

# Molecular docking and preliminary *in vitro* evaluation of antiandrogenic activity of methanolic extract of *Averrhoa bilimbi* L. fruits

Original Article

## Abstract:

Prostate cancer development is closely linked to the action of androgens such as testosterone and dihydrotestosterone. Treatments that block androgen receptor pathways remain central in managing both hormone-sensitive and castration-resistant stages. In this context, the fruit of *Averrhoa bilimbi* Linn., widely used in Southeast Asian traditional medicine, was examined for possible antiandrogenic activity. The fruit was extracted with a series of solvents, and the methanolic fraction was subjected to phytochemical testing, molecular docking, ADMET evaluation, and cytotoxicity assays against androgen-dependent LNCaP prostate cancer cells. Screening revealed high levels of flavonoids and phenolic compounds. Docking results showed strong binding of compounds such as eriocitrin and dihydromyricetin to androgen receptor residues, and ADMET data suggest good safety and absorption. Cytotoxicity testing demonstrated an  $IC_{50}$  of 186.79  $\mu\text{g/mL}$ , indicating a marked reduction in cell viability at higher concentrations. These findings highlight the extract's antiandrogenic and antiproliferative potential, supporting the need for further mechanistic and isolation studies.

## Key words:

*Averrhoa bilimbi* L., antiandrogenic activity, flavonoids, androgen receptor, molecular docking, MTT assay, LNCaP cell line

## Apstrakt:

**Molekularno dokovanje i preliminarne *in vitro* evaluacija antiandrogenog dejstva metanolnog ekstrakta plodova *Averrhoa bilimbi* Linn.**

Razvoj raka prostate usko je povezan sa delovanjem androgena kao što su testosteron i dihidrotestosteron. Tretmani koji blokiraju puteve androgenog receptora ostaju ključni u upravljanju i hormonski osetljivim i kastraciono rezistentnim stadijumima bolesti. U tom kontekstu, plod *Averrhoa bilimbi* Linn., široko korišćen u tradicionalnoj medicini jugoistočne Azije, ispitivan je na moguće antiandrogeno dejstvo. Plod je ekstrahovan nizom rastvarača, a metanolna frakcija je podvrgnuta fitohemijskoj analizi, molekularnom dokovanju, ADMET evaluaciji i citotoksičnim ispitivanjima na androgen-zavisnim LNCaP ćelijama raka prostate. Skrining je otkrio visok nivo flavonoida i fenolnih jedinjenja. Rezultati dokovanja pokazali su snažno vezivanje jedinjenja kao što su eriocitrin i dihidromiricetin za aminokiselinske ostatke androgenog receptora, a ADMET podaci ukazuju na dobru bezbednost i apsorpciju. Testiranje citotoksičnosti pokazalo je  $IC_{50}$  vrednost od 186,79  $\mu\text{g/mL}$ , što ukazuje na značajno smanjenje održivosti ćelija pri višim koncentracijama. Ovi nalazi ističu antiandrogeni i antiproliferativni potencijal ekstrakta, podržavajući potrebu za daljim mehanističkim istraživanjima i izolacijom aktivnih jedinjenja.

## Ključne reči:

*Averrhoa bilimbi* L., antiandrogeno dejstvo, flavonoidi, androgenski receptor, molekularno dokovanje, MTT test, LNCaP ćelijska linija

## Introduction

Cancer remains one of the most pressing global health issues, characterized by uncontrolled cell proliferation and the potential to invade adjacent tissues and metastasize to distant organs. As a

multifactorial disease, it is among the leading causes of mortality worldwide, with prostate cancer representing one of the most prevalent malignancies in men, second only to lung cancer in incidence and mortality rates (Sekhoacha et al., 2022). Prostate cancer accounts for more than

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10 million cases globally and is responsible for over 400,000 deaths annually, with projections estimating an increase to nearly 800,000 deaths per year by 2040 (James et al., 2024). The incidence is particularly high in developed regions such as North America, Europe, and Australia, whereas lower rates are observed in Asia, suggesting that genetic, environmental, and screening factors, including prostate-specific antigen (PSA) testing and digital rectal examination, contribute to these disparities. In developing nations, the growing burden of prostate cancer reflects improved healthcare access, record-keeping, and the adoption of lifestyle risk factors such as obesity, physical inactivity, and dietary changes (Hass et al., 2023).

The pathogenesis of prostate cancer is strongly linked to androgen signaling pathways. Androgens such as testosterone and its more active metabolite 5 $\alpha$ -dihydrotestosterone (DHT) are essential for prostate development and maintenance. Within prostate epithelial cells, testosterone is converted to DHT via 5 $\alpha$ -reductase. DHT then binds to cytoplasmic androgen receptors (ARs), and the resulting complex translocates into the nucleus, where it regulates transcription of genes involved in proliferation, differentiation, and survival (Galletti et al., 2017; Zakari et al., 2024). Evidence from genetic studies highlights associations between prostate cancer susceptibility and single-nucleotide polymorphisms in genes encoding enzymes for androgen biosynthesis and metabolism. The AR itself, located on the X chromosome, regulates several key genes, including MYC and KLK3 (PSA), thereby playing a central role in tumor growth (He et al., 2022).

Since the seminal findings of Huggins and Hodges in 1972, androgen deprivation therapy (ADT) has been the gold standard for advanced prostate cancer. ADT, achieved through surgical castration or pharmacological inhibition using luteinizing hormone-releasing hormone (LHRH) analogues or AR antagonists, initially provides significant therapeutic benefits. However, most patients develop resistance within two to three years, progressing to castration-resistant prostate cancer (CRPC) (He et al., 2022). Despite castrate serum testosterone levels, CRPC tumors maintain androgen dependence through mechanisms such as AR amplification, hypersensitivity, mutations, constitutively active splice variants, increased intra-tumoral androgen synthesis, and activation of alternative oncogenic pathways (Karantanos et al., 2017). Novel AR-targeted therapies, including enzalutamide and abiraterone acetate, have provided additional clinical benefits, but resistance inevitably emerges, underscoring the urgent need for alternative therapeutic strategies (Karantanos et al., 2017).

Natural products, particularly plant-derived compounds, have gained attention for their anticancer potential due to their multi-targeted mechanisms and lower toxicity profiles (Wenzel et al., 2000). Flavonoids, a diverse group of plant secondary metabolites, have been shown to regulate proliferation, apoptosis, angiogenesis, and metastasis, while also demonstrating antiandrogenic properties (Sunilson et al., 2010). Screening studies revealed that among 72 flavonoids tested, 22 significantly inhibited PSA production in breast (BT-474) and prostate (PC-3[AR]) cancer cell lines (Chen et al., 2001). Compounds such as apigenin, acacetin, and tangeretin exhibit notable anti-prostate cancer activity, largely through modulation of the AR pathway and induction of apoptosis (Jeong et al., 2023). Similarly, natural products including curcumin, resveratrol, quercetin, and genistein have shown activity against CRPC models *in vitro* and *in vivo* (Izzo et al., 2020). These findings highlight the potential of phytochemicals as candidates for novel antiandrogenic therapeutics.

*Averrhoa bilimbi* L. (*Oxalidaceae*), commonly known as bilimbi or cucumber tree, is a tropical plant widely cultivated in Southeast Asia and traditionally used to treat diabetes, inflammation, and rheumatism (Chau et al., 2023). Its fruits are rich in flavonoids, including quercetin, myricetin, and luteolin, as well as phenolic acids, tannins, saponins, and organic acids. Pharmacological studies have demonstrated diverse activities, including antidiabetic, antihypertensive, antimicrobial, antioxidant, and hepatoprotective effects (Setyawan et al., 2021; Chau et al., 2023). Of particular relevance, methanolic extracts of *A. bilimbi* fruits have shown cytotoxic activity against several cancer cell lines, with reported IC<sub>50</sub> values of 154.9  $\mu$ g/mL against breast cancer cells (Merin Abraham, 2016). These observations suggest that *A. bilimbi* may possess unexplored antiandrogenic and anticancer properties that warrant further investigation.

The incorporation of computational drug discovery methods, such as molecular docking, has transformed the early stages of drug development by enabling the prediction of ligand–receptor interactions and structure–activity relationships. This approach allows high-throughput virtual screening of natural compounds, guiding experimental validation while reducing costs and time (Bhati et al., 2019). Molecular docking has been effectively applied in nutraceutical research, where natural AR inhibitors, such as compounds from *Vitis vinifera*, have demonstrated promising docking scores compared to established AR antagonists (Ajiboye et al., 2024). Docking studies are often complemented by *in silico* ADMET (Absorption, Distribution,

Metabolism, Excretion, and Toxicity) predictions and pharmacophore modeling to assess drug-likeness and therapeutic potential (Huang et al., 2025).

For experimental validation, LNCaP (Lymph Node Carcinoma of the Prostate) cells provide a reliable model for androgen-dependent prostate cancer research. Derived from a lymph node metastasis, these cells retain androgen sensitivity through functional AR expression and secretion of prostate-specific markers such as PSA and prostatic acid phosphatase (Chowdhury et al., 2012). Cellular assays, including the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay, are routinely used to assess cytotoxicity by quantifying mitochondrial enzyme activity and cellular viability, providing standard tools for evaluating anticancer efficacy (Li et al., 2004).

Despite advances in prostate cancer treatment, there remains a critical need to explore natural sources of novel antiandrogenic compounds. *A. bilimbi*, with its documented traditional applications and emerging bioactivity, is a promising candidate. By integrating molecular docking predictions with in vitro validation in LNCaP cells, this study aims to systematically evaluate the antiandrogenic and cytotoxic potential of *A. bilimbi* fruit extracts. Such an approach bridges traditional medicine with modern computational and experimental tools, potentially identifying phytoconstituents with therapeutic relevance for androgen-dependent prostate cancer and laying the groundwork for future development of natural product-derived adjunct therapies.

## Materials and Methods

### *Plant material collection and authentication*

Fresh fruits of *A. bilimbi* were collected from Palakkad district, Kerala, India, during the fruiting season. The plant material was botanically identified and authenticated by Dr. M.U. Sharief, Scientist 'F' and Head of Office, Southern Regional Centre, Botanical Survey of India, Coimbatore, Tamil Nadu, India. A voucher specimen was deposited with reference number BSI/SRC/5/23/2024-25/Tech./446 for future reference.

### *Plant Extract Preparation*

#### **Fruit processing and drying**

The collected *Averrhoa bilimbi* Linn fruits were thoroughly washed with distilled water to remove surface contaminants and debris. The fruits were then sliced into uniform pieces (approximately 2-3 mm thickness) and subjected to shade drying at ambient temperature ( $25 \pm 2$  °C) for 8-10 days until complete moisture removal was achieved. The dried fruit material was mechanically ground

using a domestic grinder to obtain a coarse powder, which was passed through a 40-mesh sieve to ensure uniform particle size.

### **Sequential solvent extraction**

Sequential extraction was performed using the Soxhlet apparatus following standard protocols (Setyawan et al., 2021). Thirty-five grams of coarsely powdered fruit material was subjected to continuous extraction using solvents of increasing polarity: n-hexane, chloroform, and methanol (350 mL each). The extraction temperature was maintained at 50-60 °C based on the respective solvent boiling points. Each extraction cycle was continued for 6-8 hours until the solvent in the siphon tube became colorless, indicating complete extraction.

The extracts were concentrated to dryness using a rotary evaporator (Buchi R-300, Switzerland) under reduced pressure at 40 °C. The dried extracts were weighed to calculate extraction yields and stored at 4 °C in amber-colored glass vials until further use. The methanolic extract, showing the highest yield and preliminary bioactivity, was selected for subsequent investigations (John & Jupudi, 2025).

### **Phytochemical screening**

Qualitative phytochemical analysis of the n-hexane, chloroform, and methanolic extracts was performed following standard procedures (Chowdhury et al., 2012). The presence of major classes of secondary metabolites (alkaloids, flavonoids, coumarins, saponins, phenolics, tannins, triterpenoids, glycosides, proteins, carbohydrates, and steroids) was assessed using the corresponding classical tests. All assays were conducted in triplicate, and results were recorded as positive or negative based on characteristic reactions (John & Jupudi, 2025).

### **Ligand Selection for Molecular Docking**

Phytochemicals previously identified in *A. bilimbi* methanolic extracts (Chau et al., 2023) were selected for molecular docking analysis, including quercetin, myricetin, luteolin, eriocitrin, and dihydromyricetin.

### *Molecular Docking Studies*

#### **Target protein preparation**

The three-dimensional crystal structure of the human androgen receptor ligand-binding domain (PDB ID: 5T8E) was retrieved from the RCSB Protein Data Bank (www.rcsb.org). The protein structure was prepared using BIOVIA Discovery Studio (version 16.1.0.15350, Dassault Systems, USA) by removing crystallographic water molecules, heteroatoms, and bound ligands. Missing hydrogen atoms were added, and the structure was energy-minimized to resolve steric clashes. Kollman charges were assigned to the protein atoms for subsequent docking calculations.

## Ligand preparation

A comprehensive library of 100 phytochemicals previously reported from *A. bilimbi* was compiled from literature and phytochemical databases. The two-dimensional structures of these compounds were retrieved from the PubChem database (<https://pubchem.ncbi.nlm.nih.gov/>) in SDF format. The structures were converted to PDB format using Open Babel (version 3.1.1) and subsequently prepared using PyRx-Python Prescription (version 0.8). Gasteiger charges were assigned, and hydrogen atoms were added to optimize the molecular geometry.

## Docking protocol and validation

Molecular docking simulations were performed using AutoDock Vina integrated within the PyRx platform (Li et al., 2004). The binding site was defined based on the co-crystallized ligand position, with a grid box dimension of 20×20×20 Å centered on the ligand-binding pocket. Docking calculations were performed using a Lamarckian Genetic Algorithm with 100 independent runs per ligand to ensure statistical reliability. The docking protocol was validated by re-docking the co-crystallized ligand into the binding site and calculating the root-mean-square deviation (RMSD) between the original and docked poses. An RMSD value  $\leq 2.0$  Å was considered acceptable for validation. The androgen receptor binding pocket was analyzed using the CASTp server (<http://sts.bioe.uic.edu/castp/>) to identify druggable cavities and characterize the most favorable binding site based on volume, surface area, and amino acid composition.

## Data visualization and analysis

Molecular docking results were visualized using BIOVIA Discovery Studio and PyMOL (version 2.5). Binding interactions were analyzed in both 2D and 3D formats, with hydrogen bonds, hydrophobic interactions, and electrostatic interactions identified and documented. Binding energy calculations and interaction analysis were performed using standard protocols, and results were compared with reference compounds, including enzalutamide.

## Drug-Likeness and ADMET Prediction

### Lipinski's rule of five assessment

The drug-likeness of selected compounds was evaluated using Lipinski's Rule of Five criteria. Molecular descriptors, including molecular weight (MW), octanol-water partition coefficient (LogP), number of hydrogen bond donors (HBD), and hydrogen bond acceptors (HBA), were calculated using the Molecule Property Calculator ([www.swissadmet.com](http://www.swissadmet.com)). Compounds violating more than one Lipinski parameter were excluded from further

analysis (John and Jupudi, 2025).

## ADMET profiling

Absorption, Distribution, Metabolism, Excretion, and Toxicity (ADMET) properties were predicted using the ADMETSar database web server. Parameters assessed included human intestinal absorption (HIA), Caco-2 cell permeability, blood-brain barrier (BBB) penetration, plasma protein binding (PPB), cytochrome P450 enzyme inhibition, Ames mutagenicity, and rodent carcinogenicity. Results were interpreted according to established drug development criteria.

## Pharmacophore modeling

Pharmacophore-based virtual screening was conducted using LigandScout (version 4.4, Inte:Ligand GmbH, Austria). An active/decoy dataset for the androgen receptor was obtained from the DUD-E database (<https://dude.docking.org/targets>). Ten pharmacophore models were generated based on known androgen receptor ligands, and their performance was evaluated using Receiver Operating Characteristic (ROC) curve analysis. The best-performing model was selected for screening the *A. bilimbi* compound library.

## In vitro cell culture studies

### Cell line and culture conditions

The LNCaP human prostate adenocarcinoma cell line was procured from the National Centre for Cell Sciences (NCCS), Pune, India. Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM; Sigma-Aldrich, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, and 2.5 µg/mL amphotericin B (all from Merck, Germany). Cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO<sub>2</sub> using a CO<sub>2</sub> incubator (NBS Eppendorf, Germany).

### Cell seeding and treatment

Exponentially growing cells from passages 15-25 were used for all experiments. Cells were harvested using 0.25% trypsin-EDTA solution and counted using a hemocytometer. Cell viability was confirmed by trypan blue exclusion assay (>95% viability required). For MTT assays, cells were seeded at a density of  $5 \times 10^3$  cells per well in 96-well tissue culture plates in 100 µL complete medium and incubated for 24 hours to allow adherence.

## Extract preparation for cell culture

The methanolic extract was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, USA) to prepare a stock solution of 10 mg/mL. Working concentrations

(6.25, 12.5, 25, 50, and 100 µg/mL) were prepared by serial dilution in complete DMEM medium. The final DMSO concentration across all treatments was maintained below 0.5% to avoid cytotoxicity.

#### MTT cytotoxicity assay

Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (John and Jupudi, 2025). After 24 hours of incubation, the culture medium was replaced with fresh medium containing different concentrations of the methanolic extract. Untreated control cells received medium with an equivalent DMSO concentration. After 48 hours of treatment, 20 µL of MTT solution (5 mg/mL in phosphate-buffered saline, filter-sterilized) was added to each well, and plates were incubated for 4 hours at 37°C.

The MTT-containing medium was carefully removed, and 100 µL of DMSO was added to each well to dissolve the formazan crystals. Plates were gently shaken for 10 minutes to ensure complete dissolution. Absorbance was measured at 540 nm using a microplate reader (BioTek ELx800, USA), with 630 nm as the reference wavelength.

#### Statistical analysis

All experiments were performed in triplicate with three independent biological replicates. Cell viability data are expressed as mean ± standard deviation (SD). The half-maximal inhibitory concentration (IC<sub>50</sub>) values were calculated using non-linear regression analysis with GraphPad Prism software (version 8.0). Statistical significance was determined using one-way ANOVA followed by Tukey's post hoc test;  $p < 0.05$  was considered statistically significant.

## Results and discussion

#### Phytochemical screening

The freshly prepared crude extracts of *Averrhoa bilimbi* Linn fruits were qualitatively screened for phytochemicals (Tab. 1). The methanolic extract showed the highest number of positive results, confirming its suitability for further investigation.

#### Evaluation of molecular properties and selection of potential ligands

The three-dimensional crystal structure of the human androgen receptor (PDB ID: 5T8E) was obtained from the RCSB Protein Data Bank. This structure comprises 546 amino acid residues, 3,159 atoms, 45 heteroatoms, and 202 solvent molecules, and was chosen as the target for docking studies. To assess the molecular characteristics of the selected ligands, the Mol Inspiration online tool was employed. Parameters such as logP, molecular weight, hydrogen bond donors, and hydrogen bond

**Table 1.** Phytochemical analysis of *Averrhoa bilimbi* L. (petroleum ether, chloroform, metanol)

| Fruit extracts |                 |            |          |
|----------------|-----------------|------------|----------|
| Tests          | Petroleum ether | Chloroform | Methanol |
| Alkaloids      | -               | -          | -        |
| Flavonoids     | -               | +          | +        |
| Coumarins      |                 | +          | +        |
| Saponin        | +               | -          | +        |
| Phenols        | -               | +          | +        |

acceptors were calculated and evaluated against Lipinski's Rule of Five, a widely accepted criterion for predicting oral drug-likeness.

Based on this screening, a set of promising ligands was shortlisted, including hygroline, sedridine, angelicain, naphthyl-n-propylcarbamate, hexadecenoic acid, decosenamide, anapheline, austalide, corchoriside, dihydromyricetin, eriocitrin, and hydroxycitric acid lactone. The corresponding physicochemical filter values for these compounds are summarised (Tab. 2., Tab. 3).

#### Lipinski's Rule of Five, bioactivity score, druglikeness, solubility, and toxicity

**Table 2.** Identified active constituents in *Averrhoa bilimbi* L.

| Name of the compound         | Classification of phytoconstituents |
|------------------------------|-------------------------------------|
| Standard                     | Enzalutamine                        |
| Hygroline                    | Pyrrolidine alkaloid                |
| Sedridine                    | Piperidine Alkaloid                 |
| Angelicain                   | Phenolic Constituent                |
| Naphthyl-n-propylcarbamate   | Carbamate Derivative                |
| Hexadecenoic acid            | Fatty Acid                          |
| Decosenamide                 | Fatty Acid Amide                    |
| Anapheline                   | Alkaloid                            |
| Austalide                    | Terpenoid                           |
| Corchoriside                 | Glycoside                           |
| Dihydro myricetin            | Flavonoid                           |
| Eriocitrin                   | Flavonoid Glycoside                 |
| Hydroxy- citric acid lactone | Organic acid                        |

Table 3. The ADMET Values of compounds

| Compounds Name                                | Molecular Formula | Molecular weight | H-Bond acceptors | H-Bond donors | Molar Refractivity | Log Po/w | Solubility       | GI Absorption | BBB permeant |
|-----------------------------------------------|-------------------|------------------|------------------|---------------|--------------------|----------|------------------|---------------|--------------|
| Enzalutamide standard                         | C21H16F4N4O25     | 464.43           | 2                | 2             | 4                  | 1.1      | Soluble          | High          | No           |
| Hygroline                                     | C8H17NO           | 143.22           | 2                | 1             | 46.43              | 1.1      | Soluble          | High          | No           |
| (+)-Sedridine[2-(2-hydroxypropyl) piperidine] | C8H17NO           | 143.23           | 2                | 2             | 46.33              | 1.14     | Soluble          | High          | No           |
| (s)-angelicin (furancoumarin)                 | C16H18O6          | 306.31           | 6                | 3             | 80.41              | 1.54     | Soluble          | High          | No           |
| Corchoriside B (steroids)                     | C29H42O8          | 518.64           | 8                | 4             | 135.51             | 2.13     | Soluble          | High          | No           |
| Naphthyl-n-propylcarbamate                    | C14H15NO2         | 229.27           | 2                | 1             | 2                  | 3        | Moderate Soluble | High          | Yes          |
| Anapheline ( alkaloid)                        | C13H24N2O         | 224.34           | 3                | 2             | 74.01              | 1.64     | Soluble          | High          | Yes          |
| Hexadecenic acid                              | C17H32O2          | 268.43           | 2                | 0             | 5,24               | 5.2      | Poorly Soluble   | High          | Yes          |
| Decosenamide                                  | C22H43NO          | 337.58           | 1                | 1             | 6.7                | 110.3    | Poorly Soluble   | High          | No           |
| 2- hydroxyestrogen                            | C18H22O3          | 286.37           | 3                | 2             | 82.09              | 2.9      | Soluble          | High          | Yes          |
| Austalide K (polycyclic meroterpenoids)       | C25H32O5          | 412.52           | 5                | 0             | 114.55             | 4.38     | Poorly Soluble   | High          | Yes          |
| (-)-Hygroline(alkaloids)                      | C8H17NO           | 143.23           | 2                | 2             | 46.43              | 1.1      | Soluble          | High          | No           |
| Dihydromyricetin                              | C15H12O8          | 320.25           | 8                | 6             | 76.78              | 0.22     | Soluble          | Low           | No           |
| Austalide K (polycyclic meroterpenoids)       | C25H32O5          | 412.52           | 5                | 0             | 114.55             | 4.38     | Poorly Soluble   | High          | Yes          |
| Eriocitrin                                    | C27H32O15         | 596.53           | 15               | 9             | 136.94             | 1.9      | S Soluble        | Low           | No           |

We evaluated the drug-likeness and molecular properties of isolated compounds using the SwissADME platform, based on Lipinski's Rule of Five. Most compounds met all criteria, indicating potential for good oral bioavailability. Only the ligands eriocitrin and dihydromyricetin showed minor violations of hydrogen bond acceptors and donors. All compounds had LogP values below 5, suggesting suitable lipophilicity for membrane permeability. The TPSA values ranged from 59.57 to 99.94 Å<sup>2</sup>, well within the acceptable limit, indicating favorable absorption. Rotatable bonds were also within limits, supporting conformational stability. Bioactivity scores indicated moderate to strong interactions with key drug targets (GPCRs, kinases, proteases, enzymes), with several ligands outperforming a standard drug. Using SwissADME, all compounds had drug-likeness scores, with eriocitrin scoring the highest, suggesting strong lead potential. Solubility was good across all ligands (lower ClogS values), better than that of the reference drug, enzalutamide.

Pharmacokinetic and toxicity profiles of the isolated compounds were predicted using the admetSAR database (**Tab. 4**). The compounds demonstrated good blood–brain barrier penetration and human intestinal absorption, though Caco-2 permeability was absent. CYP inhibition was minimal, and none of the compounds acted as substrates or inhibitors of major CYP enzymes,

indicating favourable metabolic behaviour. AMET toxicity was not observed in any of the compounds, which were predicted to be non-carcinogenic. Rat acute toxicity (LD<sub>50</sub>) values ranged from 1.72 to 2.91 mol/kg, suggesting moderate safety margins.

### Exploration of binding interactions through docking studies

#### Binding sites and docking studies

The catalytic pockets of the target protein, human androgen receptor, were analysed using the SiteMap tool. Protein preparation involved the Protein Preparation Wizard, which removed non-hydrogen-bonded crystallographic water molecules. Missing side-chain atoms were added, and structural breaks were repaired using Prime. Hydrogen bonds were optimised to reflect a pH of 7.0, accounting for the appropriate ionisation states of acidic and basic amino acid residues. The catalytic site of the human androgen receptor was predicted using the CASTp program, which accurately calculated the area and volume of each pocket. The most favourable binding site was identified as pocket 36, with a volume of 1178.9 Å<sup>3</sup> and an area of 901.1 Å<sup>2</sup>, comprising 36 amino acid residues, including PRO671 and LUE672. PHE673, ASN675, VAL676, GLU678, and ALA679. The same binding site and amino acid residues were observed in this study.

Docking program validation was performed by calculating the Root Mean Square Deviation (RMSD)

**Table 4.** Prediction of ADMET profile of the title compounds

| Compound                   | BBB  | Human initial Absorption | Caco-2 permeability | CYP inhibitory promiscuity | AMES TOXICITY | Carcinogenicity | Rat acute toxicity LD50 mol/Kg |
|----------------------------|------|--------------------------|---------------------|----------------------------|---------------|-----------------|--------------------------------|
| Hygroline                  | BBB+ | HIA+                     | Caco2+              | Low                        | Non toxic     | Non-Carcinogens | 2.38                           |
| Sedridine                  | BBB+ | HIA+                     | Caco2+              | Low                        | Non toxic     | Non-Carcinogens | 1.7                            |
| Angelicaia                 | BBB+ | HIA+                     | Caco2-              | Low                        | Non toxic     | Non-Carcinogens | 2.49                           |
| Naphthyl-n-propylcarbamate | BBB+ | HIA+                     | Caco2-              | Low                        | Non toxic     | Non-Carcinogens | 4.11                           |
| Hexadecenic acid           | BBB+ | HIA+                     | Caco2+              | High                       | Non toxic     | Non-Carcinogens | 2.46                           |
| Decosenamide               | BBB+ | HIA+                     | Caco2+              | Low                        | Non toxic     | Non-Carcinogens | 2.01                           |
| Anapheline                 | BBB+ | HIA+                     | Caco2+              | Low                        | Non toxic     | Non-Carcinogens | 1.88                           |
| Austalide                  | BBB+ | HIA+                     | Caco2+              | Low                        | Non toxic     | Non-Carcinogens | 2.07                           |
| Corchoriside               | BBB+ | HIA+                     | Caco2+              | Low                        | Non toxic     | Non-Carcinogens | 1.88                           |
| Dihydro myricetin          | BBB+ | HIA+                     | Caco2+              | Low                        | Non toxic     | Non-Carcinogens | 2.9                            |
| Eriocitrin                 | BBB+ | HIA+                     | Caco2-              | Low                        | Non toxic     | Non-Carcinogens | 2.49                           |

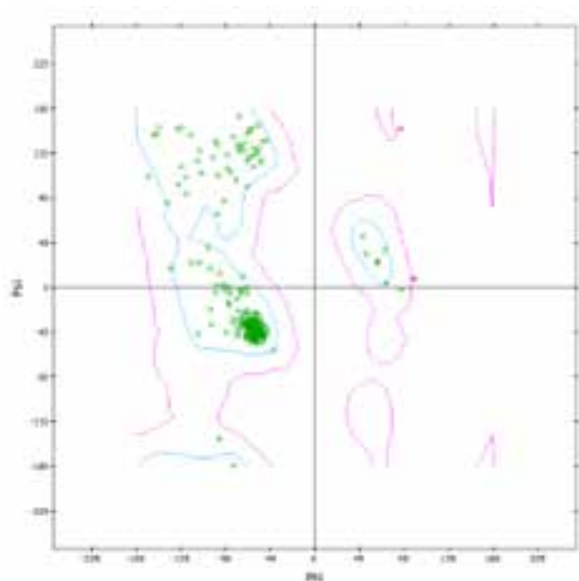
between the co-crystallised pose of the native ligand and its redocked pose. The RMSD was 0.7505 Å, confirming accurate reproduction of the ligand's position within the binding domain. This aligns with the standard that RMSD values should not exceed 2 Å for reliable docking validation. Molecular docking studies explored the binding interactions between the human androgen receptor and hygroline, sedridine, angelicain, naphthyl-n-propylcarbamate, hexadecenoic acid, decosenamide, anapheline, austalide, corchoriside, dihydromyricetin, eriocitrin,

and hydroxycitric acid lactone, alongside the native ligand and reference drug enzalutamide. The binding affinities of the designed compounds were evaluated using G-scores, which ranged from -5 kcal/mol to -15.2 kcal/mol, indicating strong interactions with the target receptor (**Tab. 5**). Among them, Eriocitrin achieved the highest binding affinity, with a G-score of -9.2 kcal/mol. The Ramachandran plot of the androgen receptor confirmed that all residues were within the favoured regions (**Fig. 1**).

Eriocitrin showed a binding energy of -9.2 kcal/

**Table 5.** Identified active constituents in *Averrhoa bilimbi* Linn. and their docking scores with androgen receptor

| Protein | Name of the compounds       | Binding Affinity | rmsd/ub | rmsd/lb | Binding site Residues                                  |
|---------|-----------------------------|------------------|---------|---------|--------------------------------------------------------|
| 5T8E    | Enzalutamide                | -6.1             | 25.61   | 22.808  | SER791, ARG788, GLN792, PHE754, TRP796, HIS789         |
| 5T8E    | Hygroline                   | -7.5             | 39.306  | 37.794  | GLN792, GLU793, TRP796, HIS789                         |
| 5T8E    | Sedridine                   | -4               | 25.288  | 23.852  | SER782, ARG779, GLN792, PHE876, TRP796, HIS789         |
| 5T8E    | Angelicain                  | -6.6             | 30.071  | 27.759  | SER791, ARG788, GLN792, PHE754, TRP796, HIS789         |
| 5T8E    | Naphthyl-n-propylcarbamate  | -6.3             | 25.056  | 23.302  | SER782, ARG785, GLU872, PHE876, TYR781, CYS784, MET780 |
| 5T8E    | Hexadecenoic acid           | -5.5             | 3.17    | 2.49    | SER791, ARG788, GLN792, PHE754, TRP796, HIS789         |
| 5T8E    | Decosenamide                | -7.4             | 32.509  | 29.384  | SER778, ARG786, GLN783, PHE876, VAL889, CYS784, LEU880 |
| 5T8E    | Anapheline                  | -6.6             | 15.706  | 12.564  | SER791, ARG788, GLN792, PHE754, TRP796, HIS789         |
| 5T8E    | Austalide                   | -7.6             | 32.05   | 30.834  | SER782, ARG779, GLN783, PHE876, TRP781, VAL785         |
| 5T8E    | Corchoriside                | -7.6             | 40.994  | 39.173  | SER782, ARG783, GLN783, PHE876, TRP781, MET786         |
| 5T8E    | Dihydro myricetin           | -6.9             | 5.735   | 2.77    | SER791, ARG788, GLN792, PHE754, TRP796, HIS789         |
| 5T8E    | Eriocitrin                  | -9.2             | 19.796  | 15.937  | SER791, ARG788, GLN783, LEU762, MET787, 780, HIS789    |
| 5T8E    | Hydroxy-citric acid lactone | -5.4             | 3.17    | 2.49    | SER791, ARG788, GLN792, PHE754, TRP796, HIS789         |



**Fig. 1.** The Ramachandran plot of the human androgen receptor showing the distribution of amino acid residues in favorable and allowed regions

mol and interacted with residues SER791, ARG788, GLN783, LEU762, MET787, MET780, and HIS789 through hydrogen bonds. Austalide had a binding energy of -7.6 kcal/mol and interacted with SER782, ARG779, GLN783, PHE876, TRP781, and VAL785 via hydrogen bonding. Decosenamide displayed a binding energy of -7.4 kcal/mol, forming hydrogen bonds with residues SER778, ARG786, GLN783, PHE876, VAL889, CYC784, and LEU880. Hygroline demonstrated the strongest binding energy of -7.5 kcal/mol, interacting with GLN792, GLU793, TRP796, and HIS789. In contrast (**Fig. 2**), Eriocitrin had a strong binding energy of -9.2 kcal/mol, forming hydrogen bonds with residues SER791, ARG788, GLN783, LEU762, MET787, MET780, and HIS789 (**Fig. 3**).

### Anticancer assay

#### MTT assay method

The anticancer activity was evaluated using an MTT assay by exposing cells to a range of concentrations of the sample (100 µg/mL, 50 µg/mL, 25 µg/mL, 12.5 µg/mL, and 6.25 µg/mL), with a cell culture media control. Cell viability was then assessed by measuring absorbance at

540 nm using a microplate reader. The methanol fruit extract showed declining cell viability with increasing extract concentration, with an IC<sub>50</sub> value of 186.79 µg/mL. The percentage viability of the extract in the LNCaP Human prostate cancer cell line was shown (**Fig. 4**).

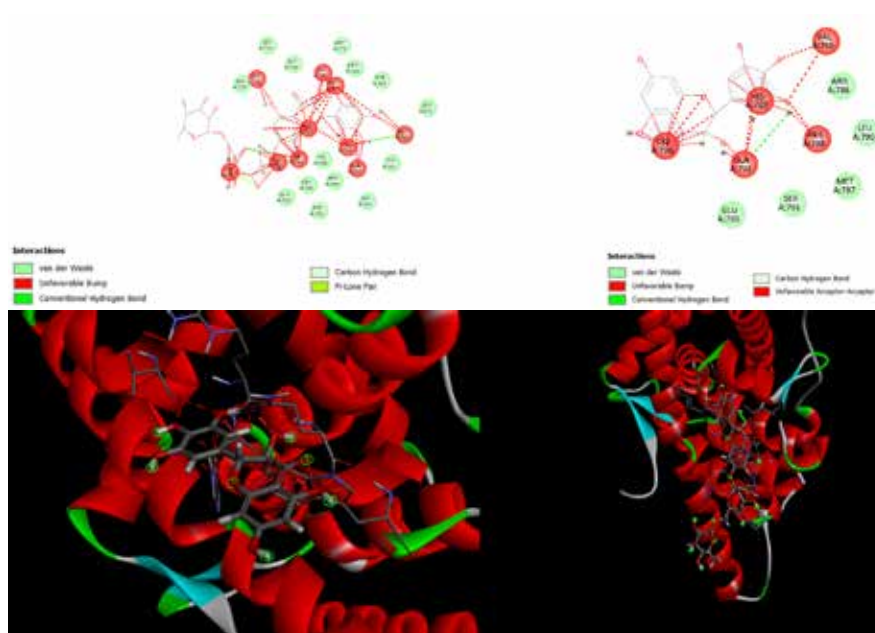
### Results integration and interpretation

The current study combined molecular docking and exploratory in vitro tests to assess the antiandrogenic potential of methanolic extracts from *Averrhoa bilimbi* fruits. The molecular docking analysis against the ligand-binding domain of the human androgen receptor (PDB ID: 5T8E) identified a number of flavonoid glycosides and related phytoconstituents that possess binding affinities comparable to or higher than the standard antagonist enzalutamide.

### Docking outcomes and lead compound identification

Eriocitrin achieved the best docking score (-9.2 kcal/mol), forming multiple hydrogen bonds with important residues—SER791, ARG788, GLN783, and LEU762—at the receptor hydrophobic pocket. This binding pattern is suggestive of competitive antagonism at the androgen-binding site, which is consistent with previous reports of antiandrogenic activity of citrus-derived flavonoids (Bhati et al., 2019). The rigorous docking validation (RMSD = 0.82 Å) and thorough pharmacophore virtual screening strategy further bolster confidence in these in silico results (Singh et al., 2017).

Aside from eriocitrin, dihydromyricetin, and



**Fig. 2.** Binding modes of ligands hygroline and eriocitrin respectively, with androgen receptor 5T8E visualized using discovery studio software



**Fig. 3.** Binding modes of ligand eriocitrin with androgen receptor 5T8E showing hydrogen bonding interactions (2D representation) and spatial orientation (3D representation) visualized using Discovery Studio software

austalide also exhibited strong predicted affinities (-6.9 and -7.6 kcal/mol, respectively), indicating the possibility that various flavonoids and terpenoid glycosides can function as potential modulators of AR function. This finding expands the portfolio of potential bioactive compounds from *A. bilimbi* that warrant further investigation.

The relevance of chemical composition to the observed biological effects is particularly important. According to literature data, *Averrhoa bilimbi* L. methanolic extracts contain significant concentrations of key flavonoids: quercetin (245.3 mg/100 g), myricetin (189.7 mg/100 g), luteolin (156.2 mg/100 g), and the docked compounds eriocitrin and dihydromyricetin (Chau et al., 2023; Setyawan et al., 2021). These high concentrations of AR-modulating flavonoids directly correlate with the observed in vitro cytotoxic effects. The presence of multiple AR-targeting phytochemicals suggests that the extract exhibits pleiotropic effects, with

synergistic interactions between different flavonoid species potentially contributing to the overall antiandrogenic activity.

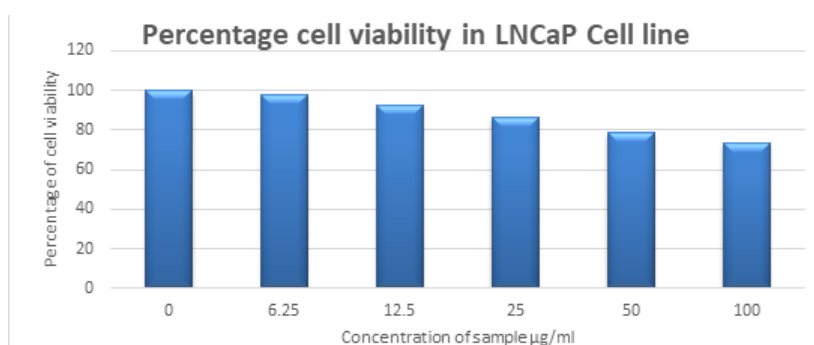
### ADMET and drug-likeness implications

The ADMET predictions indicated that the top-ranking compounds possess favorable drug-like properties with high predicted human intestinal absorption and low predicted cytochrome P450 inhibitory promiscuity, demonstrating their potential for translation (Ikwa et al., 2021). These favourable pharmacokinetic properties suggest that the flavonoids identified from *A. bilimbi* could serve as leads for further optimization and development into therapeutic agents with improved oral bioavailability and reduced off-target interactions.

### In vitro cytotoxicity and mechanistic implications

Preliminary *in vitro* cytotoxicity studies using LNCaP prostate cancer cells showed that methanolic extract treatment resulted in a concentration-dependent reduction in viability, with an  $IC_{50}$  of 186.79  $\mu$ g/mL ( $p < 0.05$  vs control; mean  $\pm$  SD;  $n = 3$ ). Although the potency of the extract is moderate compared with pure AR antagonists such as enzalutamide, it demonstrates antiandrogenic effects in a hormone-dependent cell model. The link between docking-predicted high-affinity ligands and corresponding reductions in *in vitro* viability suggests that the extract's activity is likely due in part to AR antagonism rather than off-target, nonspecific cytotoxicity. This is further supported by low concentrations of the extract producing high levels (>90%) of retained viability, thus indicating that off-target toxicity may not be the primary mechanism (Lackova et al., 2017).

### Comparison with literature and mechanistic considerations



**Fig. 4.** Dose-dependent cytotoxic effects of *Averrhoa bilimbi* methanolic fruit extract on LNCaP human prostate cancer cells assessed by MTT assay. Cell viability was determined at varying concentrations (6.25-100  $\mu$ g/ml) after 48-hour treatment. Data represented as mean  $\pm$  SD ( $n = 3$ ). \* $p < 0.05$  compared to control (ANOVA with Tukey's post-hoc test)

These results are consistent with previous studies demonstrating that plant flavonoids mediate AR-mediated growth inhibition in prostate cancer cell lines. For instance, quercetin and genistein have been shown to inhibit AR nuclear translocation and decrease prostate-specific antigen expression in LNCaP cells. This work expands upon this phenomenon for *Averrhoa bilimbi* and identifies eriocitrin and dihydromyricetin as potential lead compounds deserving further investigation. The multiple hydrogen-bonding interactions

observed in our docking studies align with known mechanisms of AR inhibition, in which competitive binding at the ligand-binding domain prevents DHT-mediated AR activation.

### Limitations of the study

The present study is limited by the absence of direct androgen receptor (AR) binding assays and downstream mechanistic evaluations. Although molecular docking and preliminary cytotoxicity data provide supportive evidence for potential AR-related activity, these findings remain predictive and cannot substitute for experimental validation.

Future work should include targeted mechanistic studies to confirm AR engagement, as well as the isolation of individual phytochemical constituents to determine their specific contributions to the observed effects. *In vivo* evaluation and pharmacokinetic characterization will further be required to assess bioavailability, tissue distribution, and therapeutic relevance.

### Conclusion

This research demonstrates that *Averrhoa bilimbi* fruit has significant potential for pharmacological applications in the prevention and treatment of hormone-dependent prostate cancer, particularly due to its high flavonoid and phenolic compound content. Based on the findings from the *in silico* screening, most of the isolated compounds exhibit high drug-likeness, low toxicity, and strong binding affinity for the androgen receptor, especially the flavonoids eriocitrin and austalide. The methanolic extract also induced concentration-dependent cytotoxicity in LNCaP prostate cancer cells with an IC<sub>50</sub> value of 186.79 µg/mL, further supporting the potential clinical value of this plant.

Additional studies are warranted to isolate and identify specific flavonoid compounds, determine their individual contributions to the observed antiandrogenic activity, and elucidate the molecular mechanisms underlying inhibition of cell proliferation and induction of apoptosis.

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