

Antioxidant and Anticancer Potency of Hydro-Alcoholic Ingudi (*Balanites aegyptiaca* Linn. Delile) Seed Extract

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Research Article

Keywords: *Balanites aegyptiaca*, Ingudi, Hingot, DPPH free radical, Anticancerous

Posted Date: March 1st, 2024

DOI: <https://doi.org/10.21203/rs.3.rs-3875562/v1>

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Additional Declarations: No competing interests reported.

Abstract

Balanites aegyptiaca is a well-known medicinal plant, which has been traditionally used by local communities to treat various human ailments such as antibacterial, skin diseases, wound healing, and antioxidant properties. This study, examined the phytochemical composition and cell toxicity of *Balanites aegyptiaca* using established methodologies. The main objectives of this research work are:

1. Evaluation of hydro-alcoholic seed extract in cell viability in breast cancer cell lines.
2. The study significance antioxidant activity.
3. Phytochemical analysis of *Balanites aegyptiaca* extract.
4. Anticancer activity of *Balanites aegyptiaca* extract.

The seeds were extracted using a hydro-alcoholic solvent (4:6 ratio), concentrated, and then lyophilized to obtain a dry crude extract. The radical scavenging capacity was determined using the DPPH method, while cytotoxicity was assessed through cell proliferation assays (MTT) involving MCF-7, MDA-MB-231, FaDu, and human PBMCs cells (2×10^3 cells). Our findings revealed that the hydro-alcoholic extract of the seeds exhibited significant antioxidant and anti-cancer properties. The novelty of this research work is the phytochemicals present in the plant extract that inhibit the migratory ability of breast cancer cells. The recommendations of this study can be adopted in the experimental and clinical trials.

Highlights

- The study shows a significant decrease in cell viability in breast cancer cell lines.
- The study shows a significant antioxidant activity.
- The result strongly recommended for clinical pilot study.

INTRODUCTION

Mutations that are inherited, brought on by environmental factors, or the result of mistakes in DNA replication are what give rise to cancer [1]. Cancer is the major leading cause of worldwide death. In the United States, it is anticipated that in 2019 there will be 1,762,450 new cancer cases and 606,880 cancer deaths [2]. Compared to USD 27 billion in 1990, the economic cost of cancer treatment in the United States was expected to be USD 161.2 billion in 2017 [3], which leads to the importance of anticancer molecule development. The *Atharva Veda* contains allusions to signs of advanced cancer as well as prayers and rituals that seek supernatural cures [4], whereas the research on complementary and alternative medicine, especially dealing with cancer management has recently received more attention. Numerous researches on plants has been done for a variety of ethno botanical reasons. Since its inception, the Indian traditional medicine known as Ayurveda has successfully avoided or suppressed a variety of tumours by employing several therapeutic modalities. Two well-known Ayurvedic classics, the

Charaka and *Sushruta* [5] Samhitas refer to cancer as either an inflammatory or non-inflammatory swelling and refer to it as either *Granthi* (benign) or *Arbuda* (malignant). These texts mention the use of surgery and herbal medications for such diseases. Approximately, 80% of the population of the world depends on herbal remedies for healthcare in different ethnic groups. In the modern age, the secondary metabolites of such described medicinal plants have served as natural resources for many incipient drug candidates due to their vast chemical diversity. The secondary metabolites of medicinal plants build up and invigorate physical systems especially immunity, which can then felicitously fight peregrine invaders and avail to destroy offending antigens without engendering any rigorous side effects. In recent years, many secondary metabolites have been reported as antioxidants that inhibit oxidative damage and prevent inflammatory conditions [6, 7]. On the other hand, naturally occurring secondary metabolites from plants are frequently used for drug discovery to save money and time. Therefore, one clever strategy to strengthen the anticancer efficacy and selectivity of these more promising chemical moieties, improve their absorption, distribution, metabolism, and excretion capabilities, and lessen their toxicity and side effects is to change their chemical structure [8]. Due to the adverse side effects of synthetic drugs, pharmaceutical industries are using traditionally used herbal drugs to develop drug candidates efficiently.

Balanites aegyptiaca Linn. Delile, more often known as *Ingudi/Hingot* locally, is a very tiny tree with glabrous or puberulous branches that have very sharp ascending spines and bifoliate leaves with two elliptical or obovate, coriaceous leaflets. Africa, the Middle East, Rajasthan, Gujarat, Madhya Pradesh, and the Deccan are the states in India where it is most prevalent [9]. In ancient times, *Ingudi* was used in traditional medicine, and their medicinal importance was mentioned in *Vedas*, *Puranas*, *Upanishads*, and Ayurvedic literature. In the Sanskrit textbook, *Abhigyan Shakuntalam* mentioned that the persons who lived in forest areas used '*Hingot*' as hair oil and for wound healing [10]. The hydro-alcoholic extract of *Ingudi* is a potential source of natural compounds containing cytotoxic and antioxidant activities. This may be the originality of this ethnopharmacological investigative work. In an earlier study, the hydro-alcoholic extract of *Ingudi* was screened for secondary metabolites having antimicrobial properties [11]. The present study showed the antioxidant and anticancer activity of the hydro-alcoholic extract of *Ingudi* which has not been investigated previously for its anticancer properties.

MATERIALS AND METHODS

Collection and extraction of plant materials

Ingudi (*Balanites aegyptiaca* Linn. Delile) fruit seeds were collected from Munaganj village, Etawah district, Uttar Pradesh, India, after being scientifically authenticated at Dravyaguna Department, Faculty of Ayurveda, Banaras Hindu University, Varanasi, with accession number DG / 1819 / 194 (seeds) (Fig. 1. A). The seed was once pulverized in the mechanical grinder to a plausible excellent powder to ascend out microscopic research and was once preserved in a nicely closed hermetic glass vessel for in-integration analysis. Now take 5 gm of seeds and air-dried at 37°C. All seeds are coarsely powdered (Fig. 1. B), macerated with one hundred ml of ethanol in a closed conical flask for twenty-four hours shaking

regularly at some factor of six hours, and allowed to stand for 18 hours. It was once quickly protected against ethanol loss by filtering it with Whatman filter paper. A 25-ml sample of the filtrate was weighed after being dried to a crisp in a shallow dish with a flat bottom at 105°C. The share of the ethanol-soluble extract was then estimated about the air-dried drug.

Further purification of the ethyl acetate fraction of extract was also carried out to isolate phytoconstituents of Ingudi using silica-gel column chromatography.

Phytochemical analysis of *Balanites aegyptiaca* extract

Take 500 µL of 5 mg/ml plant and fungal extracts of four different solvents i.e., heptane, hydro alcohol, acetone, and ethyl acetate. Thus, the filtrates obtained were used as test solutions for preliminary phytochemical screening tests described by Batool *et al.*, 2019 (Table 1) [12].

Table 1. Phytochemical screening of extract of different solvents of *Balanites aegyptiaca*

Qualitative Phytochemical Screening		hydro alcoholic extract of B. Aegyptiaca	Ethyl acetate extract of B. aegyptiaca	Heptane extract of B. aegyptiaca	Acetone extract of B. aegyptiaca
Alkaloids	Mayer's test:	++	+++	-	+
	Wagner's Tests:				
	Dragendroff's test:				
Flavonoids	Ferric chloride test:	+++	++	-	-
Steroids	Salkowski Tests:	-	++	+++	-
	Lieberman Burchardt tests:				
Phenol	Ferric chloride test:	++	-	-	-
Tannins	Ferric chloride test:	-	-	+++	-
Glycosides	Kellar Killani's test:	+++	++	-	-
Saponins	Foam test:	++	+	-	-

Note: '+' show low; '++' shows moderate and '+++ show high while '-' show absent.

Table 2
Interactions of EGFR receptor with Balanitin-6 and Gefitinib.

Compound	Binding Energy (kcal/mol)	Ligand Efficiency (kcal/mol)	Ligand Interactions (PDB ID: 6DUK)
Gefitinib	-9.590	-0.309	VAL A:726 (Pi-Sigma), MET A:766 (Pi-Sulfur), MET A:790 (Pi-Sulfur), ASP A:855 (H-Bond)
Balanitin6	-10.438	-0.168	LYS A:745 (H-Bond), ASP A:837 (H-Bond), ASP A:855 (H-Bond), PHE A:856 (H-Bond)

Antioxidant testing

The DPPH Radical Scavenging Activity Assay

The DPPH activity of the hydro-alcoholic extracts was once quantified in terms of hydrogen-donating or radical scavenging ability by utilizing the DPPH technique as described by Braca et al., 2001 with a slight modification [13]. In this experiment, 2 mL of hydro-alcoholic extracts were combined with 1 mL of DPPH (2, 2-Diphenyl-1-picrylhydrazyl) solution (0.25 mM) at concentrations ranging from 10 to 250 g/mL. The reaction mixture used to be well shaken and incubated for 30 minutes at room temperature in the dark. The previous control was yare as stated above without extract. At 518 nm, the solution's absorbance was previously measured about a blank. The free radical scavenging activity was measured as a decrease in DPPH absorbance and used to calculate the utilization of the following equation:

$$\text{Scavenging effect (\%)} = [(OD \text{ Control} - OD \text{ test}) / O.D. \text{ Control}] \times 100$$

Ascorbic acid was used as a reference standard.

Anticancer activity

Cell culture condition

The cell proliferation inhibition induced by the Hydro-alcoholic seed extract of *Ingudi* (*Balanites aegyptiaca* Linn. Delile) was determined by thiazolyl blue tetrazolium bromide (MTT) assay. The culture of cells for preparing and collecting at the lag phase was achieved by culturing the cells in a T-25 tissue culture flask in a CO₂ incubator at 37°C with 95% air and 5% CO₂ gas. The cell lines and primary cells, FaDu, MCF-7, MDA-MB-231, and human PBMCs cells were seeded in 96 well plates at a density of 2×10³ cells per well, and incubated overnight to adhere on the surface in the CO₂ incubator at 37°C. The isolation and experimentation on primary cells were approved by the Tezpur University Ethical Committee (TUEC), Tezpur University, Tezpur (Protocol no. DoRD/TUEC/10–14/4361 dated 28.03.2014) before the starting of this study. Then, the effect of plant extract on human peripheral blood mononuclear cells (PBMCs) was evaluated, using human blood, which was obtained from the volunteers with pre-informed consent. The blood was centrifuged to get peripheral blood mononuclear cells (PBMCs), followed by PBMCs isolation from the blood using density gradient centrifugation method and seeded into 96 well plates using RPMI-1640 media, supplemented with 10% FBS and 1% antibiotic-antimycotic solution, whereas MCF-7 cells were cultured in MEM medium containing 10% fetal bovine serum, MDA-MB-231 cells were cultured in Leibovitz-15 (L-15 medium containing 10% FBS, 100 U/mL penicillin, and 100mg/L streptomycin [14, 15].

In Vitro Cytotoxicity Assay

To examine the cytotoxicity of the extract, a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed. Briefly, the cells (FaDu, MCF-7, MDA-MB-231, and PBMCs cells) were harvested at log phase, counted, and seeded at 2 × 10³ cells per well of the 96-well plates for 24 h. Further, the cells were given different concentrations of plant extract and incubated for 24 and 48 hours at 37°C with 5% CO₂. After completion of incubation, 100 of 0.5 mg/mL MTT solution was added to each well and further incubated for 4 h in the dark at the same conditions, then, the solution was removed and

150 μ L of dimethyl sulfoxide (DMSO) was added to each well. Finally, the plate was read at 575 nm by an infinite M200 pro microplate reader [16].

Molecular Docking Studies

To enhance the comprehension of the molecular interaction between ligands and proteins like Balanitin-6 and epidermal growth factor receptor (EGFR) (PDB id. 6DUK), molecular docking investigations were carried out using AutoDock 4.2 [17]. Gefitinib, an approved EGFR inhibitor was used as a standard for comparison of binding. These proteins' crystal structures were obtained from the protein data bank at (<https://www.rcsb.org/>). The pdb2pqr web server was employed to ensure precise protonation statuses for the residues. After that, the analysis was done without including ions, ligands, or molecules of water. Using M.G.L Tools 1.5.6, non-polar hydrogen atoms were also eliminated, and polar hydrogen atoms and gasteiger charges were added. For the computation of maps, AutoDock employed Autogrid4.

The PLIP web server was used to identify the protein's active site (<https://projects.biotec.tu-dresden.de/plip-web/plip>) [18]. The docking study was conducted using the Lamarckian Genetic Algorithm (LGA) with specific parameters: 100 runs, 150 population sizes, 27,000 generations, and 2,500,000 energy evaluations. During the docking simulation, a 'semi-empirical free energy force field' was utilized to assess conformations. BIOVIA Discovery Studio 2021 was used to visualize the docked poses and study the interactions [18]. After the docking simulation was completed, the optimal conformation was selected using a 2.0 threshold, and the most advantageous binding energy was found within the biggest cluster.

Ethical Statement

Before beginning the study, this work received clearance from the Tezpur University Ethical Committee (TUEC), Tezpur University, Tezpur (Protocol no. DoRD/TUEC/10-14/4361 dated 28.03.2014). All experiments based on humans were according to the Ethical Guidelines of the committee.

Statistical analysis

All the results were reported as means $\pm$ SEM, $n=3$, from three independent sets of tests. ANOVA was used for statistical analysis, followed by Tukey's multiple comparisons tests. Three levels of Significant differences were considered and the sets as $ap \leq 0.001$, $bp \leq 0.01$, and $cp \leq 0.05$ (PRISM software, USA).

RESULT AND DISCUSSION

Phytochemical and Antioxidant analysis

Extraction of *Balanites aegyptiaca* Linn. Delile is done by four different organic solvents i.e., heptane, hydro-alcohol, acetone, and ethyl acetate. The qualitative analysis of the extract of all solvents is shown in Table 1. Saponins show antioxidant, antimicrobial, and anti-inflammatory properties [19].

Flavonoids exhibit antioxidant and anti-inflammatory property. tannin exhibits antimicrobial and free radical scavenging activity [20, 21]. Steroids show anti-inflammatory properties [22]. It was already documented that the major classes of antimicrobial compounds from plants have tannins, saponins, flavonoids, and glycosides [23].

The repeated purification of ethyl acetate fraction over the silica-gel column led to the isolation of Balanitin 6, a steroidal saponin. The structure of Balanitin 6 (Fig. 3) was confirmed by NMR spectroscopy. The obtained NMR spectra were compared with the reported NMR and both were in complete agreement thus, the isolated compound was confirmed as Balanitin 6 [24]. The NMR spectra of Balanitin 6 are provided in the Supplementary material (Fig. S1-S4).

Cell viability

The cytotoxicity of plant extract in MCF-7, MDA-MB 231, FaDu, and PBMCs cells was evaluated using an MTT assay and significant ($***p \leq 0.001$, $**p \leq 0.01$ and $*p \leq 0.05$) dose-dependent decrease in cell viability was observed in breast cancer cell lines (MCF-7, MDA-MB 231), head and neck cancer (FaDu) lines at 24 and 48h whereas, plant extract has minimal effect on PBMCs (Fig.4). This anti-cancer activity of plant extract might be due to the presence of flavonoids, and phenolic compounds observed in the preliminary test (Table 2). Further NMR study of *Balanites aegyptiaca* plant with fresh and dry fruit in our previous study reported the presence of compounds like oleic acid, linoleic acid, and linolenic acid [25], which has been reported to have an anti-tumorigenic effect [26, 27].

Plant extract treatment Decreases cell Migration

To study the effect of plant extract on cell migratory ability using a scratch assay and the results demonstrated that the mean scratch distance was found to be significantly larger (Fig.5) with the increasing dose of plant extract (Fig. 2) quantified by image J this may be due to the presence of phytochemicals present in the plant extract that inhibit the migratory ability of breast cancer cells. A similar result was observed by Hsu et al., 2015 where the breast cancer cells' wound area was significantly larger than to control with the increasing dose of betulin [14].

Molecular Docking

The Balanitin-6 showed important interactions with the protein viz. H-bond and π -alkyl. The binding energy, ligand efficiency, and interactions of Balanitin-6 and Gefitinib (standard) with EGFR (PDBId. 6DUK) are presented in Table 2. The Balanitin-6 showed good binding energy and ligand binding efficiency which was -10.438 kcal/mol and -0.168 kcal/mol, respectively and its binding energy was comparable to Gefitinib. Balanitin-6 showed H-bond interaction with Lys745, Asp837, and Asp855. The 2D and 3D interaction diagrams of these two compounds have been represented in Fig.6.

CONCLUSION

The results of the current investigation showed that the hydro-alcoholic extract of *Balanites aegyptiaca* seeds is a promising source of antioxidant and cancer-preventing activities. The potent anticancerous activity against MCF-7, MDA-MB 231, and FaDu breast cancer cell line is shown, due to the presence of phytochemical compounds, which are responsible for their antioxidant and anti-cancer properties. Thus, further investigations are required to study the underlying mechanism responsible for their anti-cancer property.

Declarations

ACKNOWLEDGMENTS

The authors would like to thank the Department of Dravyaguna, Ayurveda, IMS, B.H.U head. Faculty for providing a lab infrastructure and the Institute of Eminence, Banaras Hindu University, Varanasi for funding this study. The authors would also like to thank the Department of MBBT, Tezpur University, Assam, India for its support in anticancerous activity.

FUNDING

The project is funded by ICMR through an incentive grant given to the author, SK, under Development Scheme No. IIRP-2023-6092/F1 and supported by Raja Jwala Prasad-Doctoral Fellowship, IoE-BHU and IoE-BHU under Development Scheme No. 6031.

Author contribution statement

All authors have significantly contributed to this article's development and writing.

Conflict of interest

All authors have read this manuscript and have no conflict of interest.

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Figures

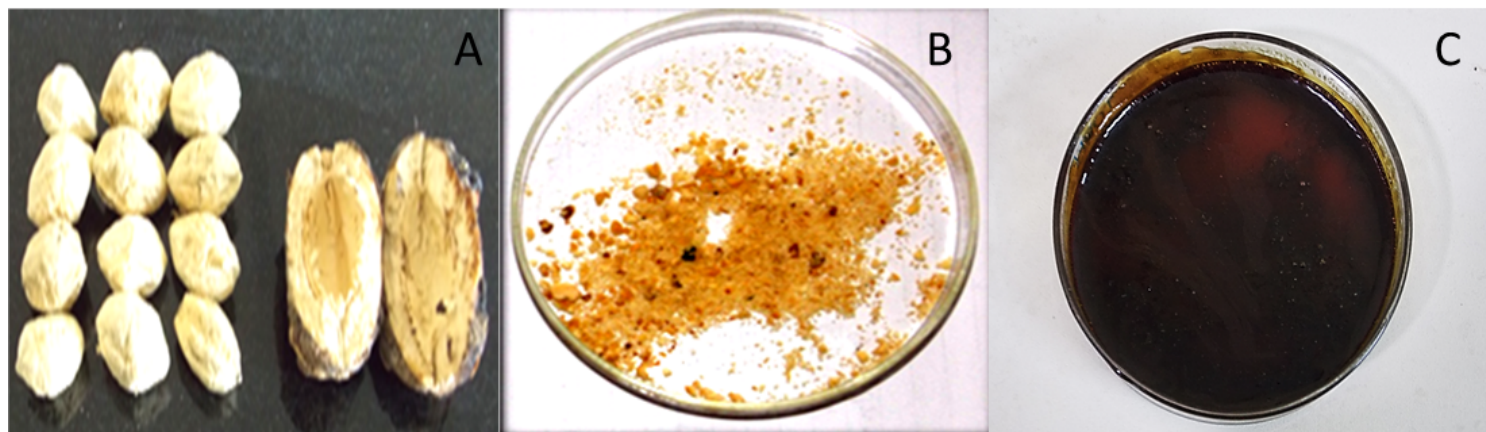


Figure 1

(A)Seeds of *Balanites aegyptiaca*(B) Seed powder of *Balanites aegyptiaca*(C)

Hydro-alcoholic extract of Seeds of *Balanites aegyptiaca*

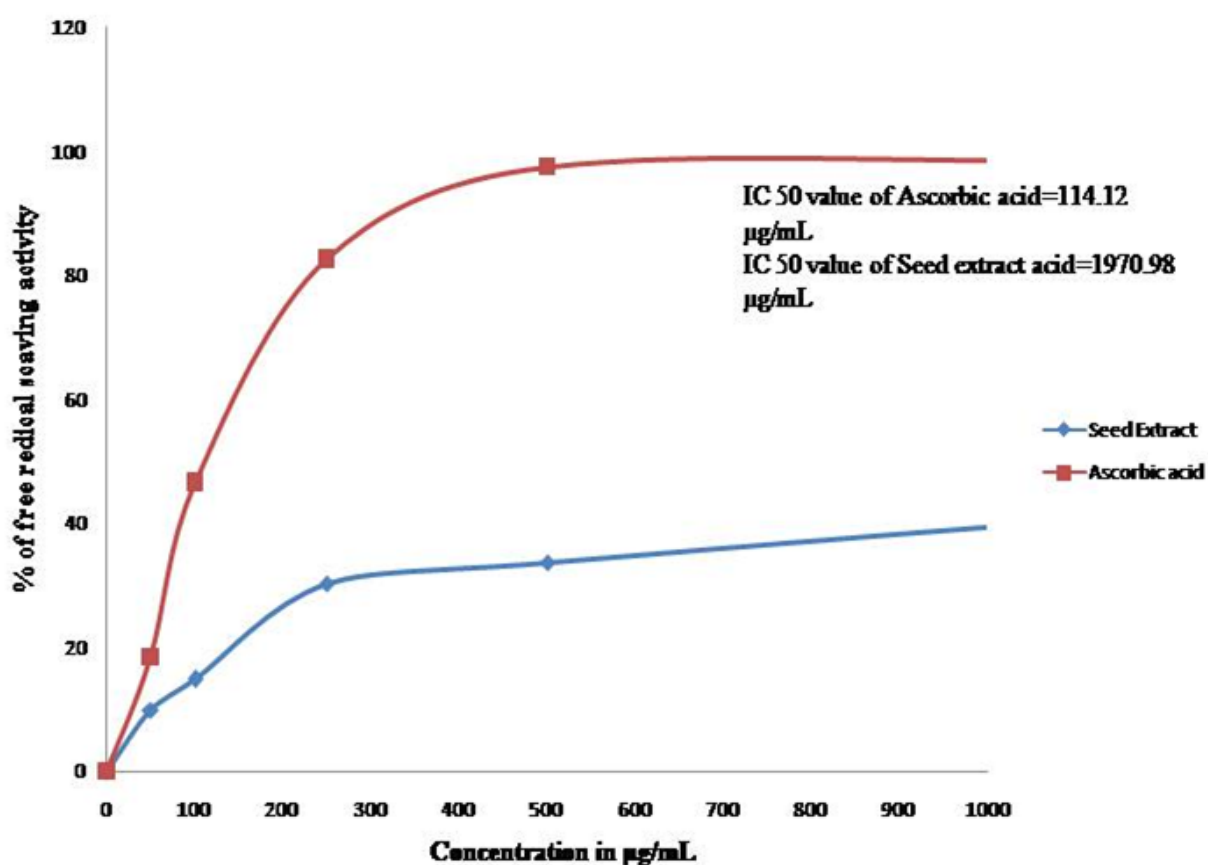


Figure 2

DPPH Free radical scavenging activity of plant extracts of *Balanites aegyptiaca*

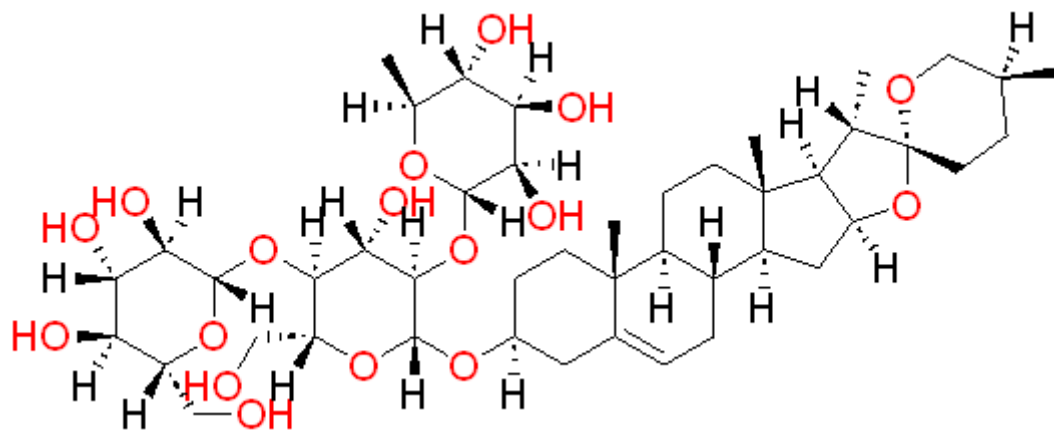


Figure 3

Structure of Balanitin 6

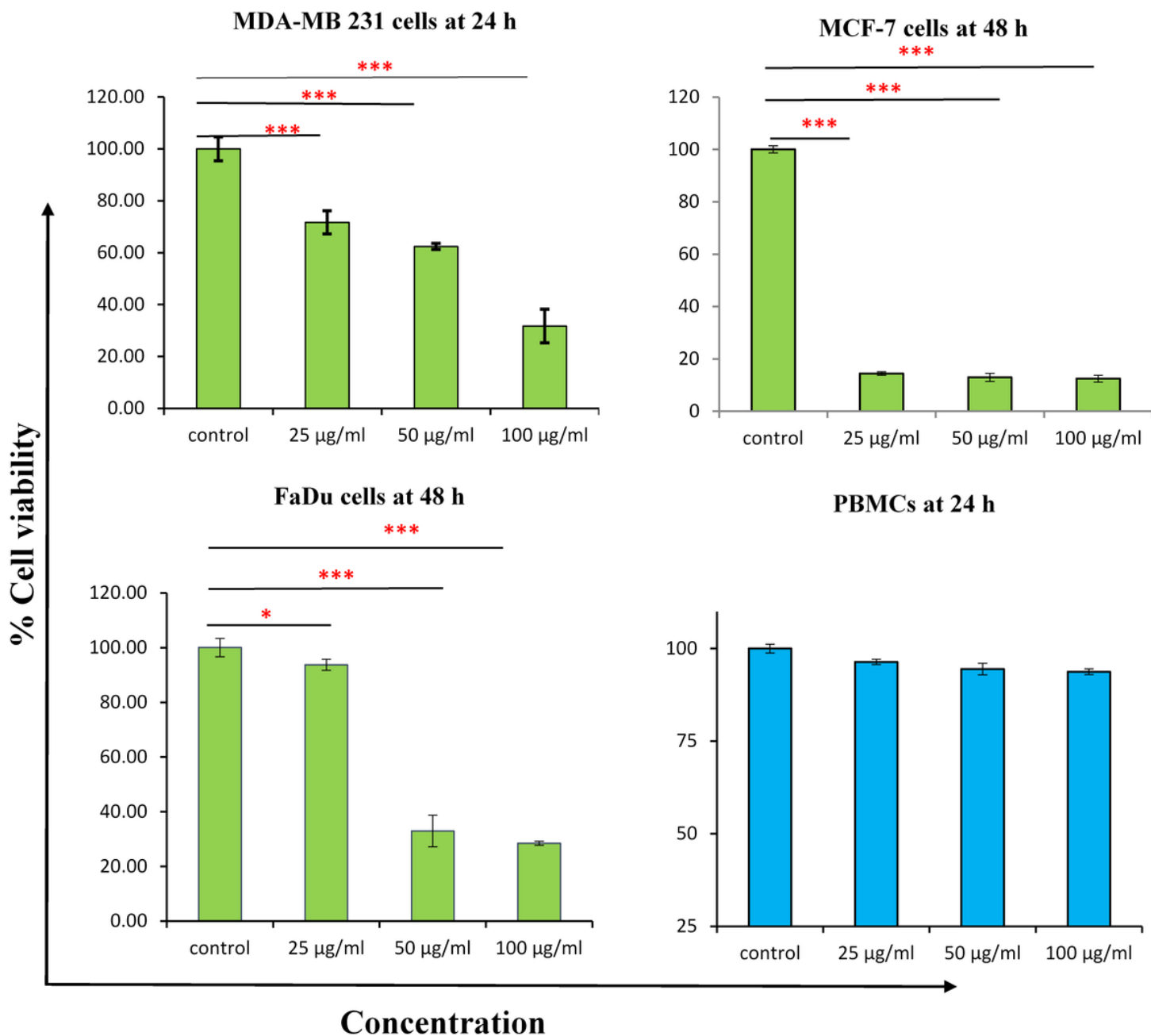


Figure 4

MTT Assay

The effect of plant extract on cell viability of cancer cell lines (MCF-7, MDA-MB 231, and FaDu) and PBMCs at 24, and 48 h, measured by MTT-based method. Values were expressed as mean $\pm$ sem; n=3; values were significant when *** $p \leq 0.001$, ** $p \leq 0.01$, and * $p \leq 0.05$.

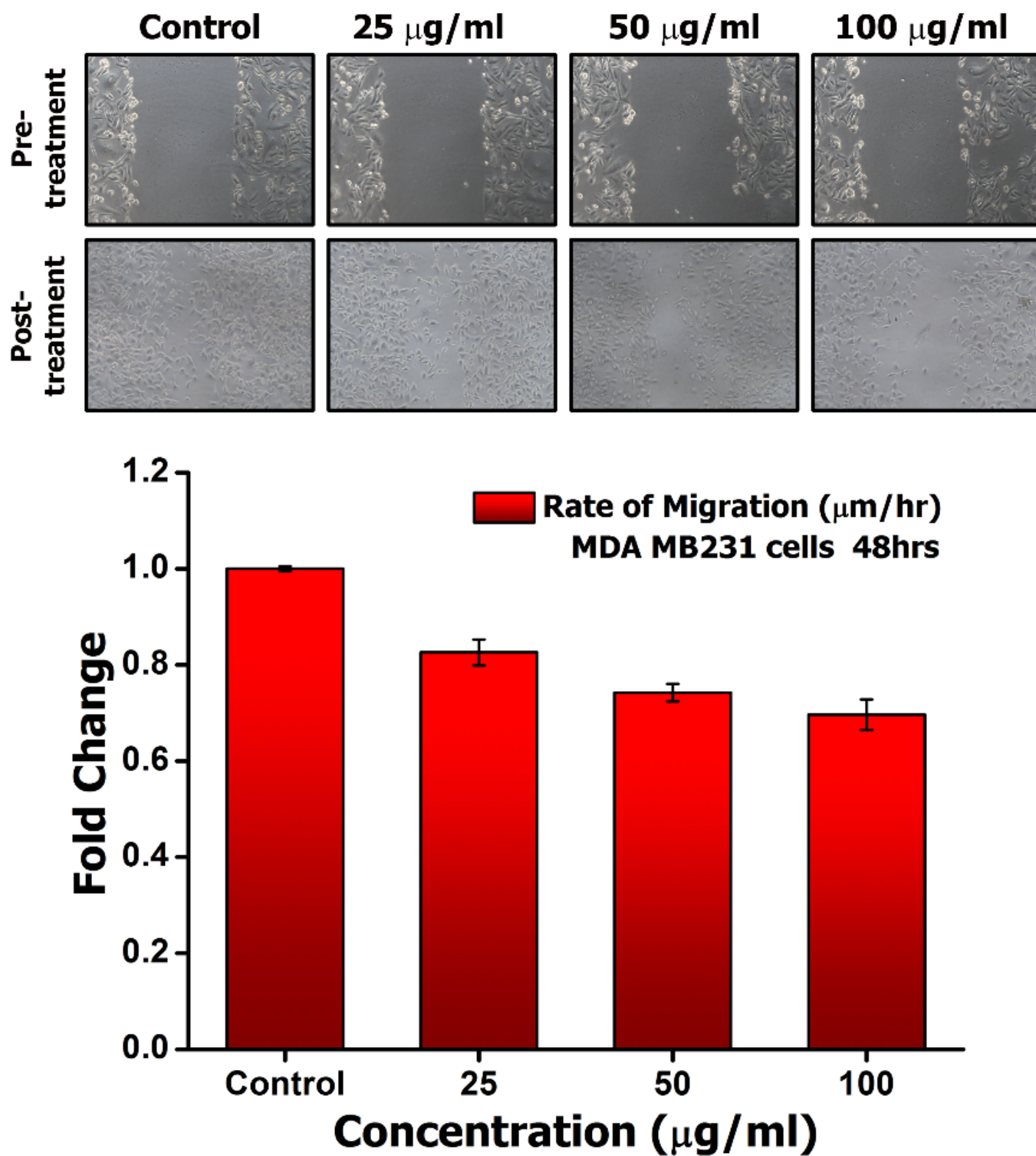


Figure 5

Anti-cancerous activity

Effects of Balanites seed extracts on cell migration of MDA-MB 231. Photomicrographs were acquired at 48 h. The applied concentrations of extracts were 25, 50, and 100 µg/mL.

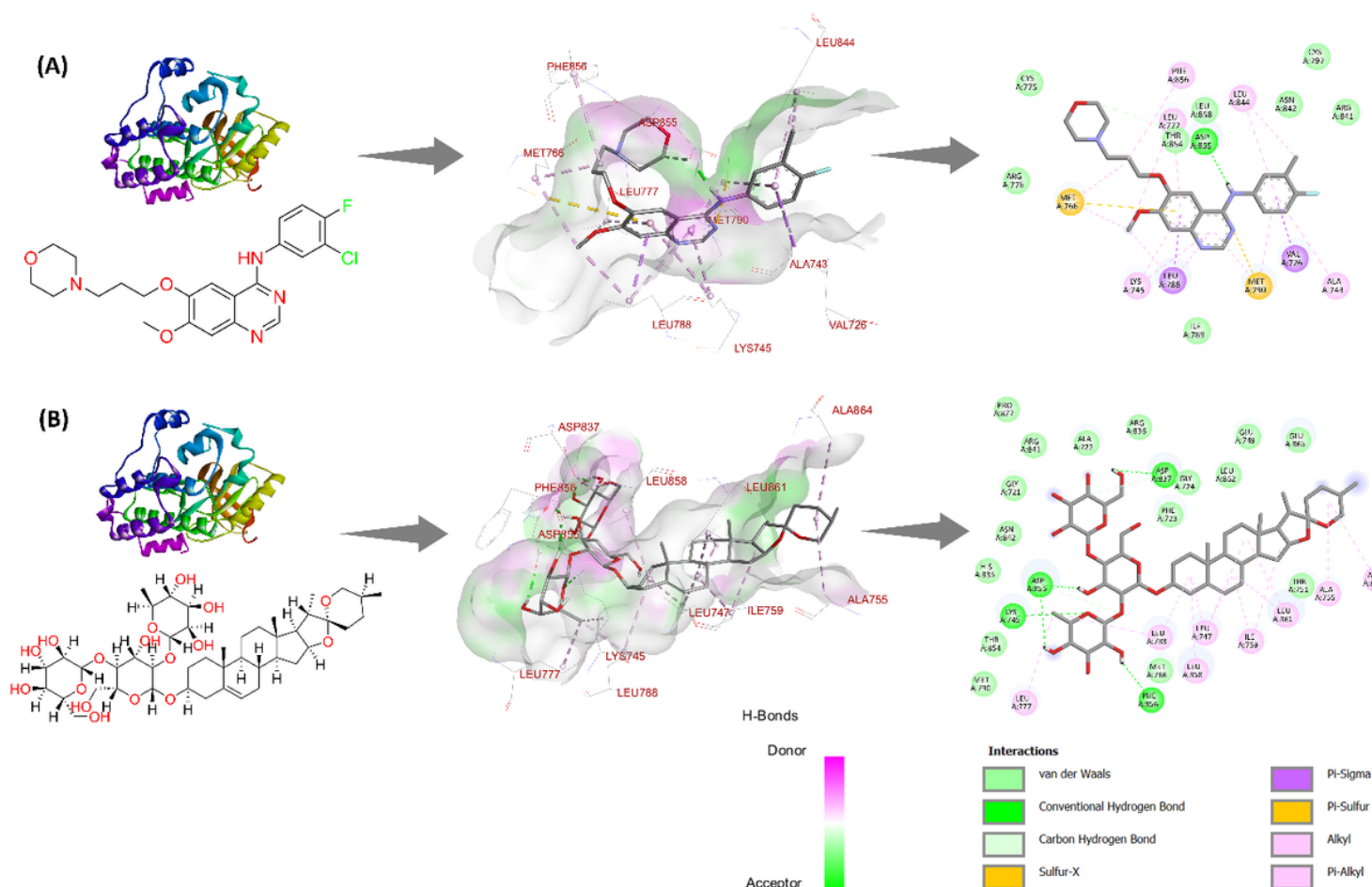


Figure 6

The protein-ligand interaction diagram of compounds **A**) Gefitinib and **B**) I against EGFR receptor protein (PDB ID: 6DUK).

Supplementary Files

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