU = 5-Fluorouracil.

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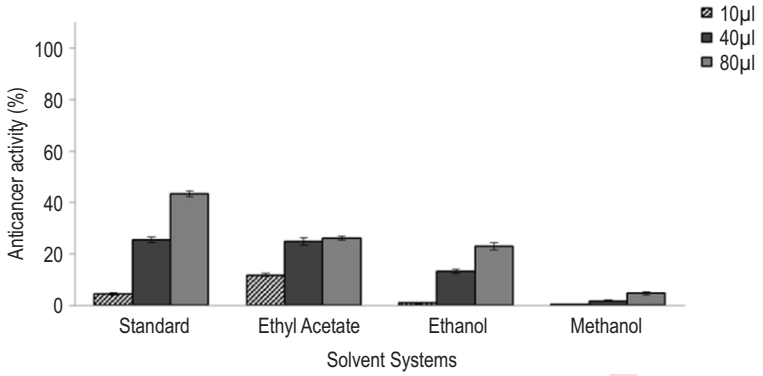

Fig. 1: Dose-dependent anticancer activity of *L. acidissima* leaf extracts against MCF-7 breast cancer cells.

that the ethyl acetate extract showed significant dose-dependent anticancer activity against MCF-7 breast cancer cells, with inhibition increasing from 11.79% at 10 µl to 24.81% at 40 µl and 26.08% at 80 µl (Fig. 1), respectively. Two-way ANOVA analysis revealed significant effects of both solvent system ($p < 0.001$) and extract concentration ($p < 0.001$), as well as a substantial solvent × dose interaction ($p < 0.001$). Linear regression analysis of ethyl acetate extract showed a significant, dose-dependent slope of 0.195% inhibition per µl ($R^2 = 0.75$, $p = 0.0026$), indicating a concentration-dependent cytotoxic mechanism. This is consistent with specific bioactive compound extraction rather than non-specific cellular toxicity. In comparison, the ethanol extract showed a moderate dose-dependent activity (0.87% at 10 µl, 13.29% at 40 µl, 22.89% at 80 µl; slope = 0.311% µl, $R^2 = 0.976$, $p < 0.001$), while methanol extract demonstrated a minimal anticancer potential (0.20% at 10 µl, 1.71% at 40 µl, 4.60% at 80 µl; slope = 0.063% µl, $R^2 = 0.991$, $p < 0.001$). Thus, the leaf extract of *L. acidissima* with ethyl acetate and ethanol showed more cytotoxicity against MCF-7 cells, where the inhibition of cell growth occured in a dose-dependent manner. Results indicate the presence of some active anticancer agents in leaf extract of *L. acidissima*. Leaves extracts of *I. purpurea* prepared with methanol and chloroform showed cytotoxic effect against A-549 and MDA-MB-23 cancer cell lines (Beheshti *et al.,* 2021). Methanol extract from *L. acidissima* fruit showed anticancer activity against SKBR3 and MDA-MB435 breast cancer cell lines (Pradhan *et al.,* 2012). Thus, for different plants with different plant parts like fruits and leaves, extraction of bioactive anticancer phytochemicals varies with the type of solvent. The findings of this study, proves that *L. acidissima* leaves can be used as a naturally available source of an anti-cancer therapeutic agent.

Notably, the ethanol extract exhibited exceptional anti-diabetic activity (75.22% α-amylase inhibition), which was statistically similar to the clinical standard acarbose (77.79, $p>0.05$) (Table 1). This adds evidence to previous studies that have reported antidiabetic potential of *L. acidissima* extracts. Alloxan-induced diabetic Wistar albino rats, when treated with methanolic extract of *Limonia acidissima* leaves exhibited a significant decrease in random blood sugar levels. This indicates the potential of *L. acidissima* leaves as a natural source of antidiabetic agents (Baheti *et al.,* 2023). Phytochemicals in *L. acidissima* leaves produce anti-diabetic effect by promoting insulin secretion and by improving the oral glucose tolerance. This also validates the traditional use of these extracts for diabetes management, suggesting improved tolerability as compared to synthetic α-glucosidase inhibitors that cause gastrointestinal side effects (Baheti *et al.,* 2023; Thakur *et al.,* 2020). Parallel results were noted for ethanolic extract (Gupta *et al.,* 2009) and methanolic extract (Priya *et al.,* 2012) of wood apple. On examining the anti-inflammatory properties of extract, it was observed that ethanol (26.19%) and ethyl acetate extracts (23.88%) demonstrated comparable and significantly higher anti-inflammatory activity in comparison to methanol (8.50%, $p<0.001$). This supports previously reported studies that have reported extract from wood apples modifying the NF-KB pathway, a key pathway in the inflammatory process and thus identifies, a natural anti-inflammatory agent (Patil *et al.,* 2025).

Notably, the methanol extract exhibited the highest antioxidant activity (78.15%, $p < 0.01$ vs. the ascorbic acid control at 62.33%), exceeding 16%. This exceptional performance indicates the presence of free radical-scavenging potential in polyphenolic compounds, and further supports previous research (Patil *et al.*, 2012) reporting similarly high antioxidant activity in wood apple extracts (Thakur *et al.,* 2020). The phenolic glycoside extract *Feronia limonia* (L.) Swingle fruit exhibited higher antioxidant activity, which was 88.7%, whereas the antioxidant activity for total phenolic contents and free phenolics was 11.8% and 3.8% respectively. These interconnected anti-inflammatory and antioxidant mechanisms support applications in chronic diseases involving oxidative stress and inflammation (cardiovascular disease, metabolic syndrome, cancer). These results validate the traditional use of *L. acidissima* across multiple therapeutic applications and provide statistically significant evidence for solvent-dependent extraction of bioactive compounds with distinct mechanisms of action and dose-dependent efficacy profiles.

Wood apple (*Limonia acidissima*) pulp exhibited higher level of total phenolics (30.67 mg GAE g^{-1}) and ascorbic acid

Table 2: Antibacterial activity of *L. acidissima* leaf extracts

Bacterial species	Streptomycin Control (mm)	Ethanol extract (mm)	Ethyl Acetate extract (mm)	Methanol extract (mm)	p-value
Bacillus subtilis	18±1.2	5±0.5	5±0.4	—	<0.001
Staphylococcus aureus	20±1.3	—	8±0.6	—	<0.001
Salmonella typhi	17±1.1	—	2±0.3	9±0.7	<0.001
Shigella spp.	19±1.2	—	2±0.2	—	<0.001

Data represent mean ± S.D. (mm, n=3). "—" indicates no detectable inhibition. Different superscript letters indicate significant differences ($p < 0.05$)

(10.42 mg 100 g^{-1}), indicating antioxidant potential of wood apple (Shukla *et al.,* 2025). Anti-inflammatory activity shown by *L. acidissima* leaf extract might be having phenolic compounds like flavonoids and phenolic acidsas potent natural anti-inflammatory agents reducingchronic inflammation by suppressing pro-inflammatory genes. The role of phenolic compounds in activating antioxidant enzymes by alleviating oxidative stress and inflammation has been demonstrated by Liu *et al.* (2023). Thus, *L. acidissima* exhibits anti-cancer, anti-diabetic, anti-inflammatory and antioxidant activity, and these properties have been previously reported (Thakur *et al.,* 2020; Wakchoure *et al.,* 2023; Amardeepa and Vijayakumar, 2025; Mohanty and Pattnaik, 2025). Antimicrobial activity against four clinically relevant bacterial species indicated that the ethyl acetate extract exhibited the broadest-spectrum inhibition, with statistically significant activity ($p < 0.001$) against all four strains (Table 2). Ethanol extract showed selective activity and was limited to *Bacillus subtilis,* the methanol extract demonstrated activity specifically against *Salmonella typhi*. From an agricultural perspective, the broad-spectrum activity of ethyl acetate extract against *Staphylococcus aureus* and other bacterial pathogens highlights its potential as a naturally available crop protection extract, offering alternatives to copper-based bactericides and chemical antibiotics that raise environmental and resistance concerns. Thus, bioactive compounds from *L. acidissima* can be promising candidates for developing an insecticide for susceptible crop plants. Therefore, a detailed study of insect gut proteases is required to explore the role of PI compounds and the mechanism of protease inhibition. Two fungal species (*Candida albicans* and *Aspergillus niger*) were not inhibited by any extract (data not shown), indicating bacterial selectivity probably due to distinct biochemical composition of fungal cell walls (chitin and β -glucans) versus bacterial structures (peptidoglycan).

Ethanol extract was selected for phytochemical fractionation based on its superior multi-target bioactivity, particularly its exceptional dose-dependent anticancer activity and anti-diabetic efficacy, as demonstrated in Fig. 1 and Table 1. Consequently, the ethanolic extract was subjected to purification via flash chromatography to isolate individual bioactive components. Three distinct components were successfully separated and collected Peak 1 (fractions 7–9), Peak 2 (fractions 10–11), and Peak 3 (fractions 12–54) (Fig. 2). All collected fractions were combined, concentrated under vacuum, and individually

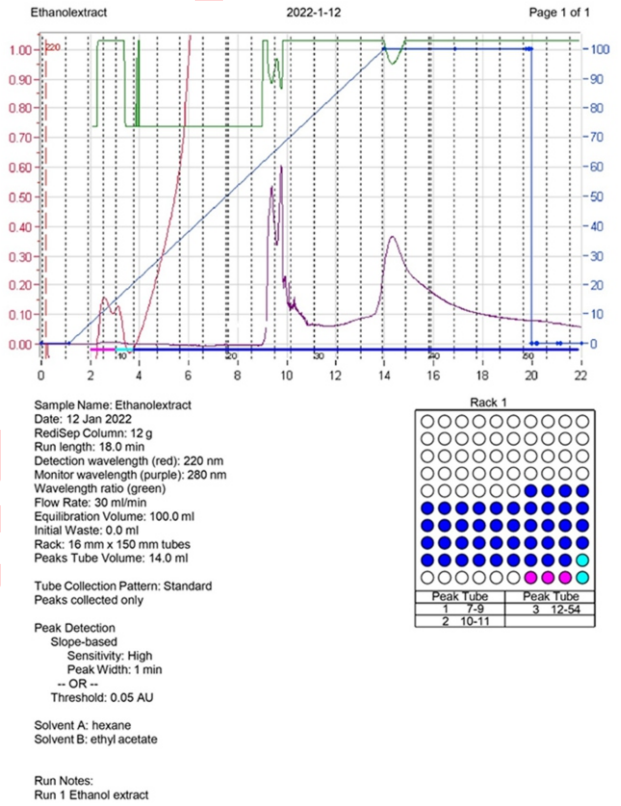

Fig. 2: Flash chromatographic separation of bioactive compounds from L. acidissima ethanolic extract. Three major peaks were resolved using gradient elution on Combifash equipment. Peak 1 (fractions 7–9) eluted at early retention time, Peak 2 (fractions 10–11) at intermediate retention, and Peak 3 (fractions 12–54) at later retention time, indicating differential polarity-based separation. Separated components were collected individually and concentrated for downstream LC-HRMS analysis.

analyzed by HR-LCMS (Fig. 3). The compounds eluted at different retention times were identified by mass spectra comparing with the authentic m/z spectra available in HR-LCMS data library. Distinctive mass spectra of *L. acidissima* bioactives isolated are shown in Fig. 3. High-resolution liquid chromatography–mass spectrometry (HR-LCMS) analysis of three separated components from ethanolic extract revealed the molecular

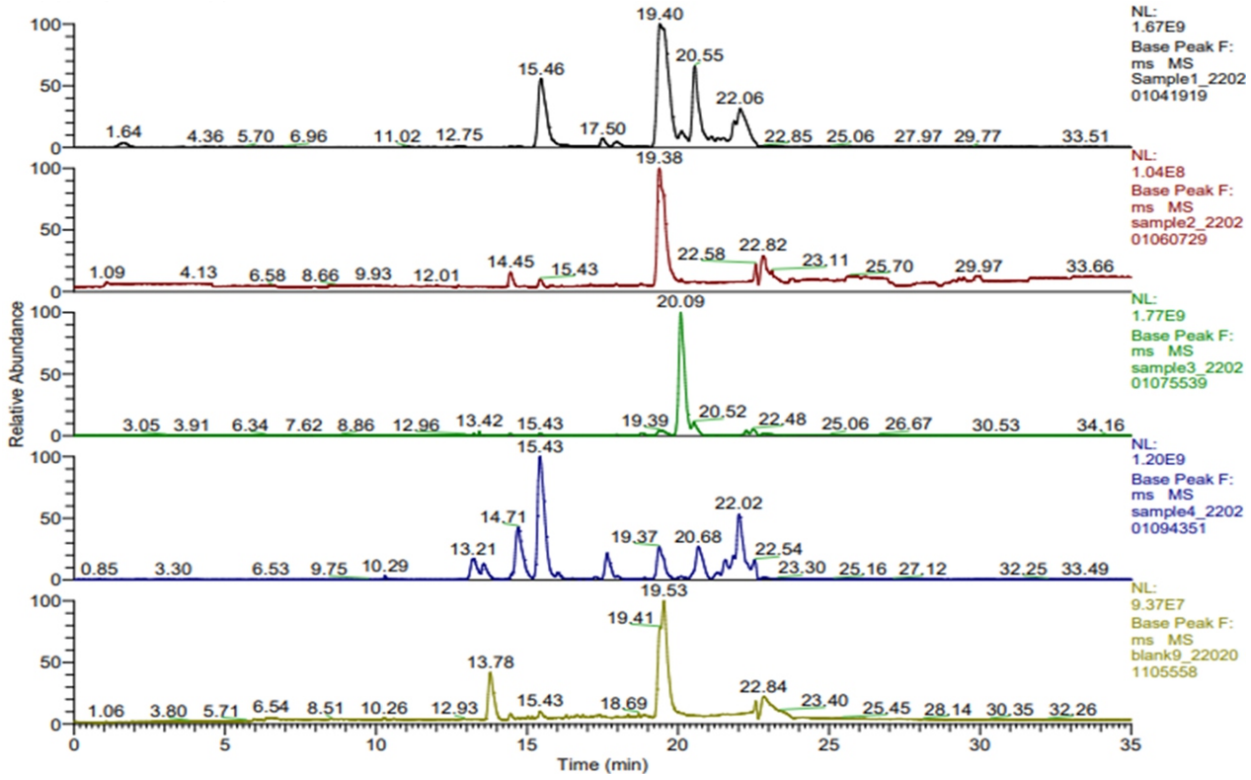

Fig. 3: High-resolution LC-MS chromatographic analysis of bioactive compounds isolated from L. acidissima ethanolic extract. Five replicate samples demonstrate consistent baseline separation of three major peaks: Component 1 (Rt 15.46 min, Methyl palmitate + Trans-3-Hexenoic acid; m/z 270); Component 2 (Rt 19.40 min, Oleanolic acid; m/z 456); Component 3 (Rt 20.55 min, Bergapten; m/z 216). Analysis performed using Q-Exactive Plus Biopharma mass spectrometer with Hypersil Gold column (3 µm, 100 × 2.1 mm). Mass accuracy ±5 ppm; detection range m/z 100–1000.

identities of bioactive compounds. Chromatographic baseline separation ascertained three prominent peaks with retention times of 15 min (Component 1), 19.40 min (Component 2), and 20.55 min (Component 3) (Fig. 3). Five independent chromatographic runs showed consistent peak separation and compound stability. In total seven phytochemicals were identified in the ethanolic extract (including abietic acid, adipic acid and Dipentyl phthalate), three compounds—(Fig. 3): Component 1 (Rt 15.46 min): Methyl palmitate ($C17H_34O_2$, m/z 270) and Trans-3-Hexenoic acid; Component 2 (Rt 19.40 min): Oleanolic acid ($C_{30}H_{48}O_3$, m/z 456); Component 3 (Rt 20.55 min): Bergapten ($C_{11}HO_3$, m/z 216). Methyl palmitate, Oleanolic acid and Bergapten were selected for subsequent molecular docking investigation. This selection was conducted on the basis of retention time precision, molecular formula accuracy, and mass spectral fragmentation pattern that matched literature values, thus prioritizing compounds with the highest protease inhibitory activity (21%, 11% and 9%).

Molecular docking analysis revealed oleanolic acid as the most potent multi-target compound having superior binding affinities across critical therapeutic targets (Table 3, Fig. 4). It exhibited multi-target potential: strong trypsin inhibition via Gly216 and Tyr94 H-bonds and π-alkyl interactions implicated in biocontrol applications; estrogen receptor antagonism through Tyr 526 H-bond explaining the anticancer activity observed in Fig. 1; and enhanced insulin receptor signalling via Arg1000 interactions implicating GLUT4 translocation for dual-mechanism glycemic control, complementing α-amylase inhibition. Bergapten exhibited strong estrogen receptor binding (-6.34 kcal mol^{-1}) with the ability to reduce ER-α protein in MCF-7 cells, and COX-2 binding (-7.80 kcal mol^{-1}), signifying its selective anti-inflammatory potential. Methyl palmitate exhibited the strongest COX-2 binding affinity (-8.07 kcal mol^{-1}) due to hydrophobic interactions, which corroborates its strong anti-inflammatory effects through selective COX-2 inhibition. Characterization of *L. acidissima,* in this study, indicates remarkable multifunctionality, with distinct solvent systems demonstrating its efficient targeting of different therapeutic objectives. Ethyl acetate extract excelled in protease inhibition (67.45%) and broad-spectrum antimicrobial activity; ethanol extract in anti-diabetic (75.22%) and anti-inflammatory activities, with the strongest dose-dependent anticancer response (Fig. 1); methanol extract in antioxidant activity (78.15%, exceeding positive control). Moreover, oleanolic acid emerged as a particularly promising multi-target compound with superior binding affinities across trypsin (-8.61 kcal mol^{-1}),

Table 3: Molecular docking binding affinities with therapeutic targets

Receptor Target	Oleanolic Acid	Bergapten	Methyl Palmitate	Clinical Relevance
Trypsin	-8.61 kcal/mol*	-6.31 kcal/mol	-5.21 kcal/mol	Insect digestive protease inhibition
— H-bonds	Gly216 (2.15 Å), Tyr94 (2.45 Å)	Ser214 (2.11 Å)	Hydrophobic	Catalytic site targeting
Estrogen Receptor	-6.80 ± 0.29 kcal/mol*	-6.34 ± 0.27 kcal/mol	—	ER+ breast cancer inhibition
— H-bonds	Tyr526 (2.01 Å)	His524 (2.13 Å), Leu525 (2.81 Å)	—	Hormone-binding pocket
Insulin Receptor	-6.99 ± 0.28 kcal/mol*	-5.06 ± 0.26 kcal/mol	—	Type-2 diabetes glucose control
— H-bonds	Arg1000 (3.01 Å, 2.07 Å)	Arg1000 (2.35 Å)	—	ATP-binding site critical
COX-2	-6.93 ± 0.30 kcal/mol	-7.80 ± 0.28 kcal/mol	-8.07 ± 0.31 kcal/mol*	Selective COX-2 inhibition
— H-bonds	Gln203 (2.18 Å)	Thr206 (2.21 Å), His388 (2.17 Å)	Phe210 (2.38 Å)	Hydrophobic channel

*** denotes highest affinity per receptor. Values in kcal/mol with H-bond distances in Ångströms (Å)

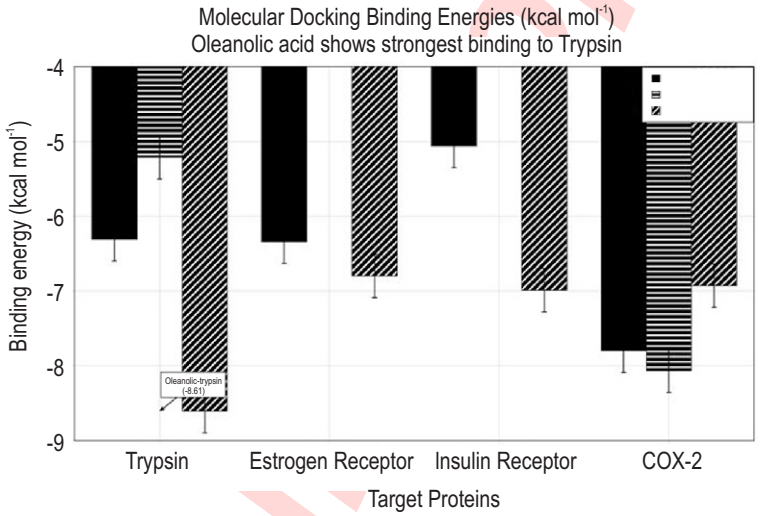

Fig. 4: Comparative molecular docking binding affinities across therapeutic targets bar graph comparing binding affinities (kcal/mol) of three identified compounds (oleanolic acid, bergapten, methyl palmitate) across four therapeutic targets (trypsin, estrogen receptor, insulin receptor, COX-2). Oleanolic acid demonstrates superior multi-target inhibition with highest binding affinities for trypsin (-8.61 kcal mol^{-1}), estrogen receptor (-6.80 kcal mol^{-1}), and insulin receptor (-6.99 kcal mol^{-1}), indicating particularly promising therapeutic potential. Methyl palmitate shows selective strength for COX-2 (-8.07 kcal mol^{-1}) inhibition, supporting potent anti-inflammatory effects. *** denotes highest affinity per receptor target.

estrogen receptor (-6.80 kcal mol^{-1}), and insulin receptor (-6.99 kcal mol^{-1}) targets, suggesting potential for complex multifactorial diseases targeting (breast cancer with concurrent metabolic syndrome; diabetes with inflammatory complications) (Table 3). The documented PI activity, combined with antimicrobial and multi-target therapeutic properties, ascertains *L. acidissima* as a natural resource for pharmaceutical and environmentally sustainable practices. As natural biocontrol agents, the extracts reduce reliance on harmful chemical insecticides, which disrupt ecosystems and contaminate soil. *L. acidissima* is commonly found in the tropical regions, especially in low socio-economic areas, where value-added extraction processes could generate income as well as benefits for sustainable land management. The antimicrobial activity of this plant extract suggests its potential as an herbal insecticide, which in turn would lead to reduced chemical bactericide application. Thus, supporting an integrated pest control strategy that aligns with ESG frameworks and sustainable development goals. Additionally, reports have emerged of the use of *L. acidissima* extracts as a natural mosquito repellent. These studies have reported the use of *L. acidissima* extracts causing larvicidal, developmental and adulticidal toxicity on Aedes aegypti mosquitoes. This can be a useful, non-toxic alternative to synthetic DEET (N, N-diethyl-meta-toluamide), which is currently the most widely used mosquito repellent (Chellappandian *et al.,* 2022; Qotrinnada *et al.,* 2024). Bovine trypsin represents only serine protease activity;

evaluation against multiple insect digestive proteases would strengthen biocontrol claims. MCF-7 cells represent only one cancer type; broader evaluation would clarify anticancer mechanisms. The dose-response studies conducted at three concentrations (10, 40, 80 µl); extended concentration ranges would refine EC_{50} determination and dose–response modelling.

In-vivo validation studies are essential to confirm the therapeutic efficacy, bioavailability, pharmacokinetics, and safety profiles through animal models and field trials. *L. acidissima* leaf extracts have multiple bioactive components with significant therapeutic potential and insecticidal properties. Bioactive compounds derived from natural plant sources with protease-inhibition potential and therapeutic activity play a significant role in agriculture and medicine for novel drug discovery. In view of this, such compounds can be explored in the sustainable management of herbivory and pathogenesis.

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