

IN VITRO AND IN SILICO APPROACH TO EVALUATE THE CYTOTOXICITY OF *Carica papaya* EXTRACTS AGAINST Saos-2 CELL LINE

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Abstract

Osteosarcoma is the predominant form of bone cancer, which affects mostly adolescents and young adults. Despite therapeutic options, the prognosis for patients suffering with recurrent or advanced osteosarcoma remains poor. *Carica papaya*, a herbaceous plant, is well-known for its medicinal properties in traditional medicine. This study explores the therapeutic potential of *Carica papaya* leaf and seed extracts against osteosarcoma through an integrated *in silico* and *in vitro* approach. An MTT assay was performed to evaluate the apoptotic and cytotoxic effects against Saos-2 osteosarcoma cell line which demonstrated an IC₅₀ value of 0.91% and 0.78% for leaf and seed extract respectively. *In silico* validation was carried out using network pharmacology to identify the top 10 hub genes. From molecular docking studies, we identified major bioactives such as flavones and androst-4-en-3-one, 17-methoxy from leaf extract with good binding affinity to SRC, and 2-methyl-5H-dibenz[b,f]azepine and supraene from seed extract with ESR1, PARP1, AKT1, ERBB2. This study serves as a preliminary investigation into the anticancer property of *Carica papaya* against osteosarcoma and the results suggest its promise as a natural therapeutic agent for osteosarcoma.

Keywords: *Carica papaya*; Anti-cancer analysis; Osteosarcoma

Introduction

Carica papaya Linn., commonly known as papaya, is a tropical evergreen plant that belongs to the family Caricaceae (Aradhya *et al.*, 1999). Traditionally valued as both a fruit crop and medicinal plant, *Carica papaya* has long been used in various cultural medicinal practices. The medicinal importance of *Carica papaya* is largely

attributed to its diverse phytochemical constituents. *Carica papaya* is popular as a rich source of essential nutrients, including vitamin C, vitamin A, vitamin E, pantothenic acid (vitamin B5), magnesium, potassium, folates, and fiber (Vij and Prashar, 2015). The leaves are reported to be rich in vitamins A, C, and B12, along with bioactive

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compounds such as carposide, the alkaloids carpain and pseudocarpain, and folic acid (Krishna *et al.*, 2008; Imaga *et al.*, 2010). The seeds are known as a source of triterpenoids, steroids, flavonoids, carpaine, benzyl isothiocyanate, benzyl glucosinolate, benzylthiourea, hentriacontane, and caricin (Kermanshai *et al.*, 2001; Krishna *et al.*, 2008). Given the rich phytochemical profile of *Carica papaya*, particularly its leaves and seeds, there is significant interest in exploring their potential anticancer properties, which forms the basis of the present study (Haber *et al.*, 2023). Phenols, carotenoids, and glucosinolates in papaya have interested researchers for their anticancer properties (Nguyen *et al.*, 2013). It has been observed that these compounds act on multiple pathways to exert their anti-cancer effect such as angiogenesis, carcinogen elimination, cancer cell signaling, apoptosis, migration, invasion and proliferation (Fauziya and Krishnamurthy, 2013). Osteosarcoma (OS) is a rare yet aggressive form of bone cancer that predominantly affects adolescents and young adults, with a higher incidence in males. OS accounts for 20% to 40% of all bone cancers with 5.6 cases per million children under the age of 15 seen annually (Papachristou and Papavassiliou, 2007). It commonly targets the long bones such as the distal femur and proximal tibia and is defined by rapid progression, recurrence, and distant metastasis. Histological findings of OS show osteoid production associated with malignant mesenchymal cells (Misaghi *et al.*, 2018).

Despite available treatment options such as chemotherapy, surgical interventions, and drugs like doxorubicin, methotrexate, and cisplatin, the prognosis for advanced OS remains poor. Clinicopathological factors such as older age, size of the tumor, proximal tumor locations, poor response to chemotherapy, lack of surgery and amputation instead of limb-salvage procedures contribute to poor prognosis of patients (Xin and Wei, 2020).

Recent studies have highlighted the potential of *Carica papaya* as a source of bioactive secondary metabolites with anticancer properties. Compounds such as phenols, carotenoids, and glucosinolates have been found to act on multiple pathways, including angiogenesis, apoptosis, cancer cell signaling, migration, invasion, and proliferation (Fauziya and Krishnamurthy, 2013). While *Carica papaya* extracts have shown activity against various cancer cell lines, such as those of the stomach, breast, colon, liver, and lung, limited research has been conducted on their effects against OS. In particular, there is a lack of studies that combine *in vitro* and *in silico* approaches to identify

the molecular targets and mechanisms through which papaya-derived phytochemicals may exert anti-osteosarcoma effects. Therefore, the present study was designed to address this by evaluating the anticancer potential of ethanolic leaf and seed extracts of *Carica papaya* against the human osteosarcoma cell line Saos-2 using an integrated *in vitro* and *in silico* approach. Cytotoxic activity was assessed through MTT-based cell viability assays, while network pharmacology, protein-protein interaction analysis, functional enrichment analysis, molecular docking, and molecular dynamics simulations were used to identify potential therapeutic targets and bioactive compounds.

Materials and methods

Plants

Carica papaya was collected from the local market in Bangalore, Karnataka. The plant was authenticated in the nursery of The University of Trans-Disciplinary Health Sciences and Technology.

Leaf and seed extract preparation

The leaves and seeds of *Carica papaya* were collected and sun-dried. The dried samples were then ground to obtain a fine powder. 10 g of the powdered *Carica papaya* leaves and seeds were each soaked in 100 mL of 70% ethanol for 48 hours. After extraction, the mixtures were filtered using Whatman filter paper. The total volume of the filtered extract was 100 mL and three replicates were performed for the *in vivo* testing.

Phytochemical tests

The extracts were tested for the following classes of phytochemicals. The tests carried out were Alkaline reagent test for Flavonoids, Iodine test for Phenolic Compounds, NaOH test for Tannins, Iodine test for Alkaloids, Foam test for Saponins, Salkowski Test for Sterols, Copper Acetate Test for Diterpenes (Shaikh and Patil, 2020).

Alkaline reagent test: 1 mL of the extract was taken to which 2 mL of 2% NaOH and a few drops of diluted HCl was added. Formation of intense yellow color which upon addition of dilute HCl acid disappears indicates the presence of flavonoids.

Phenolic Compounds

Iodine test: To 1 mL of extract, few drops of dilute Iodine solution was added. A temporary red color indicates the presence of phenolic compounds.

Tannins

NaOH test: To the ethanolic extract, 1% gelatin and 10% NaCl was added. Formation of white precipitate indicates the presence of tannins.

Alkaloids

Iodine test: 3 mL of extract was taken and a few drops of iodine solution was added. The solution turning into blue color and disappearing on boiling and reappearing on cooling indicates the presence of alkaloids.

Saponins

Foam test: 2 mL of water was added to 0.5 mL of the extract. Upon vigorous shaking. Persistent foam for 10 minutes indicates the presence of saponins.

Sterols

Salkowski Test: 2 mL of extract was used, into which 2 mL dichloromethane, 2mL concentrated sulphuric acid in drops along the walls of the test tube were added. Appearance of a red layer due to dichloromethane and fluorescent greenish yellow layer due to the acid, indicates positive result for sterols

Diterpenes

Copper Acetate Test: 0.5 mL of the extract and few drops of copper acetate. Formation of emerald green colour indicates the presence of diterpenes.

Cell Culture

Saos-2 cell (ATCC: HTB-85) line was procured from American Type Culture Collection (ATCC), stock cells were cultured in RPMI media (Invitrogen) with 10% inactivated Fetal Bovine Serum (FBS) (Invitrogen), penicillin (100 IU/mL), streptomycin (100µg/mL) (Invitrogen) in a humidified atmosphere of 5% CO₂ at 37°C until confluent. The cells were dissociated using 0.05% trypsin (Invitrogen) and centrifuged at 1000 rpm for 5 mins. The culture media was discarded and the cell pellet was gently re-suspended using 2 mL RPMI (Invitrogen) media. The viability of the cells was checked and a single cell suspension of 5.0 x 10⁵ cells/mL was prepared.

Sample Preparation: Leaf and Seed Extract

For cytotoxicity studies, the extracts were considered to be 100%. Dilutions were prepared using plain media (Figure 1).

Test Procedure

To each well of the pre-labelled 96-well microtiter plate, 100 µL of the prepared cell suspension (50,000 cells/well) was added and

incubated at 37°C with 5% CO₂. After 24 hours of incubation, the supernatant was removed and the monolayer was rinsed with media. To each pre-designated well, 100µL of test drugs at various concentrations were added and incubated for 24 hours. After incubation, the test solutions in the wells were discarded and 100 µL of MTT reagent (4 mg/10 mL of MTT in PBS) was added to each well. The plates were incubated for 4 hours at 37°C in 5% CO₂. The supernatant was removed and 100 µL of DMSO was added and the plates were gently shaken to solubilize the formazan crystals. The absorbance was measured using a microplate reader at 590 nm wavelength using a multimode plate reader, Spectra max i3X, Molecular devices. The percentage growth inhibition was calculated using the following formula and the concentration of test drug to inhibit cell growth by 50% (IC₅₀) values is generated from the dose-response curves for each cell line using GraphPad Prism 5.0 (GraphPad Software, Inc).

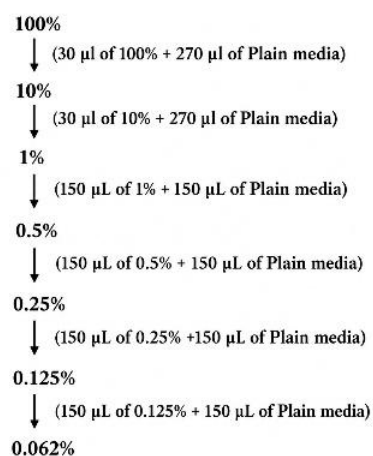


Figure 1. Dilutions of extracts prepared for cytotoxicity studies

Calculating Inhibition

% Inhibition = $\frac{[(\text{O.D of control} - \text{O.D of sample}) \div \text{O.D of control}] \times 100}{1}$.

Ligands present in ethanolic leaf and seed extract

The ligands of *Carica papaya* ethanolic leaf extract and seed extract were obtained from literature (Raj *et al.*, 2022; Iyappan *et al.*, 2014).

Identifying the Common Targets Using Venn Diagram

OS targets were retrieved from the DisGeNET database (<https://disgenet.com/>). Similarly, *Carica papaya* ethanolic leaf and seed

extract targets were predicted using Swiss Target Prediction (<http://www.swisstargetprediction.ch/>). There were a total of 2283 OS genes, 765 leaf ligand targets and 256 seed extract targets. A Venn diagram was constructed using Venny 2.1. (<https://bioinfogp.cnb.csic.es/tools/venny/>). A total of two Venn diagrams were constructed to get the common targets between OS genes and *Carica papaya* extract targets.

Construction and Investigation of Protein-Protein Interaction (PPI) Network

Protein-protein interaction network was constructed using the String database (<https://string-db.org/>). The leaf extract common targets and seed extract common targets were uploaded onto the database, with species set to "Human" and a minimum interaction score of "medium confidence" (0.400). The results were exported as TSV files for further analysis. The PPI network was loaded in Cytoscape (Version 3.10.2), where the network was analyzed using a network analyzer based on different parameters like Betweenness centrality, Closeness centrality and Degree. Using the CytoHubba plugin of cytoscape tool, top 10 hub genes were predicted and ranked by Maximal Clique Centrality (MCC) topological analysis.

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Enrichment Analysis

SR Plot (<https://www.bioinformatics.com.cn/en>) was used to construct the dot plots for various gene ontology terms like Biological Process (BP), Cellular Components (CC), Molecular Function (MF) and KEGG pathways. The dot plots summarize top 10 statistically significant and enriched terms.

Molecular Docking

PyRx was used to perform multi-ligand docking and Biovia Discovery Studio was used to visualize the interactions between the ligands and the hub genes. The 2D SDF structures of ligands were downloaded from the PubChem database. The hub genes structures were downloaded from the PDB database. The hub genes structures were cleaned in Biovia Discovery Studio and saved as PDB files. Docking was performed in PyRx and the interactions were analyzed in Biovia Discovery Studio (Lolok *et al.*, 2022).

Molecular dynamic (MD) simulation and MM-PBSA studies

Molecular Dynamics simulations were carried out using the GROMACS package with the CHARMM27 force field and TIP3P water model.

The protein-ligand complex structure, which was generated from molecular docking, was parameterized by adding hydrogen atoms, and ligand topologies were created using SwissParam. The system was solvated in a periodic box, neutralized with counter-ions, and scaled to an ionic concentration of 0.1 M. Energy minimization was done using the steepest descent algorithm, followed by equilibration and a 100 ns production run under standard conditions. Trajectory analysis was done to assess the stability of the system and the dynamics of interactions (Van Der Spoel *et al.*, 2005).

Results and Discussion

Phytochemical Tests

Previous studies indicate the cytotoxicity of *Carica papaya* extracts against various cancer cell lines such as MCF-7 (breast cancer) (Hamed *et al.*, 2022), PC-3 (prostate cancer) (Alotaibi *et al.*, 2017), HT-29 (colon cancer) (Rifaath *et al.*, 2022), TK10 (kidney cancer) (Rashed and Fouche, 2013) PC14, H2452, H226, MESO-4, and LU-1 (lung cancer) (Otsuki *et al.*, 2010) and Caco-2 (colorectal cancer) (Mahrous and Noseer, 2023). Earlier studies with GC-MS analysis have evidenced the presence of compounds belonging to the classes of phytochemicals present in the extracts used in this study. In the present study, the leaf extract tested positive for phenolic compounds, tannins, saponins, sterols, and diterpenes, but lacked flavonoids and alkaloids. The seed extract revealed the presence of all phytochemicals. The results indicated a varied distribution of phytoconstituents between the leaves and seeds of *Carica papaya* as represented in Table 1.

Table 1. Results of phytochemicals test for leaf and seed extracts

Sl no.	Phytochemicals	Test Results	
		Leaf Extract	Seed Extract
1.	Flavonoids	-	+
2.	Phenolic Compounds	+	+
3.	Tannins	+	+
4.	Alkaloids	-	+
5.	Saponins	+	+
6.	Sterols	+	+
7.	Diterpenes	+	+

Cytotoxic activity

The anticancer activity of *Carica papaya* leaf and seed ethanolic extracts was evaluated using the MTT assay on Saos-2 cell lines. Cells were treated

with increasing concentrations of leaf and seed extracts (i.e. 0.03%, 0.06%, 0.13%, 0.25%, 0.5%, 1.00%). Both extracts demonstrated a dose-dependent decrease in cell viability. The half maximal inhibitory concentrations (IC₅₀) were determined to be 0.91% (Figure 2a) and 0.78% (Figure 2b) for leaf and seed extract respectively. Both extracts demonstrated a dose-dependent decrease in cell viability. These findings were supported by morphological changes such as reduction in cell density, less rounded cells, and showed signs of rounding and detachment. This proved that the extracts displayed a cytotoxic effect against the Saos-2 cells. (Figure 3) The study utilized a crude extract of *Carica papaya* leaf and seed with 10 g of plant material added to 100 mL of ethanol. Ethanol was used for extraction, however, other solvents such as chloroform which is non-polar can be used for preparing the extract. The choice of solvent may also impact the cytotoxic potential of the extract and this can be investigated further.

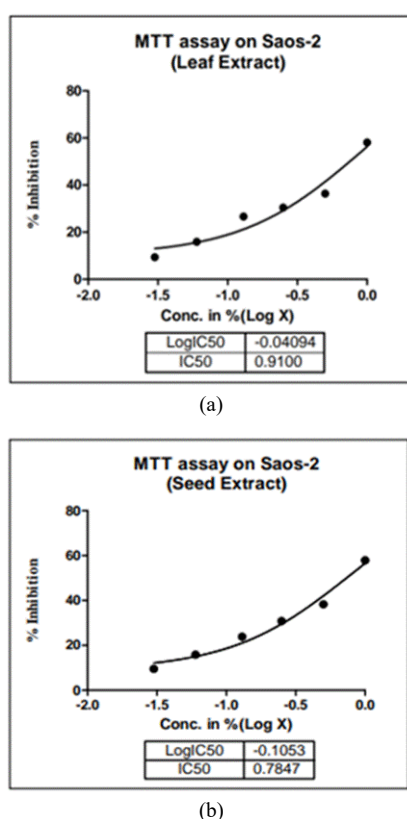


Figure 2. Box-and-whisker plot showing the contents of PC, PS, and PA in fingerroot powders obtained from various online stores. PC, PS, and PA refer to pinocembrin, pinostrobin, and panduratin A, respectively

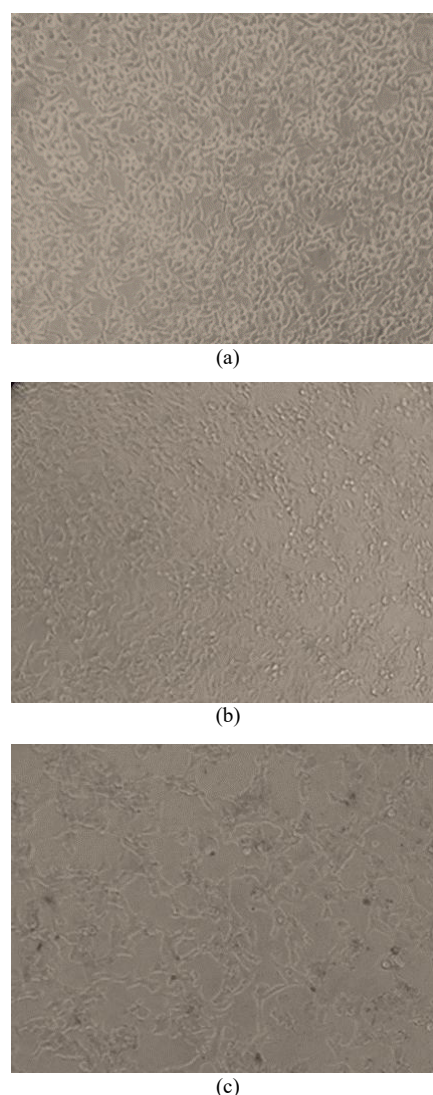


Figure 3. Cell morphological study of *Carica papaya* extracts induced signs of rounding and detachment and reduced the cell density compared to the control - A) Control; B) Leaf extract; C) Seed extract

Ligands present in ethanolic leaf and seed extract

The following 16 ligands in the leaf extract were identified from existing literature (Raj *et al.*, 2022) and selected for our study. The compounds are listed along with their PubChem ID. (Table 2).

Moreover, the following 18 ligands in the seed extract were identified from existing literature (Iyappan *et al.*, 2014) and selected for our study. The compounds are listed along with their PubChem ID. (Table 3).

Table 2. Ligands identified in ethanolic leaf extract

Sl no.	Ligand name	PubChem ID
1.	Coumarine3-(2,4-dinitrophenol)	1493
2.	Alpha-lardeine (Cyclohexaamylose)	320760
3.	3,7-Dimethyl-6-nonen-1-ol acetate (Methyl tridecanoate)	15608
4.	Dodecanoic acid, 10-methyl-, methyl ester	7800
5.	Hexadecanoic acid, ethyl ester	5281
6.	Phytol	145386
7.	9,12-Octadecadienoic acid, ethyl ester	11001
8.	5,8-Octadecadienoic acid, methyl ester	549020
9.	Flavone	10680
10.	Androst-4-en-3-one,17-methoxy, 3-methoxime	90895
11.	2-methylbutyl 4-(4- pentylphenyl)benzoate	622673
12.	Delta-Iraldeine	97996
13.	2,6,10,14,18-Pentamethyl- 2,6,10,14, C ₂₅ H ₄₂ 342.6 16.52 18-eicosapentaene	550101
14.	Dodecyl acrylate	(75084)
15.	d-Thionodecalactone	61982
16.	Neodecanoic acid	62838
17.	Dasycarpidan-1-methanol, acetate(ester)	550072

Table 3. Ligands identified in ethanolic seed extract

Sl no.	Ligand name	PubChem ID
1.	1-(2'-Methoxy-5-nitrophenyl) ethanone	18615104
2.	Phenol, 2-methyl-5-(1,2,2- trimethylcyclopentyl)-, (S)	11790670
3.	Tributyl acetylcitrate	6505
4.	2-Propenoic acid, 3-(4- methoxyphenyl)-, 2- ethylhexylester	5355130
5.	2-Methyl-5H- dibenz[b,f]azepine	12498159
6.	9-Octadecenamide	1930
7.	Hexadecanoic acid, methyl ester	8181
8.	Heptadecanoic acid, ethyl ester	26397
9.	Hexadecanoic acid, phenylmethyl ester	13908925
10.	cis-11-Eicosenoic acid, methyl ester	5463047
11.	Supraene	638072
12.	Hexadecanoic acid, ethyl ester	12366
13.	Dodecanoic acid, ethyl ester	7800
14.	i-Propyl 9-octadecenoate	66981
15.	9-Octadecenoic acid (Z)-, methyl ester	5364509
16.	Tetradecanoic acid, ethyl ester	31283
17.	Ethyl tetracosanoate	141135
18.	L-(+)-Rhamnopyranose, tetrakis(trimethylsilyl) ether	525317

Identifying the Common Targets Using Venn Diagram

A total of 2283 OS genes from DisGeNET, 765 leaf extract targets and 256 seed extract targets from Swiss Target Prediction were predicted. Venn diagrams constructed for each extract revealed 239 overlapping genes between OS gene targets and leaf extract targets, and 88 overlapping genes between OS gene targets and seed extract targets. These overlapping genes are the common targets that were further analyzed for PPI network construction.

PPI Network

PPI network of common targets between OS and *Carica papaya* leaf extract targets had a total of 238 nodes, 4315 edges, average local clustering coefficient of 0.608 and a PPI enrichment p-value of <1.0e-16. In comparison the PPI network of common targets between OS and *Carica papaya* seed extract targets had a total of 87 nodes, 724 edges, average local clustering coefficient of 0.631 and a PPI enrichment p-value of <1.0e-16. Using this PPI network, the top 10 hub genes were identified using the Cytohubba plugin on Cytoscape. The hub genes predicted based on MCC topological analysis are tabulated in Table 4. According to which, STAT3 and TP53 of leaf extract and CASP3 and CCND1 of seed extract have higher rank implying higher degree of significance. The leaf extract network featured TP53, STAT3, AKT1, BCL2, and IL6. These genes are key regulators of apoptosis, cell cycle progression, and survival signaling. The activation or inhibition of these genes can influence cellular fate, suggesting that the cytotoxic effect observed may be partially driven by modulation of the TP53-STAT3 axis, suppression of pro-survival genes like BCL2, and interference with cytokine signaling (IL6). The seed extract network had a few overlapping hub genes as well as distinct ones including STAT3, CASP3, EGFR, MAP2K1, and PPARG. CASP3, a crucial effector of apoptosis, along with growth signaling regulators such as EGFR and MAP2K1, suggests that seed extract may exert its effects through both apoptotic and proliferative control mechanisms.

GO and KEGG Pathway Enrichment Analysis

To gain insights into functions of the identified hub genes in OS, SR plot was used to summarize the top 10 significantly enriched GO terms such as BP (Biological Process), CC (Cellular Component) and MF (Molecular Function) as well as top

10 significantly enriched KEGG pathways. For leaf extract a total of 1499 BPs, 39 CCs, 68 MFs and 113 KEGG pathways were significantly enriched. These genes were found to be involved in biological processes such as T cell lineage commitment, negative regulation of production of miRNAs involved in gene silencing by miRNA and other terms as depicted in Figure 4A. These genes were also involved in cellular components such as nuclear envelope, nuclear membrane and others (Figure 4B); and molecular functions such as protein phosphatase 2A binding, general transcription initiation factor binding (Figure 4C). They were also enriched in pathways such as endocrine resistance, proteoglycans in cancer and other pathways (Figure 4D).

In parallel for seed extract a total of 1026 BPs, 28 CCs, 93 MFs and 112 KEGG pathways were significantly enriched. These genes were found to be involved in biological processes such as epithelial cell proliferation, regulation of production of miRNAs involved in gene silencing by miRNA and other terms as depicted in Figure 4E. These genes were also involved in cellular components such as transcription regulator complex, basal plasma membrane and others (Figure 4F), in molecular functions such as protein phosphatase binding, steroid hormone receptor binding as shown (Figure 4G) and in pathways such as proteoglycans in cancer, EGFR tyrosine kinase inhibitor resistance and other pathways (Figure 4H).

The enrichment analysis of hub genes from *Carica papaya* leaf and seed extracts unveiled both shared and unique biological effects appropriate to osteosarcoma. The leaf extract impacted a wider range of biological processes (1499 BPs), while the seed extract revealed a more targeted enrichment in molecular functions (93 MFs), suggesting systemic versus focused modulation, respectively.

Key GO terms enriched by both extracts such as T cell lineage commitment, epithelial cell

proliferation, and regulation of miRNA production involved in gene silencing highlight roles in immune regulation, cell proliferation, and epigenetic control, all critical to osteosarcoma pathogenesis (Wang *et al.*, 2019; Czarnecka *et al.*, 2020). Enriched molecular functions-protein phosphatase 2A binding, steroid hormone receptor binding, and general transcription initiation factor binding further specify dysregulation of signaling and hormone-mediated pathways (Rhim *et al.*, 1975; Bland *et al.*, 1997). Notably, both extracts significantly enriched KEGG pathways such as proteoglycans in cancer, endocrine resistance, and EGFR tyrosine kinase inhibitor resistance, all of which are known to drive OS progression and treatment resistance (Nikitovic *et al.*, 2008; Wang *et al.*, 2019; Hinoi *et al.*, 2024).

The KEGG pathway enrichment analysis of both the extracts revealed a range of cancer-associated and viral infection-related pathways such as Hepatitis B, Kaposi sarcoma-associated herpesvirus infection, prostate cancer, Human cytomegalovirus infection, Non-small cell lung cancer, Pancreatic cancer, Endometrial cancer, Breast cancer, Hepatitis C and Colorectal cancer. While these pathways are not specific to OS, their enrichment likely reflects the shared molecular mechanisms. This is expected, as many oncogenic pathways are broadly conserved and annotated across multiple diseases in KEGG databases. Notably, within the seed extract pathway analysis dataset, the KEGG pathway ‘osteoclast differentiation’ was specifically related to OS due to its role in bone remodeling and tumor-bone microenvironment interactions. The presence of this OS-relevant pathway, alongside more general cancer and viral related signatures, underscores the complexity of OS biology.

Table 4. Top 10 hub genes identified for leaf and seed extracts

Leaf extract			Seed extract		
Rank	Gene	PDB ID	Rank	Gene	PDB ID
1	STAT3	6NJS	1	CASP3	6BG0
2	TP53	5O1H	2	CCND1	6P8E
3	BCL2	6O0K	3	STAT3	6NJS
4	IL6	1ALU	4	ESR1	1A52
5	AKT1	7NH5	5	AKT1	7NH5
6	ESR1	1A52	6	PARP1	5DS3
7	SRC	1Y0J	7	MAP2K1	6NYB
8	CCND1	6P8E	8	EGFR	4WD5
9	MTOR	5GPG	9	PPARG	2PRG
10	MMP9	1GKC	10	ERBB2	3PP0

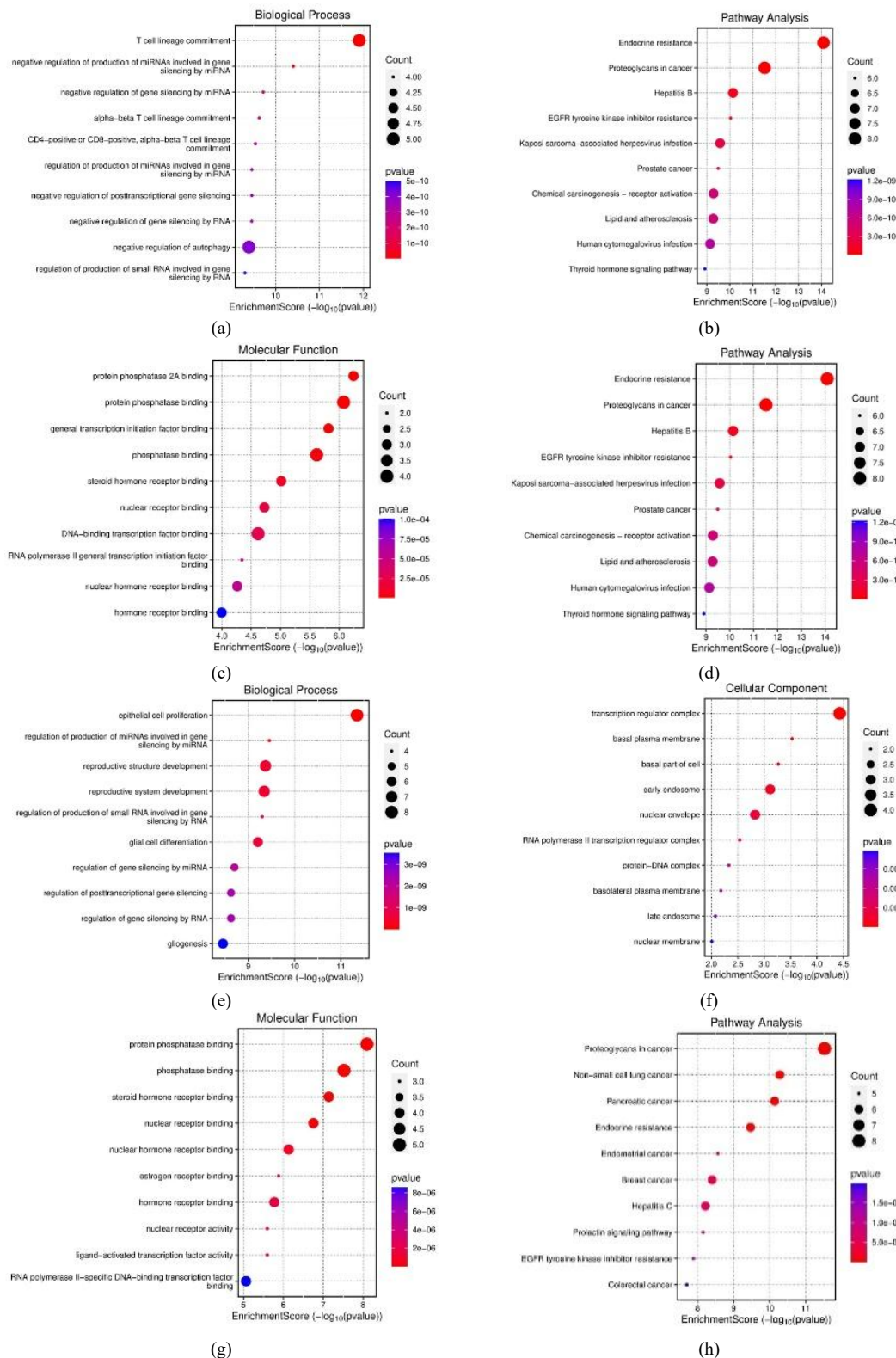


Figure 4. Top 10 significantly enriched GO terms and KEGG pathways associated with hub genes derived from leaf and seed extract - (A to 4) Leaf extract hub genes, A. Biological Process, B. Cellular Component, C. Molecular Function and D. Pathway Analysis; (E to H) Seed extract hub genes, E. Biological Process, F. Cellular Component, G. Molecular Function and H. Pathway Analysis

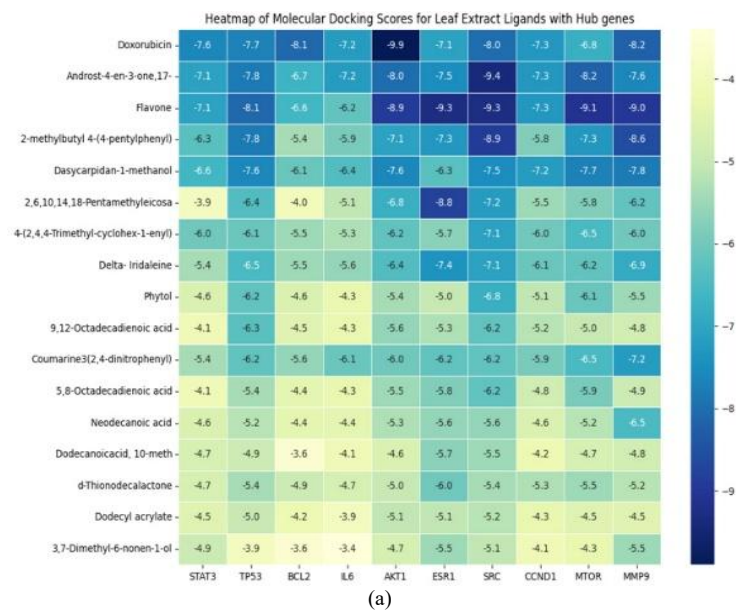
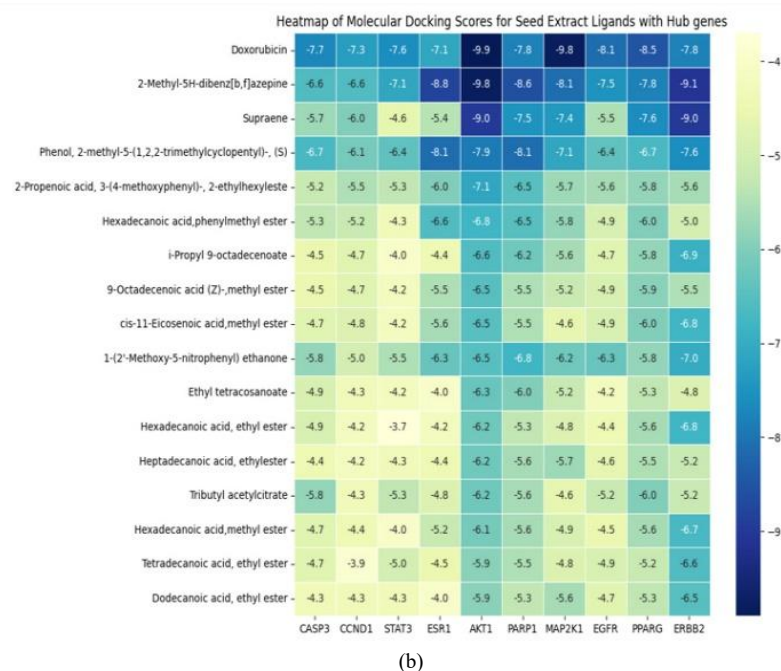


Figure 5. (a) Heatmap of Molecular Docking Scores for Leaf Extract Ligands with Hub genes (b) Heatmap of Molecular Docking Scores for Seed Extract Ligands with Hub genes

Molecular Docking

To identify the binding interactions between the identified hub genes and ligands, molecular docking was performed using PyRx (Figure 6). Typically, more negative docking scores indicate stronger binding affinities between ligands and genes. Among leaf extract ligands, Flavone exhibited a binding affinity of -9.3 kcal/mol for ESR1 and SRC and Androst-4-en-3-one, 17-

SRC (Figure 5A). Overexpression of SRC is observed in aggressive and metastatic forms of OS. This leads to proliferation, migration and resistance to apoptosis. Inhibitors like dasatinib, saracatinib, and SI-83 have validated SRC as a drug target in OS (Chen *et al.*, 2015). ESR1 is a receptor for hormone and DNA binding and activation of transcription. Inhibition of ESR1 expression can suppress cancer cell proliferation (Hu *et al.*, 2019). Targeting SRC and ESR1 could be two possible pathways through

which *Carica papaya* leaf extract displays cytotoxicity. Similarly for seed extract ligands, 2-Methyl-5H-dibenz[b,f]azepine exhibited a binding affinity of -9.8 kcal/mol for AKT1 and -9.1 kcal/mol for ERBB2 and ESR1 (-8.8 kcal/mol). Supraene exhibited a binding affinity of -9.0 kcal/mol for AKT1 and ERBB2 (Figure 5B). AKT1 promotes tumor cell proliferation, survival, migration, and metastasis and plays a crucial role in OS progression. Increased expression of AKT1 corresponds to poor response to chemotherapy and low survival rates (Zhu *et al.*, 2017). AKT1 is also involved in the mediation of RANK and HIF-1 signalling pathways, facilitating tumorigenesis and invasion (Zhang *et al.*, 2015). ERBB2 codes for a transmembrane tyrosine kinase receptor and is involved in tumor proliferation, migration, invasion, and metastasis in several cancers. Overexpression of ERBB2 showed poor survival rates in OS patients (Liu *et al.*, 2014). AKT1, ERBB2 and ESR1 show potential as gene targets in the treatment of OS.

MD Simulation

A molecular dynamics simulation of 100 ns was carried out to assess the stability of the plant-derived ligand in complex with the cancer-associated hub protein (Figure 7). The total potential energy was stable around -8.1×10^5 kJ/mol, signifying correct equilibration. RMSD values showed larger deviations in the first 50 ns, but then settled down to 3.5-4.0 nm in the final 40 ns of the simulation, indicating convergence into a dynamically stable conformational state. The radius of gyration remained between 2.6 and 4.8 nm, with smaller deviations in the second half of the simulation, as expected for structural stabilization. Hydrogen bond numbers showed stable bonds (usually 1-3 bonds) in the final phase of the simulation. RMSF values (0.6-2.6 nm) indicated localized flexibility without any signs of global destabilization. The complex showed conformational adaptation and subsequent stabilization into a dynamically stable bound state.

The molecular docking and MD simulation results were found to be consistent with the observed *in vitro* cytotoxic activity of the extracts against Saos-2 cells. Seed extract ligands and leaf extract ligands demonstrated strong docking affinities with key osteosarcoma-associated proteins. Similarly, MD simulation of the SRC-Androst-4-en-3-one,17-methoxy,3-methoxime complex demonstrated stable protein-ligand interactions throughout the 100 ns trajectory, supported by stabilized RMSD, radius of gyration, and persistent hydrogen bonding patterns.

We believe this shows that *Carica papaya* shows strong anticancer activity against Osteosarcoma cells and warrants further investigation into its mechanism. Future directions of this study include further investigation into apoptosis-related signaling pathways, and gene and protein expression analysis of identified hub genes. We present this study as a preliminary investigation and further *in silico* validation using MM-PBSA binding free energy calculations can help strengthen the understanding of the stability and binding efficiency of the identified ligand-protein complexes, thereby supporting their potential role as therapeutic candidates against osteosarcoma.

Conclusions

With a low overall survival rate and a poor prognosis, it is essential to explore additional treatment methods for OS. This study investigates the apoptotic property of the leaf and seed extract of *Carica papaya*, tested against the Saos-2 OS cell line. Among the ethanolic extracts, the seed extract was more cytotoxic (IC₅₀ = 0.78%) than the leaf extract (0.91%) towards Saos-2 cells. From *In silico* study, we identified major bioactives such as Flavones and Androst-4-en-3-one,17-methoxy from leaf extract with good binding affinity to SRC, and 2-Methyl-5H-dibenz[b,f]azepine and Supraene from seed extract with ESR1, PARP1, AKT1, ERBB2. Further study in drug designing and targeted medicinal chemistry can pave the way towards development of therapeutic agents for OS treatment.

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Author Contributions

Conceptualization: S.S., S.P., S.B., A.D.B.; Investigation: S.S., S.P., S.B.; Data Curation: S.S., S.P., S.B.; Writing - Original Draft: S.S., S.P., S.B.; Writing - Review & Editing: S.S., S.P., S.B., A.D.B.; Supervision: A.D.B. All authors have read and agreed to the published version of the manuscript.

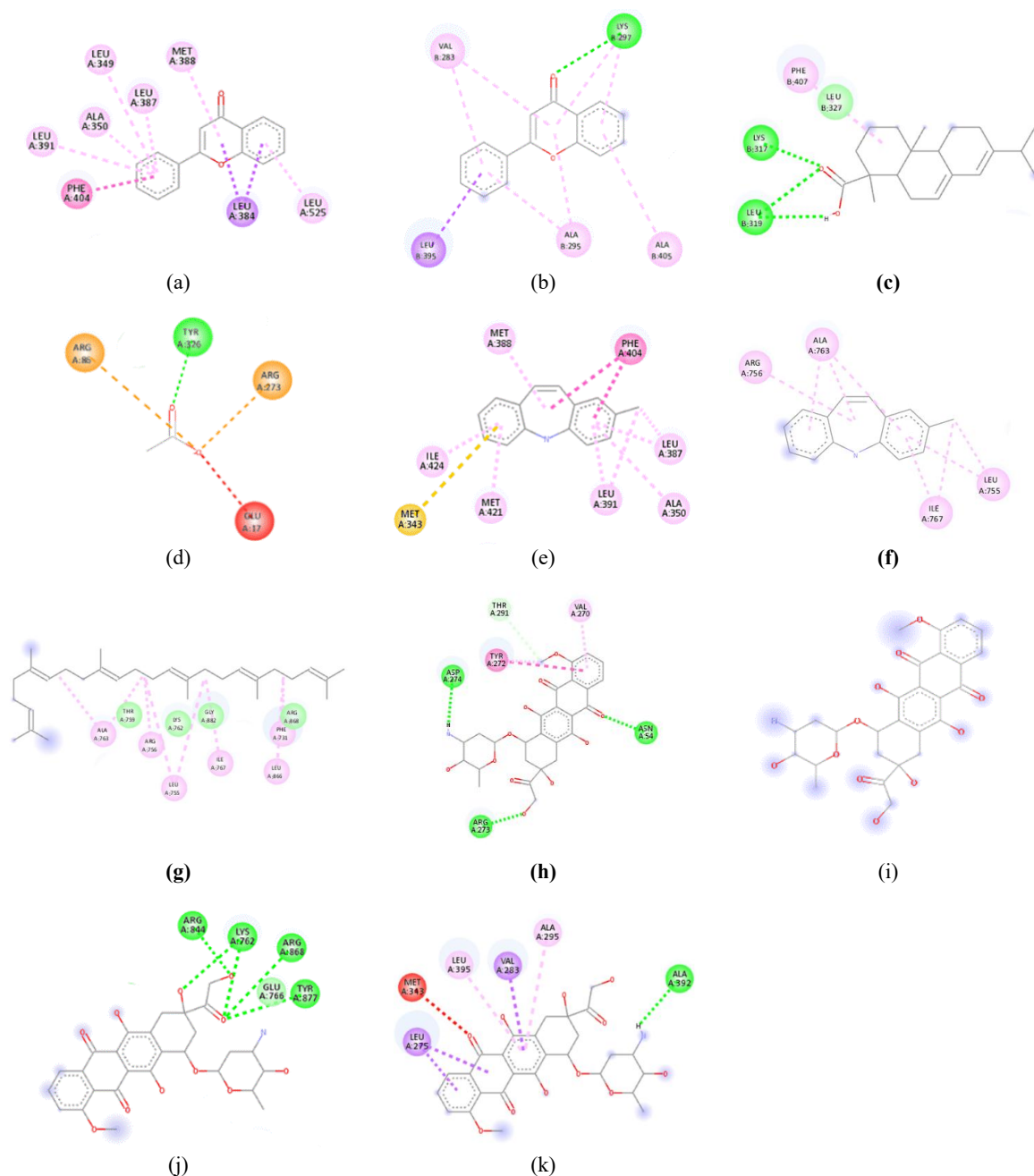


Figure 6. 2D Interaction Diagrams of Various Ligands with Target Proteins. A. Flavone with ESR1; B. Flavone with SRC; C. Androst-4-en-3-one,17-methoxy, 3-methoxime with SRC; D. 2-Methyl-5H-dibenz[b,f]azepine with AKT1; E. 2-Methyl-5H-dibenz[b,f]azepine with ESR1; F. 2-Methyl-5H-dibenz[b,f]azepine with ERBB2; G. Supraene with ERBB2; H. Doxorubicin with AKT1; I. Doxorubicin with ESR1; J. Doxorubicin with ERBB2; K. Doxorubicin with SRC

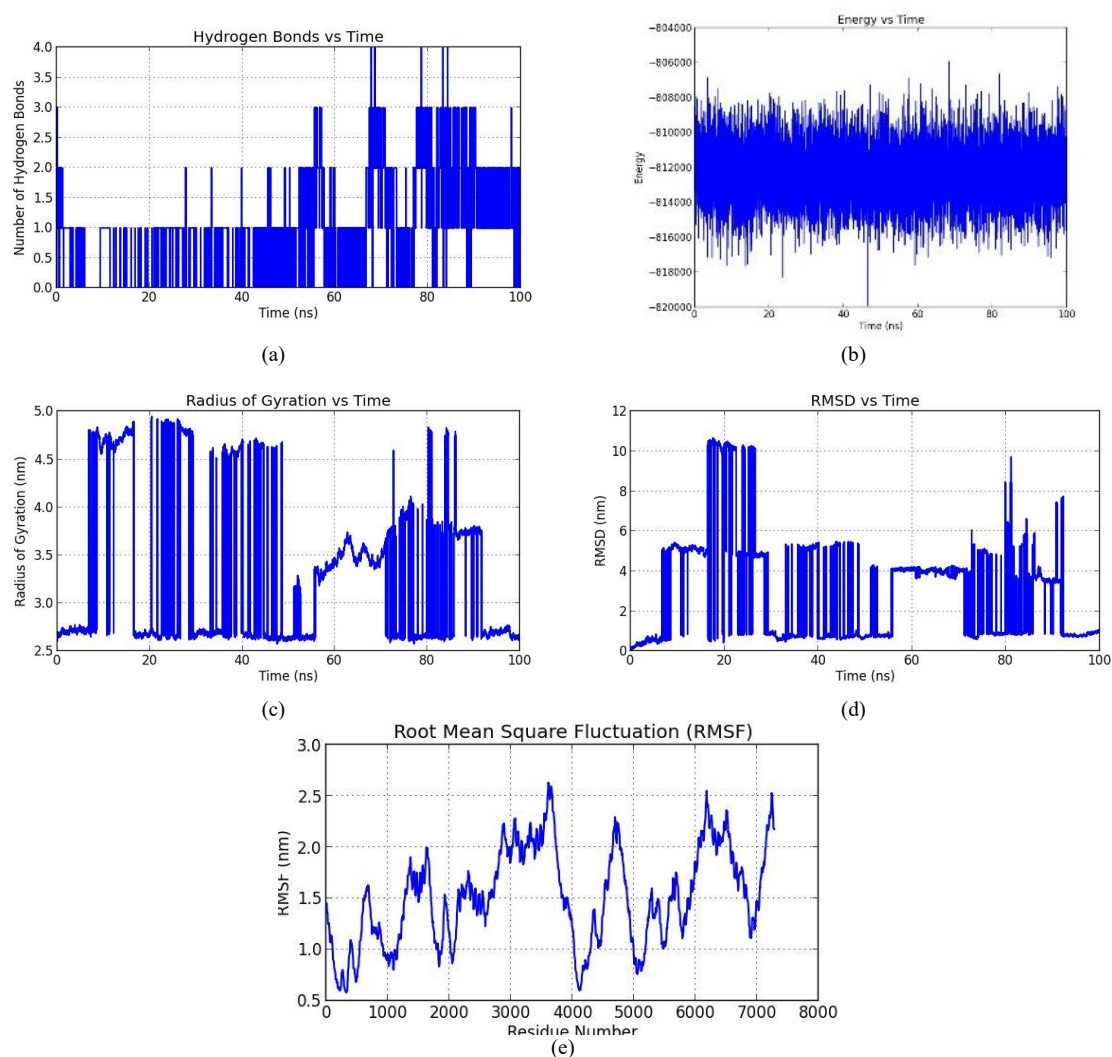


Figure 7. Molecular dynamics simulation analysis of the ligand Androst-4-en-3-one,17-methoxy, 3-methoxime in complex with the hub gene SRC (PDB ID: 1yoj) over a 100 ns trajectory. Shown are (A) number of hydrogen bonds as a function of simulation time. (B) total potential energy, (C) radius of gyration (Rg), (D) root mean square deviation (RMSD), (E) root mean square fluctuation (RMSF)

Ethics Statement

Ethical approval was not required for this study because it did not involve human participants, animals, identifiable personal data, or materials requiring institutional ethical review.

Conflict of Interest

We know of no conflicts of interest associated with this publication, and there has been no significant financial support for this work that could have influenced its outcome.

Declaration of Generative AI Use

The authors declare that no Generative AI tools were used in the preparation of this manuscript.

Data Availability Statement

The datasets analyzed during the current study are available in the PubMed and Google Scholar bibliographic databases.

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