

SUPPLEMENTARY MATERIAL

In vitro and *In silico* Study of *Aloe vera* Leaf Extract against Human Breast Cancer

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Abstract: *Aloe vera* leaf contains some bioactive compounds that have a strong binding affinity toward estrogen receptor as compared to standard drug tamoxifen. In this study, we have found that the IC₅₀ of *Aloe vera* leaf extract against breast cancer cell line MCF-7 is 23 µg/mL which is much lower than the IC₅₀ (332 µg/mL) of *Aloe vera leaf extract* against non-cancerous cell line NIH-3T3. We have also calculated the total concentration of phenolic acid (385.662 µg/mL), flavonoids (160.402 µg/mL) and alkaloids (276.754 µg/mL) in *Aloe vera* leaf extract. The free radical scavenging activity of *Aloe vera* leaf extract is 67 % to 84% (at 50 to 300 µg/ml). Our virtual molecular docking study suggests that bioactive compounds like Aloe-emodin (-8.8 Kcal/mol), 7-hydroxy-2,5 dimethylchromone (-7.5 Kcal/mol), Beta-sitosterol (-7.3 Kcal/mol) etc. have a greater binding affinity toward estrogen alpha receptor as compared to standard drug Tamoxifen (-6.4 Kcal/mol).

Keywords: *Aloe vera*, Estrogen receptor, MTT assay, Virtual molecular docking, MCF-7

1. Experimental

1.1. Sample collection and preparation of extracts:

Fresh Aloe vera leaf was collected and identified (Scientific Name: Aloe vera (L.) Burm.f., Family: Xanthorrhoeaceae, Specimen No. RM-2310) by BOTANICAL SURVEY OF INDIA, CENTRAL NATIONAL HERBARIUM, HOWRAH – 711103. The Aloe vera whole leaf was blended using mixer grinder to extract the bioactive compounds. The freshly produced extract was filtered under vacuum pressure using Whatman Filter paper. Filtrate extract solution was lyophilized for 24 h at -80 °C. Lyophilized powder was obtained as a test sample Alo and stored at 4 °C till use.

1.2. Phytochemical Screening of Plant Extracts:

1.2.1. Determination of total Phenolic content

The total phenolic content was determined by taking 20 µL extract in 1580 µL of distilled water. Then 100 µL of Folin reagent (1%) was added and left to stand for 2 min. 300 µL of Na₂CO₃ (7.5%) was added and mixed thoroughly and left to stand for 2 hat 20 °C. The absorbance was measured at 650 nm with a UV-Vis spectrophotometer. All results were determined using a standard curve of gallic acid and a linear equation was used to estimate the total amount of phenols present in the extract.

1.2.2. Determination of total Flavonoid content

The total flavonoid content (mg/mL) was determined using aluminum chloride (AlCl₃) method. The assay mixture consisting of 0.5 mL of the plant extract, 0.5 mL distilled water, and 0.3 mL of 5% NaNO₂ was incubated for 5 min at 25°C. This was followed by addition of 0.3 mL of 10% AlCl₃ immediately. Two mL of 1 M NaOH was then added to the reaction mixture, and the absorbance was measured at 510 nm. All results were determined using a standard curve of quercetin and a linear equation was used to estimate the total amount of flavonoids present in the extract.

1.2.3. Determination of total Alkaloids content

Reagent Solutions: Dragendorff's Reagent was prepared by dissolving bismuth nitrate pentahydrate (0.8044g) in 40 mL of water, 10 mL of glacial acetic acid and combined with a solution of 8.0 g of potassium iodide in 20 mL water. Ten mg bismuth nitrate pentahydrate in 5 mL concentrated nitric acid was diluted to 100 mL by adding distilled water to make a standard bismuth nitrate solution. 3.00 g of thiourea was dissolved in 100 mL of water to form a thiourea solution and a stock solution of disodium sulfide was prepared by dissolving 1.00 g of disodium sulfide in 100 mL of water.

Method: The extract solution was brought to a pH of 2.5 with dilute HCl, vortexed to dislodge the precipitation. 1.5 mL was centrifuged for 20 minutes at 13000 rpm. 0.5 mL of the supernatant was transferred to a new tube, 0.2 mL of Dragendorff's reagent was added and the tube was vortexed to dislodge any precipitate from the wall of the tube. This solution was then centrifuged for 40 minutes at 13000 rpm. The residue was dissolved in 0.2 mL concentrated nitric acid and 0.8 mL water. 0.2 mL was pipetted out and added to 1 mL of the thiourea solution. The absorbance was measured at 464 nm against a blank containing nitric acid and thiourea. All results were determined using a standard curve of 40% Bismuth nitrate pentahydrate and a linear equation was used to estimate the total amount of alkaloid present in the extract.

1.3. Quantification of Antioxidant activity using the 1, 1-diphenyl-2-picrylhydrazyl (DPPH) method:

The free radical scavenging activity (RSA) was performed using DPPH method. Different concentration of the aloe extracts (0, 50, 100, 200, 300 µg/mL) were used. A volume of 2.5 mL of 0.04% DPPH solution was mixed with 0.5 mL of all concentrations of extracts separately. After 30 min incubation at room temperature in dark, the absorbance (abs) was taken at 517 nm in triplicates for each concentration. Citric acid was used as a standard antioxidant. Water was used as a control. The percent inhibition of free radical formation was calculated as follows

$$RSA = \frac{(Control\ abs - Sample\ abs)}{Control\ abs} \times 100$$

1.4. In-vitro antitumor activity:

1.4.1. Cell culture

Mouse fibroblast cell line (NIH-3T3) and human breast cancer cell line MCF-7 were collected from National Centre for Cell Science, Pune. The growth medium was prepared using Dulbecco's Modified Eagle's Medium, supplemented with 10% fetal bovine serum (Gibco) and 2% penicillin-streptomycin and maintain at 37°C in a humidified incubator with 5% CO₂. The cells were grown in T-15 flasks till about 80% confluence.

1.4.2. Drug and test samples treatment

Test samples were prepared to dissolve lyophilized *Aloe vera* powder in 1% DMSO solution as a drug vehicle in various concentration. Tamoxifen (100 µg/mL) was used as a standard anticancer drug as well as anti-estrogenic drug for breast cancer treatment.

1.4.3. MTT assay: Cell Viability test

The antitumor potential of *Aloe vera* leaf extract was carried out by MTT assay on NIH-3T3 and MCF-7 cell line. Cells were seeded in 96 well plates and cultured at 37 °C for 24 h in a humidified incubator with 5% CO₂. After 24 h cells were treated with *Aloe vera* test samples as well as Tamoxifen (100 µg/mL) and the cells were incubated for 37 °C for 24 h in a humidified incubator with 5% CO₂. MTT (5 mg/mL in PBS) was added to the cells of each well and incubated for 3-4 h. After that, the MTT was removed and DMSO (100 µL/well) was added to dissolve the formazan crystal. Absorbance was measured at 590 nm. Later on, the percentage of cell viability was calculated.

$$\% \text{ Viable Cell} = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of untreated cells}} \times 100$$

1.5. Statistical Analysis:

Data are presented as mean SD. Statistical comparisons between groups were performed by Student's t-test. P< 0.05 was considered to be statistically significant.

1.6. Software and Data Sources:

All the software (i.e. UCSF Chimera, Biovia Discovery Studio, Ligplot+ and PubChem Sketcher V2.4) have used for virtual screening are freely available for academic use. The source of data is given in Table S1.

The Protein Data Bank (PDB) (www.rcsb.org) is worldwide repository for the processing and distribution of 3D biological macromolecular structure data (Berman et al.,

2000). The protein 3D structures can be downloaded from the database with specific keywords or a PDB ID.

The PubChem (pubchem.ncbi.nlm.nih.gov) is a database of chemical compound structures. The database system is maintained by the National Centre for Biotechnology Information (NCBI). All the chemical compound structures in this database can be downloaded freely.

UCSF Chimera (www.cgl.ucsf.edu/chimera) is a free Graphical User Interface (GUI) to perform molecular docking experiment and result analysis. It is an extensible molecular modeling system.

Biovia Discovery Studio 2017R2 is free academic software used for visualization of receptor-protein interaction.

AutoDock: The virtual screening was performed using the docking software AutoDockVina. It is an open-source program for performing molecular docking (<http://vina.scripps.edu>). AutoDock is an automated docking tool designed to predict how small molecules/ ligands such as drug candidates, bind to receptor/protein of known 3D structure.

1.7. Virtual Molecular Docking:

1.7.1. Preparation of target receptor protein and determination of Active Site: The estrogen receptor alpha structure was downloaded from RCSB PDB with code 1ERE, which has an endogenous co-crystal ligand 17- β -estradiol. The downloaded structure had six identical copies of the receptor, out of that five were removed using UCSF Chimera. After removing endogenous co-crystal ligand 17- β -estradiol and water molecules, hydrogen, Gasteiger charge was added using dock prep option of UCSF Chimera to the estrogen receptor alpha receptor protein (1ERE). The active site residues of estrogen receptor were found by using BIOVIA Discovery Studio 2017R2 software and the residues at the active site are ARG394, LEU387, LEU391, LEU525, LEU346, HIS524, GLU353 (Figure S1).

1.7.2. Preparation of ligands: All the ligands/chemical compounds 3D structures were built using canonical smile, obtained from PubChem and Zinc database through UCSF Chimera built structure option. Later, hydrogen, Gasteiger charge were added to using dock prep option of UCSF Chimera. The compounds thus obtained were tested for absorption, distribution, metabolism, excretion, and toxicity (ADME) on the basis of its properties like molecular weight, log p-value, number of rotatable bonds, number of hydrogen donor, number of hydrogen acceptor using SwissADME web tool.

1.7.3. AutoDockVina running: AutoDockVina was run using UCSF Chimera tool after the preparation of target receptor and ligands, targeting the active sites of the receptor through receptor search volume options (Center: 8.10, 46.85, 128.72. Size: 18.3, 18.85, 17.41). The other parameters were Number of binding modes: 10, Exhaustiveness of search: 8 and Maximum energy difference (Kcal/mol): 3 used in running of AutoDockVina through UCSF Chimera graphical user interface.

1.7.4. Validation of molecular docking: Validation was done by performing re-docking of endogenous co-crystal ligand. The root mean square deviation (RMSD) value of the co-crystal and re-dock crystal ligand was evaluated.

Reference

Berman, H. M., Westbrook, J., Feng, Z., Gilliland, G., Bhat, T. N., Weissig, H., ... Bourne, P. E. (2000). The Protein Data Bank. *Nucleic Acids Research*, 28(1), 235–242. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/10592235>

Supplementary Image

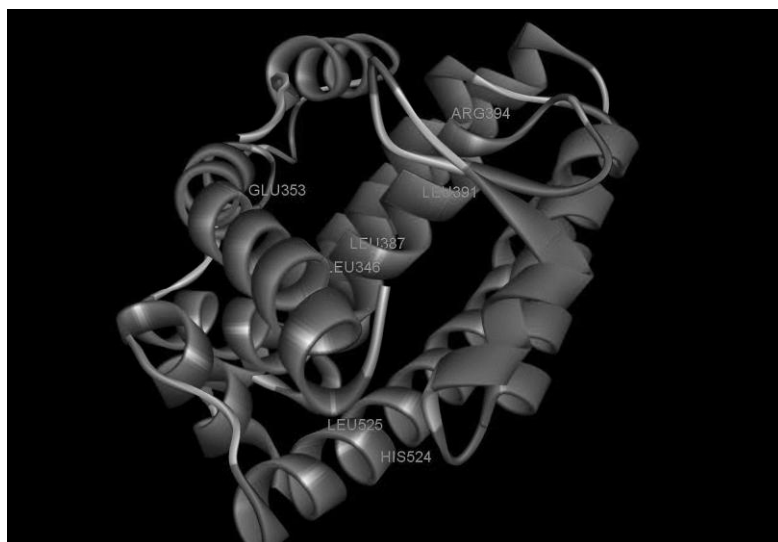


Figure S1

Supplementary image caption

Figure S1. Active sites (yellow) of estrogen alpha receptor (red ribbons)

Supplementary Image

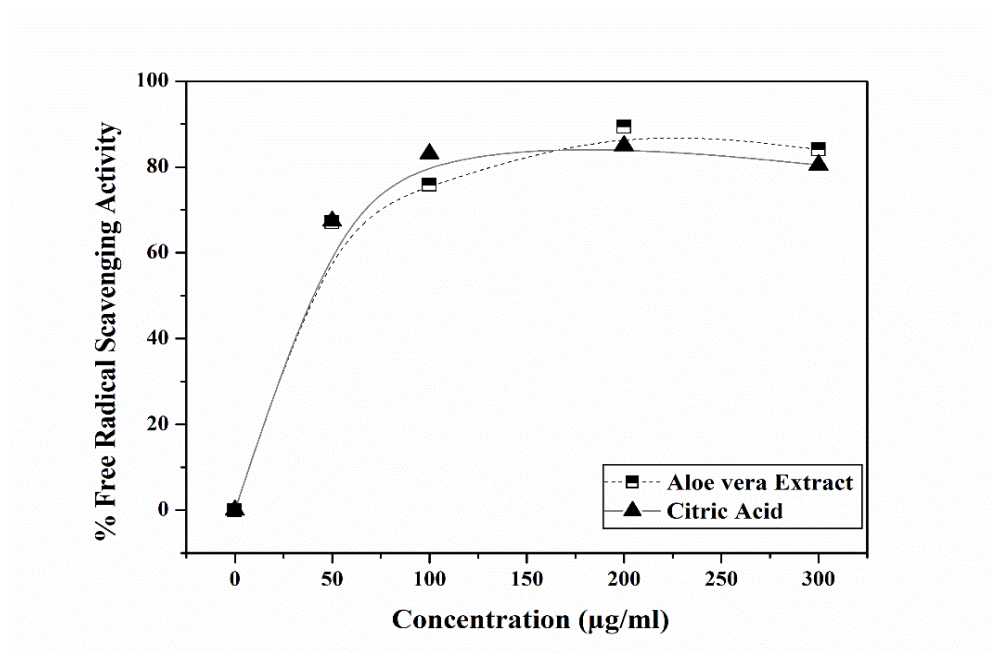


Figure S2

Supplementary image caption

Figure S2. Antioxidant Activity of Aloe vera Leaf Extract

Supplementary Image

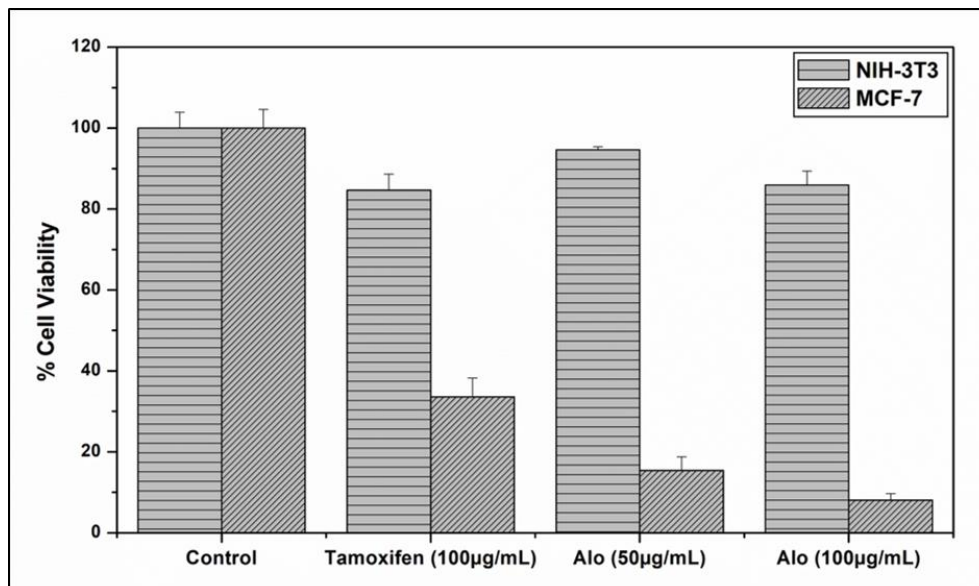


Figure S3

Supplementary image caption

Figure S3. Effect of Aloe vera leaf extract on cell viability

Supplementary Image

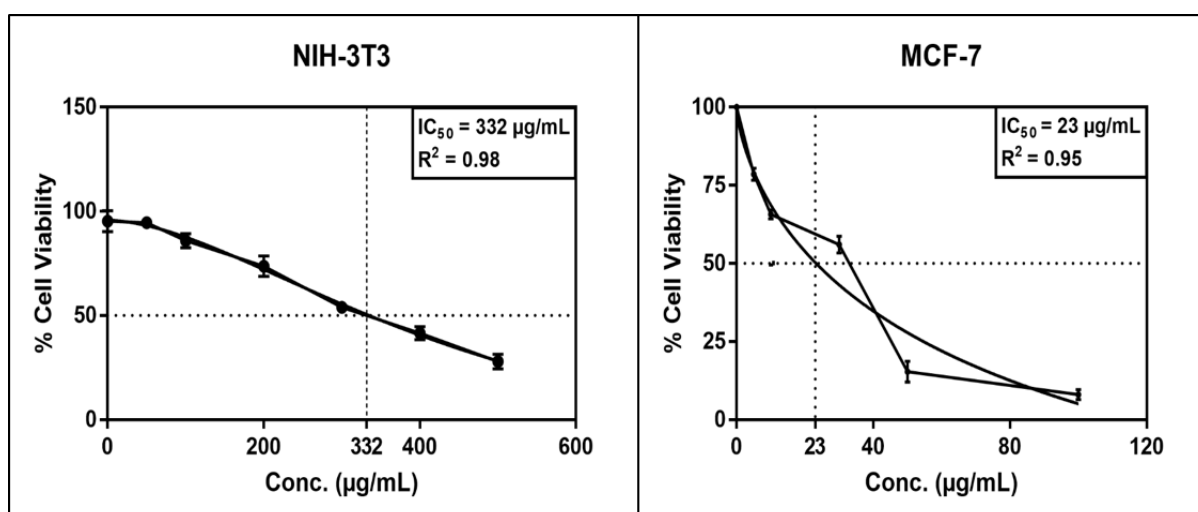


Figure S4

Supplementary image caption

Figure S4. Dose – response curve of *Aloe vera* samples against NIH-3T3 and MCF-7

Supplementary Image

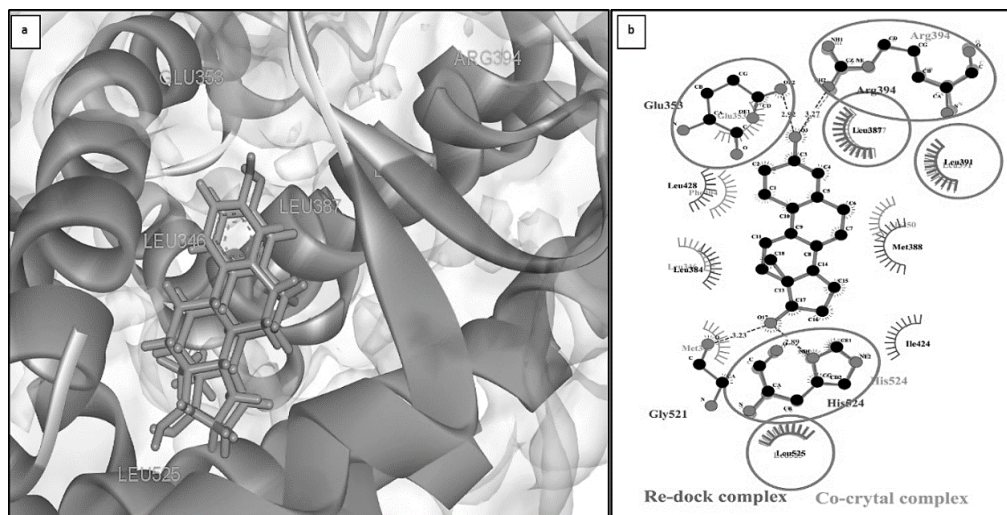


Figure S5

Supplementary image caption

Figure S5. (a) Co-crystal ligand (green) and re-docked ligand (red) at active site and the RMSD value between them is 0.0591. (b) Superimpose interaction of the co-crystal and re-docked ligand with estrogen alpha receptor active site residues (1ERE)

Supplementary Image

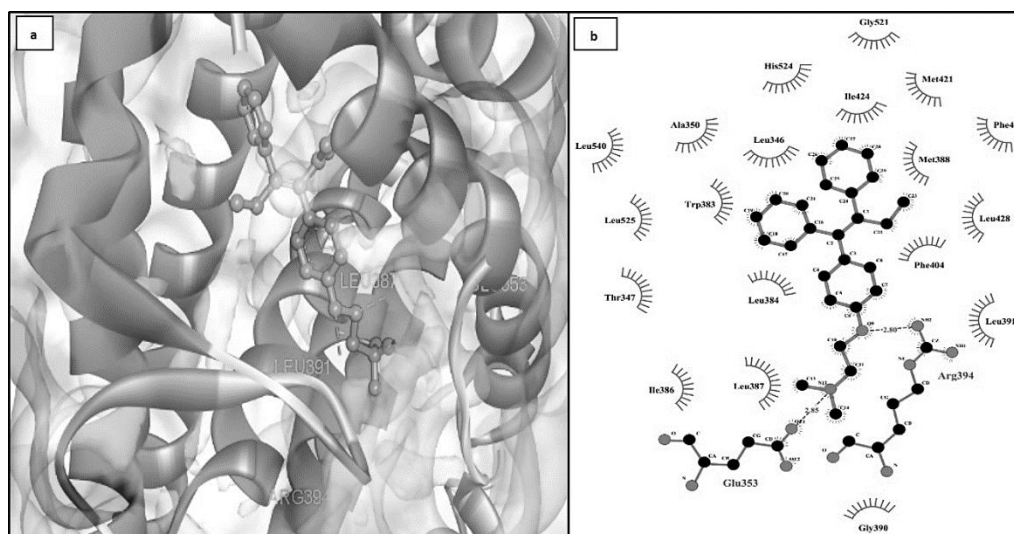


Figure S6

Supplementary image caption

Figure S6. (a) Docked pose of *Tamoxifen* (Standard drug) at active site of estrogen alpha receptor (1ERE) and the binding affinity is -6.4 Kcal/mol. (b) Interaction between Tamoxifen and active site residues of estrogen alpha receptor (1ERE)

Supplementary Image

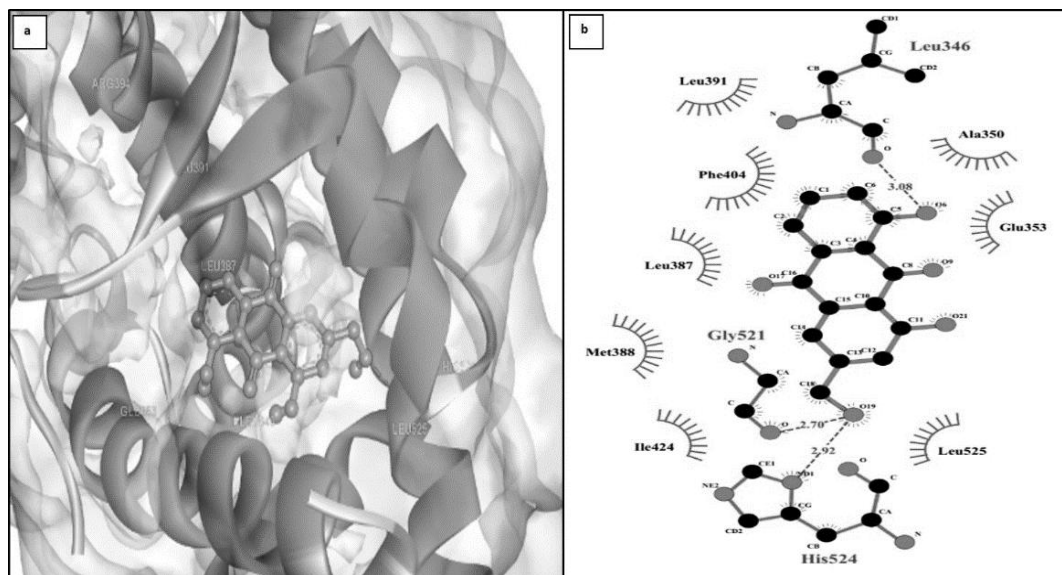


Figure S7

Supplementary image caption

Figure S7. a) Docked pose of Aloe-emodin at active site of estrogen alpha receptor (1ERE) and the binding affinity is -8.8 Kcal/mol. (b) Interaction between Aloe-emodin and active site residues of estrogen alpha receptor (1ERE).

Protein or Chemical compounds	Source	Chemical Formula
Estrogen Alpha receptor	PDB ID 1ERE	N/A
Aloin	PubChem CID 313325	C ₂₁ H ₂₂ O ₉
Aloe-Emodin	PubChem CID 10207	C ₁₅ H ₁₀ O ₅
Elgonica	PubChem CID 21582596	C ₃₆ H ₃₀ O ₁₄
Aloesin	PubChem CID 160190	C ₁₉ H ₂₂ O ₉
Beta- Sitosterol	PubChem CID 222284	C ₂₉ H ₅₀ O
Esculetin	PubChem CID 5281416	C ₉ H ₆ O ₄
Umbelliferone	PubChem CID 5281426	C ₉ H ₆ O ₃
<i>Tamoxifen</i>	PubChem CID 2733526	C ₂₆ H ₂₉ NO

Supplementary Table

Table S1

Veratric acid	PubChem CID 7121	C ₉ H ₁₀ O ₄
Vanillic acid	PubChem CID 8468	C ₈ H ₈ O ₄
Succinic acid	PubChem CID 1110	C ₄ H ₆ O ₄
7-hydroxy-2,5-dimethylchromone	ZINC14447816	C ₁₁ H ₁₀ O ₃

Supplementary Table caption

Table S1. Protein PDB ID and compound IDs retrieved from Pubchem and Zinc database.