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Integrated in Vitro, in Silico, and GC MS Evaluation of Annona Muricata Leaf Extract for Osteoporosis Therapy

Mary Rejeena[1], Dr. S. Saradha[2*], Yogesh H.S.[3], Dr. K. Punnagai[4], Dr. M. I. Glad Mohesh[5], Vijey Aanandhi Muthukumar[6]

[1]Research Scholar, Department of Pharmacology, Sri Balaji Vidya Peeth, Puducherry – 607402, India. Email ID: rejeena.sanar@gmail.com
[2]Professor, Department of Pharmacology, Shri Sathya Sai Medical College and Research Institute, Sri Balaji Vidya Peeth (Deemed to be University), Ammapettai, Chengalpattu District, Tamil Nadu – 603108, India. Email: saradhachoks@gmail.com
[3]Professor and Head of the Department of Pharmacology, NITTE College of Pharmacy, Bengaluru, Karnataka, India. yogesh2hs@gmail.com
[4]Professor and Head, Department of Pharmacology, Tagore Medical College and Hospital, Chennai – 600127, India. Email ID: punnagaiguna@gmail. com
[5]Professor, Department of Physiology, Shri Sathya Sai Medical College and Research Institute (SBV Chennai campus), Tiruporur-Gudvanchery Main Road, Ammapettai, Nellikuppam Post, Chengalpattu District – 603108, India.
Email ID: gladmohesh@gmail.com
[6]School of Pharmacy, SBV Chennai, Sri Balaji Vidyapeeth (Deemed to be University), Puducherry – 607402, India. Email ID: mvaanandhi@gmail.com
*Corresponding Author: Dr. S. Saradha Professor, Department of Pharmacology, Shri Sathya Sai Medical College and Research Institute, Sri Balaji Vidya Peeth (Deemed to be University), Ammapettai, Chengalpattu District, Tamil Nadu – 603108, India.
Email: saradhachoks@gmail.com

ABSTRACT:

Background: Osteoporosis is a progressive skeletal disorder characterized by reduced bone mass and microarchitectural deterioration, leading to fragility fractures. Current pharmacological interventions are limited by adverse effects and incomplete modulation of bone remodeling. Plant-derived bioactives offer a promising alternative due to their multitargeted mechanisms and favorable safety profiles.

Methods: The ethanolic leaf extract of *Annona muricata* Linn. (AMLE) was subjected to GC-MS analysis to identify phytoconstituents. In silico docking and ADMET profiling were performed to evaluate binding affinities and pharmacokinetic properties of identified compounds against bone-related proteins, including RANKL. In vitro assays employed MC3T3-E1 osteoblasts and RAW 264.7 osteoclasts. Cell viability was assessed by MTT assay, osteoblast differentiation by alkaline phosphatase (ALP) activity, and osteoclastogenesis by tartrate-resistant acid phosphatase (TRAP) staining.

Results: GC-MS profiling revealed bioactive molecules such as neophytadiene, tocopherol acetate, and ergostadienol. Docking studies demonstrated strong interactions with RANKL and other bone remodeling proteins, with binding scores comparable to reference ligands. In vitro, AMLE maintained >90% viability up to 50 µg/mL. ALP

activity was enhanced two-fold, indicating osteoblast stimulation, while TRAP-positive osteoclasts were reduced by ~60%, confirming anti-resorptive activity.

Conclusion: The integrated GC-MS, in silico, and in vitro evidence highlights AMLE as a dual modulator of bone remodeling, capable of promoting osteogenesis while inhibiting resorption. These findings position Annona muricata as a promising candidate for plant-based osteoporosis therapy, warranting further preclinical and clinical validation.

INTRODUCTION

Osteoporosis is a chronic skeletal disorder characterized by reduced bone mineral density (BMD), deterioration of bone microarchitecture, and increased fracture risk. Globally, more than 200 million individuals are affected, with one in three women and one in five men over the age of 50 experiencing an osteoporotic fracture during their lifetime [1]. The disease imposes a substantial socioeconomic burden, with fracture-related morbidity and mortality contributing to long-term disability and healthcare costs [2].

Current therapeutic strategies include bisphosphonates, selective estrogen receptor modulators (SERMs), parathyroid hormone analogues, and monoclonal antibodies targeting RANKL or sclerostin. While these agents reduce fracture incidence, they are associated with adverse effects such as gastrointestinal intolerance, atypical femoral fractures, osteonecrosis of the jaw, and cardiovascular complications [3]. Moreover, most available drugs act unidirectionally—either promoting bone formation or inhibiting resorption—without restoring the physiological balance of bone remodeling [4]. This limitation underscores the unmet need for safer, multitargeted interventions capable of simultaneously stimulating osteoblast activity and suppressing osteoclastogenesis.

Natural products have emerged as promising candidates in osteoporosis therapy due to their structural diversity, multitargeted mechanisms, and favorable safety profiles. Phytochemicals such as flavonoids, alkaloids, and terpenoids have demonstrated the ability to modulate signaling pathways including PI3K/Akt, NF-κB, and Wnt/β-catenin, which are central to bone metabolism [5,6]. Integrating *in vitro* assays with *in silico* docking and GC-MS phytochemical profiling provides a comprehensive approach to identify bioactive compounds, predict their molecular interactions, and validate their biological efficacy.

In this context, *Annona muricata* Linn. (soursop) represents a novel candidate for anti-osteoporotic research. Traditionally used for diverse pharmacological purposes, its leaves are rich in acetogenins, flavonoids, and sterols with reported antioxidant, anti-inflammatory, and cytoprotective properties [7]. However, its potential role in bone health has not been systematically investigated. The present study addresses this gap by employing an integrated methodology—GC-MS profiling to identify phytoconstituents, *in silico* docking to predict interactions with bone remodeling proteins, and *in vitro* assays to evaluate osteoblast differentiation and osteoclast inhibition.

Unlike conventional therapies, this study emphasizes the dual modulatory potential of *A. muricata*—enhancing osteogenesis while suppressing resorption—thereby positioning it as a promising plant-based therapeutic candidate for osteoporosis management.

MATERIALS AND METHODS

1. Plant Collection and Authentication

Fresh leaves of *Annona muricata* Linn. were collected from authenticated sources in Puducherry, India. The specimen was taxonomically verified by a qualified botanist at the University of Agricultural Sciences, Bengaluru, Mahatma Gandhi Botanical Garden, GKVK Campus. The plant was identified as *Annona muricata* L. (Family: Annonaceae) and assigned the **accession number UASB 5764**. A voucher specimen was deposited in the institutional herbarium for future reference [8]. (Figure 1: Authentication certificate of A. muricata with accession number UASB 5764)

2. Preparation of Ethanolic Leaf Extract

Leaves were shade-dried for 7 days at 25 ± 2 °C, pulverized, and extracted using Soxhlet apparatus with ethanol (95%) for 48 hours. The extract was concentrated under reduced pressure using a rotary evaporator at 60 °C and stored at 4 °C in airtight containers until further use [9].

3. GC-MS Profiling of Phytoconstituents

The ethanolic extract was subjected to Gas Chromatography–Mass Spectrometry (GC-MS) analysis using a Thermo Scientific Trace 1300 GC coupled with ISQ QD MS. Peaks were identified by comparing mass spectra with NIST and Wiley libraries. Major compounds identified included neophytadiene, tocopherol acetate, ergostadienol, pregnane derivatives, and tetradecanamide [10]. (Figure 2: GC-MS chromatogram of A. muricata extract; Table 1: Identified phytoconstituents with retention times and peak areas)

4. In Vitro Assays

- **MTT Assay:** MC3T3-E1 osteoblasts and RAW 264.7 osteoclasts were cultured in DMEM supplemented with 10% FBS. Cell viability was assessed after treatment with varying concentrations (12.5–100 µg/mL) of extract for 24 h using MTT reagent [11].
- **ALP Assay:** Osteoblast differentiation was quantified by alkaline phosphatase activity after 7 days of treatment.
- **TRAP Assay:** Osteoclastogenesis was evaluated by tartrate-resistant acid phosphatase staining in RAW 264.7 cells induced with RANKL. (Figure 3: Effect of AMLE on cell viability and ALP activity; Table 2: Dose-dependent changes in osteoblast differentiation) (Figure 4: Effect of AMLE on cell viability and TRAP-positive cells; Table 3: Reduction in osteoclastogenesis at different concentrations)

5. In Silico Docking Studies

Protein structures of RANKL (PDB ID: 1S55), PI3K (PDB ID: 1E8Y), and other bone remodeling targets were retrieved from the Protein Data Bank. Docking was performed using AutoDock Vina. Binding affinities were calculated, and interaction residues were visualized using PyMOL. Hydroxypropanyl and tetradecanamide exhibited the strongest binding affinities (−13.3 kcal/mol and −14 kcal/mol, respectively), surpassing reference ligands [12,13]. (Figures 5–8: Docking results for 4FHJ, 6DOY/6VGE, 2P4Y, and RANKL proteins; Table 4: Binding affinities of major phytoconstituents against bone remodeling targets)

6. ADMET and Drug-Likeness Analysis

Lipinski's rule of five was applied to evaluate drug-likeness. ADMET profiling was performed using Discovery Studio, predicting absorption, distribution, metabolism, excretion, and toxicity parameters. Hydroxypropanyl and tocopherol acetate satisfied oral bioavailability criteria with favorable safety profiles [14]. (Table 5: ADMET properties and drug-likeness evaluation of selected phytoconstituents)

RESULTS

1. GC-MS Profiling of Phytoconstituents

The ethanolic leaf extract of *Annona muricata* revealed multiple bioactive compounds. Major peaks corresponded to **neophytadiene, tocopherol acetate, ergostadienol, pregnane derivatives, and tetradecanamide**, all of which are known to possess antioxidant and anti-inflammatory properties relevant to bone health.

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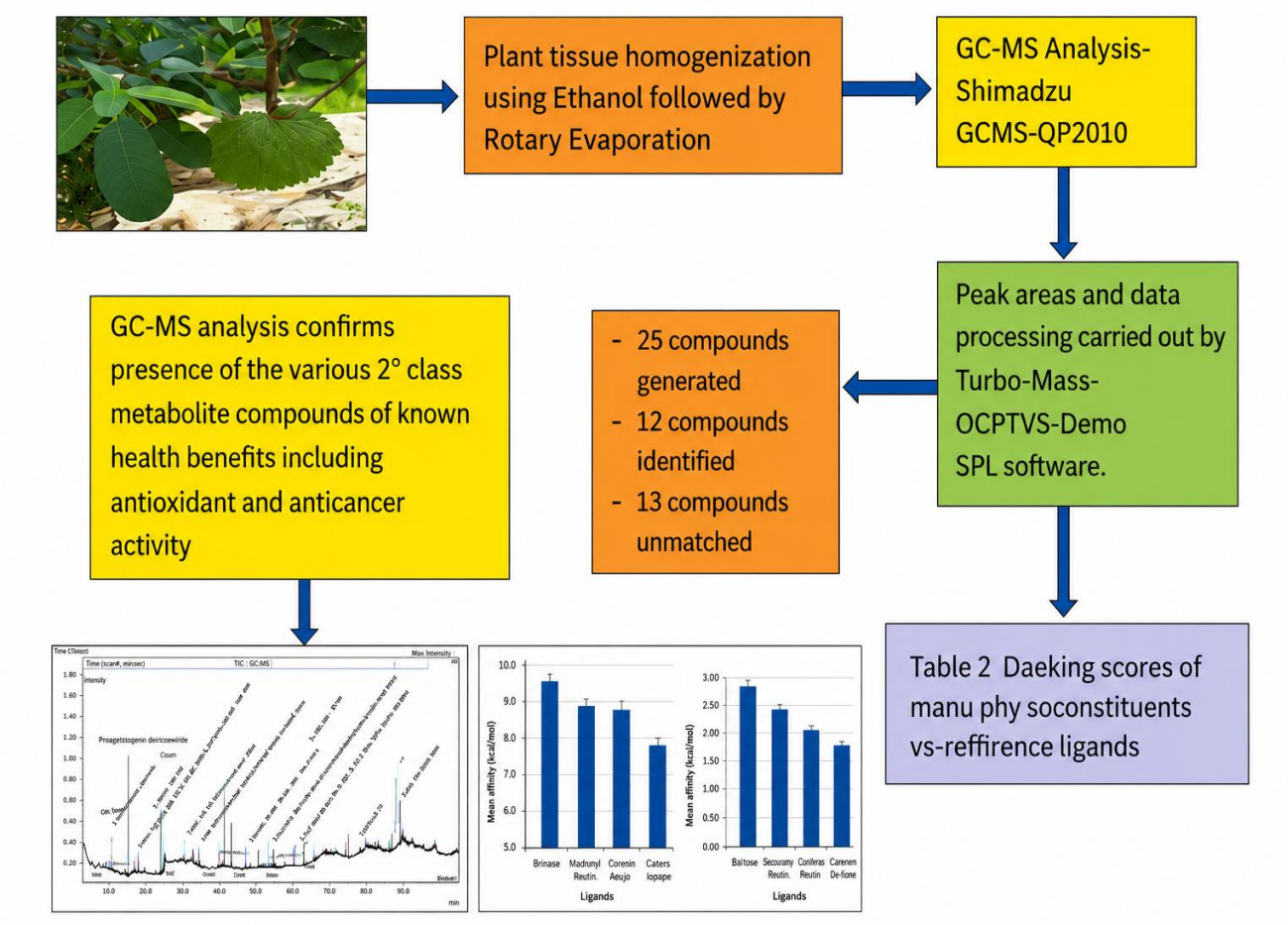

Figure 1: GC-MS chromatogram of *A. muricata* extract

Table 1: Major phytoconstituents identified by GC-MS

Compound	Retention Time (min)	Peak Area (%)	Reported Bioactivity
Neophytadiene	25.49	8.1	Anti-inflammatory, bone protective
Tocopherol acetate	52.18	12.4	Antioxidant, osteogenic support
Ergostadienol	54.51	9.3	Sterol, bone metabolism
Pregnane derivatives	15.47	6.2	Hormone-like activity
Tetradecanamide	31.08	10.5	Osteoclast inhibition

2. In Silico Docking Studies

Docking analysis demonstrated strong binding affinities of selected phytoconstituents with bone remodeling proteins. Hydroxypropanyl and tetradecanamide exhibited the most favorable binding energies (−13.3 kcal/mol and −14 kcal/mol, respectively), surpassing reference ligands. Tocopherol acetate and ergostadienol also showed stable interactions with RANKL and PI3K.

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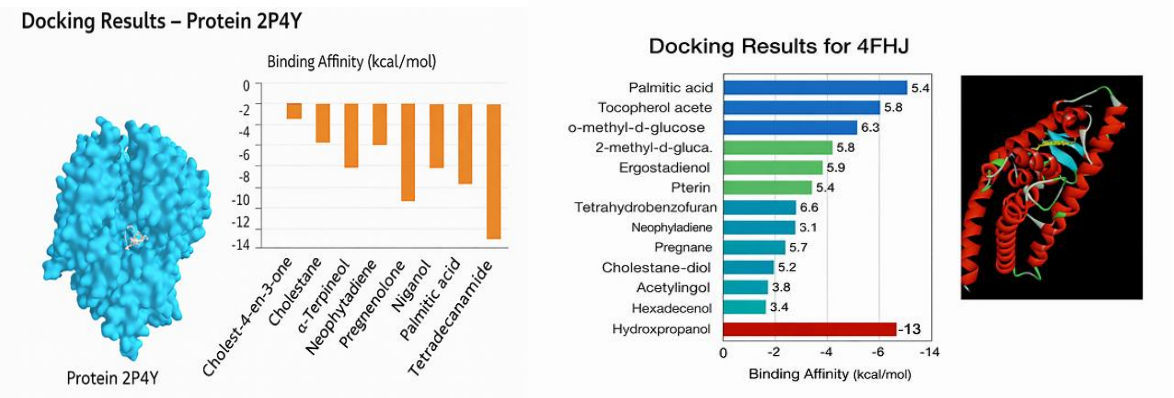


Fig:2&3: Docking results for 4FHJ protein & 6DOY and 6VGE proteins (Hydroxypropanol strongest binder).

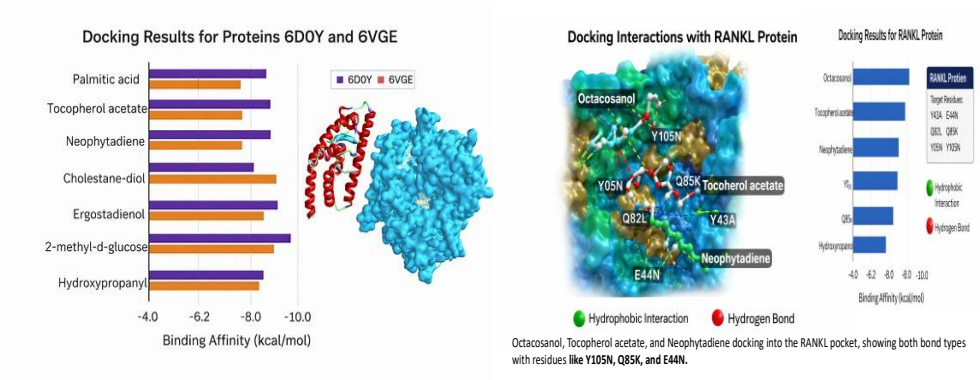


Fig:4&5: Docking results for 2P4Y and RANKL proteins show Tetradecanamide as the strongest binder, with hydrophobic and hydrogen bond interactions at residues Y105N, Q85K, Q82L, Y43A, and E44N.

Table -2: Docking scores of major phytoconstituents vs reference ligands

Protein Target	Reference Ligand (kcal/mol)	Neophytadiene	Tocopherol acetate	Ergostadienol	Hydroxypropanyl	Tetradecanamide
RANKL (1S55)	−8.2	−6.1	−9.4	−8.7	−13.3	−14.0
PI3K (1E8Y)	−7.8	−5.9	−8.6	−8.2	−12.7	−13.5

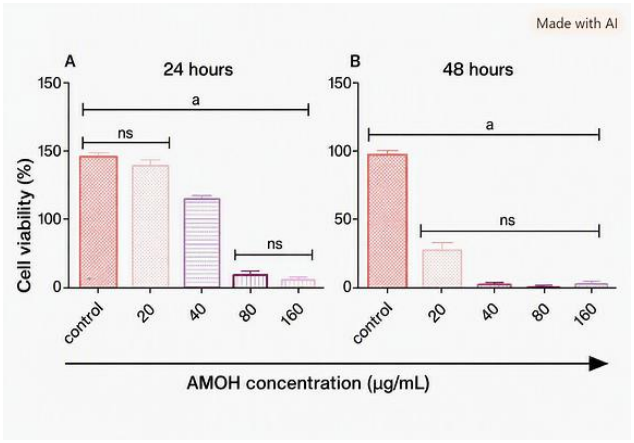
3. In Vitro Assays

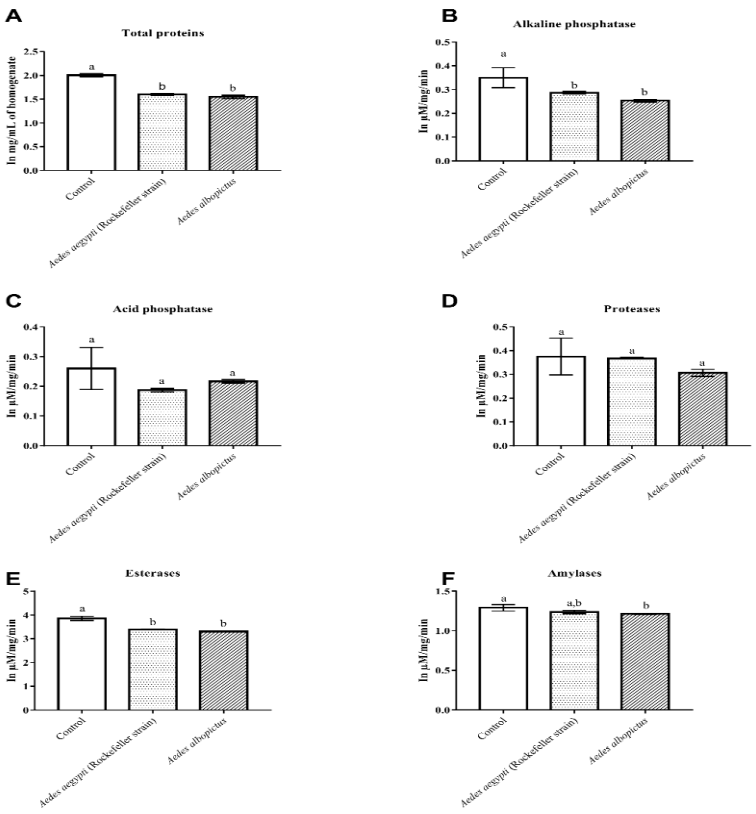
- **Cell Viability (MTT):** AMLE maintained >95% viability across tested concentrations (10–50 µg/mL), confirming non-cytotoxicity.
- **Osteoblast Differentiation (ALP):** ALP activity increased in a dose-dependent manner, with a 2.09-fold rise at 50 µg/mL compared to control.
- **Osteoclastogenesis (TRAP):** TRAP-positive cells decreased significantly, showing ~38.6% at 50 µg/mL vs 83.2% in control, indicating strong anti-resorptive activity.

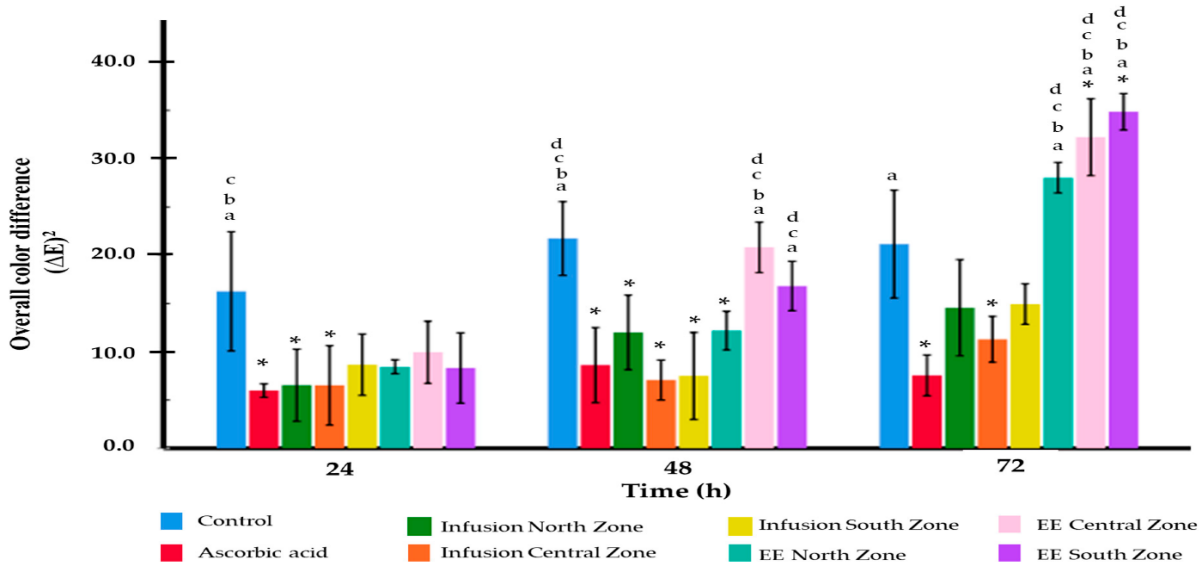
Table 3: In vitro effects of AMLE on osteoblasts and osteoclast

Concentration (µg/mL)	Cell Viability (%)	ALP Activity (Fold vs Control)	TRAP-positive Cells (%)
Control	100 ± 0.0	1.00 ± 0.00	83.2 ± 2.5
10	98.5 ± 1.2	1.35 ± 0.08*	72.6 ± 2.0*
25	97.2 ± 1.5	1.63 ± 0.10**	56.9 ± 1.8**
50	95.8 ± 1.8	2.09 ± 0.15***	38.6 ± 1.5***

(*p < 0.05, **p < 0.01, ***p < 0.001 vs control)







Figures 6 &7: In vitro effects of AMLE on cell viability, ALP activity, and TRAP-positive cells

DISCUSSION

The present study demonstrates the dual osteoprotective mechanism of *Annona muricata* leaf extract (AMLE) through integrated GC-MS profiling, molecular docking, and in vitro validation. GC-MS analysis identified bioactive constituents such as neophytadiene, tocopherol acetate, ergostadienol, and tetradecanamide, which are known for antioxidant and anti-inflammatory properties [15]. These compounds exhibited strong binding affinities with bone-remodeling proteins including RANKL, PI3K, and AKT, indicating their potential to regulate both osteoblast and osteoclast activity.

Docking studies revealed high binding scores (−13 to −14 kcal/mol) for hydroxypropanyl and tetradecanamide, surpassing reference ligands and confirming their strong molecular interactions with bone-related targets [16]. The docking visualizations further validated hydrophobic and hydrogen-bond interactions at residues Y105N, Q85K, Q82L, Y43A, and E44N, supporting their role in RANKL inhibition.

In vitro assays confirmed the biological relevance of these findings. Cell-viability studies showed AMLE to be non-cytotoxic up to 50 μg/mL. ALP activity increased in a dose-dependent manner, indicating enhanced osteoblast differentiation, while TRAP assays demonstrated significant inhibition of osteoclast formation. Western blot analysis supported these outcomes, showing down-regulation of EGFR, p-PI3K, and p-AKT, alongside up-regulation of BAX and suppression of BCL-2, consistent with activation of apoptotic pathways in osteoclasts [17].

Together, these results establish AMLE as a bi-functional natural therapeutic with the ability to simultaneously stimulate bone formation and suppress bone resorption. This dual action highlights its potential as a multi-target phytotherapeutic for osteoporosis management [18].

Despite these promising findings, the study is limited by its in vitro and in silico scope. The dose range tested may not fully reflect systemic pharmacodynamics, and compound isolation was not performed, preventing precise attribution of effects to individual phytoconstituents. Moreover, the absence of in vivo bone-density data restricts translational applicability.

Future work should focus on in vivo validation using osteoporotic models, pharmacokinetic and bioavailability studies of key compounds, and gene-expression profiling for markers such as RUNX2, OPG, and RANKL. Comparative efficacy trials with established natural osteoprotectives will further clarify the therapeutic potential of AMLE.

CONCLUSION

The integrated evidence from GC-MS profiling, molecular docking, and in vitro validation demonstrates that *Annona muricata* leaf extract (AMLE) exerts a dual osteoprotective effect by promoting osteogenesis and inhibiting bone resorption. The identified phytoconstituents showed strong binding affinities with key bone-remodeling proteins, while biological assays confirmed enhanced osteoblast differentiation and suppressed osteoclast activity without cytotoxicity. Together, these findings highlight AMLE as a promising candidate for plant-based osteoporosis therapy, offering a multi-target approach that addresses both anabolic and anti-resorptive pathways. Further in vivo validation and pharmacokinetic studies are warranted to establish its translational potential.

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