Studies on *in silico* estrogenicity and prediction of drug ability of Genistein and Coumestrol with respect to human estrogen receptors

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**Abstract**

Genistein and coumestrol are plant-derived non-steroidal compounds found in many fruits, beans and vegetables having affinity for binding to the estrogen receptors and thus mediate estrogenic activity. Although many *in vivo* and *in vitro* studies have shown comparable estrogenic activity of these compounds with respect to endogenous estrogens, reports are rare with regard to their *in-silico* estrogenicity. In the present study, their estrogenic potential has been studied keeping 17β-estradiol (E2), estrone (E1) and estriol (E3) as reference endogenous estrogens. Binding affinity to the hERα and hERβ has been studied by Molecular Docking using BioSolveIT/LeadIT (FlexX). Quantitative Structure activity Relationship (QSAR) approach (Easy QSAR 1.0) and ADME-Tox screening (Mobyle@RPBS) have been used to prepare activity-IC50 and toxicity profile respectively of the compounds. In addition, the drug likeness scores of the compounds were recorded using Molsoft. Both genistein and coumestrol passed the ADME-Tox screening. Both compounds exhibited higher binding affinity than E2 for both hERα and hERβ and also shared the same binding cavity with most potent estrogen E2 for binding to both the receptor subtypes. Interestingly, both compounds exhibited relatively higher activity with hERβ than hERα. However, only genistein exhibited ideal positive drug-likeness score. The results clearly indicate strong but receptor specific estrogenic activity of the selected dietarily derived compounds. Therefore, estrogenic activity together with the toxicity and drug likeness profiles of phytoestrogens may provide important clues as a first step towards exploring their potential for designing receptor-specific drugs in preventing estrogen-dependent cancers.

**Keywords:** Genistein, Coumestrol, 17β-estradiol, Docking, QSAR, ADME-Tox, Drug-likeness.
INTRODUCTION

Genistein, an isoflavone (4’, 5, 7-trihydroxyisoflavone) and coumestrol, a coumestan (7, 12-dihydroxy coumestan) are two potent dietary phytoestrogens found richly in soybean and other legumes [1]. They have captured much attention in recent years due to their ability to activate both genomic as well as non-genomic mechanism of actions and their differential interaction with estrogen receptors (ERα & ERβ) [2] and transactivation [3,4] which mediate comparable estrogenic activity. In vitro competitive binding assays revealed that some isoflavones have relatively greater binding affinity for ERβ than for ERα but are $10^2$ to $10^3$ times less active than steroidal estrogens [5, 6, 7].

In human, phytoestrogens have been reported to have influence on reproductive cycle, menstrual behaviour [8] estrogenic effects on pre-menopausal women [9, 10]. These are also reported to have reduced breast, endometrial and ovarian cancer [11, 12, 13, 14, 15, 16] by inducing cell cycle arrest [17, 18, 19] or by inducing cellular differentiation [20, 21, 22]. Soy food and phytoestrogens have beneficial effect on prostate cancer [23, 24, 25] reducing postmenopausal syndrome [26, 27].

The ERα and ERβ are similar in their architecture to the other members of the steroid-thyroid hormone superfamily of nuclear receptors in that they are composed of independent but interacting functional domains: the N-terminal A/B domain, the least conserved among nuclear receptors, enables the receptor to interact with the members of the transcriptional apparatus; C domain, devoted to binding to DNA, contains two zinc-binding motifs and a dimerization interface that mediates cooperativity in DNA binding; D domain, also referred to as a “hinge region”, necessary to give the receptor some degree of flexibility between the DNA and the ligand binding domains, binds heat shock protein hsp90 and probably harbours the sequence representing the nuclear localization signal; E/F multifunctional domain recognizes and binds ligand and is involved in receptor dimerization and interaction with transcription factors and cofactors. Although the DNA-binding domains of ERα and ERβ in both human and mouse show a high degree of homology (97%, only three amino acids differ in human), the ligand-binding domain shows only 59% homology [28] (Fig.1).

![Figure 1. Schematic structure of human ERα and ERβ. Numbers above each receptor represent amino acid number, whereas numbers inside the respective boxes represents percentage of amino acid identity. The N-terminal ‘extension’ of the ERβ protein is marked with a dotted line (B). DBD, DNA binding domain; LBD, ligand binding domain. (After Gustafsson, J.A., 1999).](image-url)
Figure 2. Schematic representation of anatomical distribution of expressing ERα and ERβ in the major human tissues, in female (right) and male (left). (After Gustafsson, J.A., 1999).

Therefore, the association of dietarily derived compounds and multitude of health beneficial effects in human via involvement of estrogen receptors present in different vital cells and tissues of the body is an enormous area of research to understand the molecular interactions between hormone receptor and plant-derived compounds.

Molecular docking and QSAR studies explored the therapeutic potential of 8-prenyl naringenin and Isoxanthohumol against osteoporosis [29]. Molecular docking studies have also been carried out to identify estrogen mimics from dietary herbal supplements [30]. Molecular dynamics (MD) simulation has been used to understand the agonistic behaviour of resveratrol on ERα [31]. In silico docking studies also revealed the potential of isoflavonoids as natural healing agent against breast cancer [32].

However, in spite of the enormous in-vitro and in-vivo study reports of the possible health beneficial effects of phytoestrogens on humans, computer-aided studies of the molecular interactions with human estrogen receptor alpha and -beta and prediction of the toxicity, activity and drug-likeness of potent dietary phytoestrogens like genistein and coumestrol have not been studied. Therefore, the present work aims at screening of genistein and coumestrol for their estrogenicity by studying their interaction with human estrogen receptors (hERα & hERβ) by molecular docking. Screening has also been done for adsorption, digestion, metabolism, excretion and toxicity properties by ADME-Tox filtering. In addition, activity-IC50 and drug-likeness of the selected compounds have been studied in order to predict their potential as lead molecules for these receptors.
MATERIALS & METHODS

Experiment 1:

To study molecular interaction of genistein and coumestrol with hERα & hERβ by molecular docking.

Table 1. Selected dietary phytoestrogens and their major food sources

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Dietary compounds</th>
<th>Class</th>
<th>Major Food Sources</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Genistein</td>
<td>Isoflavone</td>
<td>Soybeans and soy products, Red clover leaf, Kudzu root, Mung bean sprouts</td>
</tr>
<tr>
<td>2</td>
<td>Coumestrol</td>
<td>Coumestan</td>
<td>Red clover sprouts, Round split peas, Kala chana, Mature alfalfa, Lucerne sprouts, Alfalfa sprouts, Pinto bean seeds, Large lima bean seeds-dry-raw, Kudzu leaf, Soybean sprouts</td>
</tr>
</tbody>
</table>

Table 2. The Pubchem ID & Structure of reference endogenous estrogens and selected dietary compounds

<table>
<thead>
<tr>
<th>Sl No.</th>
<th>Compound (PubchemCID)</th>
<th>Mol Formula/Mol wt</th>
<th>Structure/ SMILES Notation/IUPAC Name</th>
</tr>
</thead>
</table>
| 1      | 17-beta estradiol /Estradiol/ Dihydrofolliculin (5757) | C\(_{18}\)H\(_{24}\)O\(_2\) 272.381 | **SMILES:** CC12CCC3C(C1CC(C2O)O)CCC4=C3C=CC(=C4)O  
IUPAC: (8R,9S,13S,14S,17S)-13-methyl-6,7,8,9,11,12,14,15,16,17-decahydrocyclopenta[a]phenanthrene-3,17-diol |
| 2      | Estriol/ Ovestin (5756) | C\(_{18}\)H\(_{24}\)O\(_3\) 288.381 | **SMILES:** CC12CCC3C(C1CC(C2O)O)CCC4=C3C=CC(=C4)O  
IUPAC: (8R,9S,13S,14S,16R,17R)-13-methyl-6,7,8,9,11,12,14,15,16,17-decahydrocyclopenta[a]phenanthrene-3,16,17-triol |
<table>
<thead>
<tr>
<th></th>
<th>Compound</th>
<th>Molecular Formula</th>
<th>Molecular Weight</th>
<th>Structure</th>
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<tr>
<td>3</td>
<td>Estrone/ Folliculin (5870)</td>
<td>C\textsubscript{18}H\textsubscript{22}O\textsubscript{2}</td>
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<td>SMILES:CC12CCC3C(C1CCC2=O)CCC4=C3C=CC(=C4)O</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>IUPAC:(8R,9S,13S,14S)-3-hydroxy-13-methyl-7,8,9,11,12,14,15,16-octahydro-6H-cyclopenta[a]phenanthren-17-one</td>
</tr>
<tr>
<td>4</td>
<td>Genistein/ 4’,5,7-Trihydroxyisoflavone (Also produced from Biochanin A by colon bacteria) (5280961)</td>
<td>C\textsubscript{15}H\textsubscript{10}O\textsubscript{5}</td>
<td>270.2369</td>
<td><img src="image" alt="Genistein/ 4’,5,7-Trihydroxyisoflavone" /></td>
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<td></td>
<td></td>
<td></td>
<td>SMILES:C1=CC(=CC=C1C2=OC(C3=C2=CC(=CC(=C3C2=O)O)O)O)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>IUPAC:5,7-dihydroxy-3-(4-hydroxyphenyl)chromen-4-one</td>
</tr>
<tr>
<td>5</td>
<td>Coumestrol/ 7,12-Dihydroxycoumestan (5281707)</td>
<td>C\textsubscript{15}H\textsubscript{8}O\textsubscript{5}</td>
<td>268.221</td>
<td><img src="image" alt="Coumestrol/ 7,12-Dihydroxycoumestan" /></td>
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<tr>
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<td></td>
<td></td>
<td></td>
<td>SMILES:C1=CC2=C(C=C1O)OC3=C2C(=O)OC4=C3C=CC(=C4)O</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>IUPAC:3,9-dihydroxy-[1]benzofuro[3,2-c]chromen-6-one</td>
</tr>
</tbody>
</table>
Figure 3. Showing the human estrogen receptor alpha and beta protein used for docking (Source: Protein data bank)

Figure 4. Showing the hERα proteins (Chain A-LBD) selected for Docking (Source: Protein data bank); LBD: Ligand Binding Domain
The targets of these compounds have also been confirmed by using PharmMapper online server tool (Target Fishing). The protein structures of the human Estrogen Receptor alpha (PDBID: 1X7R) and human Estrogen Receptor beta (PDBID: 1X7J) have been retrieved from Protein Data Bank (PDB). Then the SMILES notations of the compounds including estrogens have been saved in notepad from PubChem Compound Database along with their PubchemID. The SMILES notations (.smi format) of the compounds have been converted to .sdf format by using OpenBabel Software. Docking of the selected compounds (.sdf format) with the Chain A, Ligand Binding Domain (LBD) of two receptor subtypes (.pdb format) (after removing the reference ligand) has been carried out using BioSolveIT/LeadIT (FlexX) Software. The Docking scores (Rank 1) of the compounds and the Hydrogen Bond Properties have been noted down for further analysis.

The binding pockets of the compounds and endogenous estrogens in the receptors have been identified from 3D snapshot (snipping tool) of docking interaction to examine the binding similarities with the most potent estrogen, 17-beta estradiol.
Table 3: Description of the Ligand Binding Domain (LBD) of hERα & hERβ protein

<table>
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<th>Human Estrogen Receptor Alpha (ESR1)</th>
<th>Human Estrogen Receptor Beta (ESR2)</th>
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<td>Chain</td>
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<td>A</td>
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<tr>
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</tr>
<tr>
<td>Length</td>
<td>245 residues</td>
<td>240 residues</td>
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<tr>
<td>Chain Type</td>
<td>Polypeptide(L)</td>
<td>Polypeptide(L)</td>
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<tr>
<td>Ligand Binding Domain</td>
<td>Amino acid 305-549</td>
<td>Amino Acid 261-500</td>
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<tr>
<td>Reference</td>
<td>UniProtKB (P03372)</td>
<td>UniProtKB (Q92731)</td>
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</table>

Experiment 2:

To study and screen genistein and coumestrol for adsorption, digestion, metabolism, excretion and toxicity properties by ADME-Tox filtering.

Toxicity of the selected compounds has been studied through ADME-Tox filtering by using Mobyle@RPBS online server. For this, the ‘.smi’ formats of all the compounds have been noted from Pubchem Compound Database and then these ‘.smi’ formats have been loaded in openbabel to convert into .sdf format. In the Mobyle@RPBS, the .sdf formats have been loaded to get the result.

Experiment 3:

To study the activites of genistein and coumestrol

Quantitative Structure Activity Relationship (QSAR) models have been used to find out the activity of the selected compounds based on their IC$_{50}$ values. For this, a total of 44 known inhibitors of both hERα and hERβ have been retrieved from Binding DB Database. The IC$_{50}$ values of these compounds were noted down and the corresponding activity has been calculated using the formula

$$\text{Activity} = -\log (\text{IC}_{50})$$

The different molecular descriptors of all the inhibitors, genistein, coumestrol and endogenous estrogens have been noted down by using ChemSketch Software with the help of SMILES notations. Then, QSAR model equation has been generated for inhibitors of both hERα and hERβ by using different molecular descriptors of the inhibitors and their activity has been determined using Easy QSAR 1.0 Software. Thereafter the similar descriptors of the test compounds (genistein, coumestrol and endogenous estrogens) have been used to predict their activity. The predicted activity values have been used to determine the IC$_{50}$ values of all the compounds including endogenous estrogens by using the formula

$$\text{IC}_{50} = \text{Power} (10^{-\text{Activity}})$$
Table: 4. Activity and descriptors (5) of 20 selected Inhibitors of hERα used for QSAR Analysis

<table>
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<th>Sl No</th>
<th>PubchemID</th>
<th>LogP</th>
<th>MolarVol</th>
<th>Index of Refraction</th>
<th>Polarizability</th>
<th>Nominal Mass</th>
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Table: 5. Activity and descriptors (6) of 24 Selected Inhibitors of hERβ used for QSAR Analysis

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<tr>
<th>Sl No</th>
<th>PubchemID</th>
<th>HB</th>
<th>Parachor</th>
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<th>Density</th>
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### Experiment 4:

**To study drug-likeness of genistein and coumestrol.**

For studying drug-likeness score, the .smi formats of all the compounds have been loaded in the Molsoft online software. The scores alongwith different molecular properties like LogP, HBA, HBD etc. have been recorded. The snapshot of Molsoft results for the compounds was taken using snipping tool.
RESULTS

Experiment 1:
To study molecular interaction of genistein and coumestrol with hERα & hERβ by molecular docking.

From the docking result presented in the Table: 6 & Figure: 6 it is seen that both genistein and coumestrol as well as endogenous estrogens exhibit higher docking scores (negative) for hERα than hERβ indicating preferential binding to hERα. Interestingly, higher (negative) docking scores were recorded for the plant-derived compounds than endogenous reference estrogens for both receptor subtypes suggesting stronger binding affinity than estrogens. The higher score found for genistein for hERβ indicate differential binding affinity of the tested compounds.

Table: 6. Docking scores and Hydrogen bond characteristics recorded for docking genistein, coumestrol and endogenous estrogens with hERα & hERβ. (*BE=Bond Energy  BL=Bond Length)
Figure 6. Graphical representation of Docking Scores of genistein, coumestrol and endogenous strogens with Chain A protein of hERα and hERβ.

The involvement of similar amino acids (Bold in Table 6) of hERα & hERβ (Chain A-Ligand Binding Domain) protein with which phytoestrogens and estrogens form hydrogen bonds suggests their binding similarity i.e., the compounds share similar binding pockets with estradiol or estrone in both hERα & hERβ (chain A-Ligand Binding Domain) protein. These molecular interactions are also shown as two dimensional (2D) pictures in Fig. 7.

From docking study it has been revealed that both genistein and coumestrol share the same binding cavity with estradiol for binding to both hERα & hERβ.

Figure 7. 2D pictures showing Hydrogen bonds formed between amino acids of hERα and hERβ (chain A) proteins and compounds under study.
Experiment 2:

To study and screen the compounds for adsorption, digestion, metabolism, excretion and toxicity properties by ADME-Tox filtering.

The results of ADME-Tox study are presented in the Table 7. All the tested compounds passed ADME-Tox screening.
Table 7. Results of ADMETox study obtained for the compounds selected in the study.

<table>
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<tr>
<th>S N</th>
<th>Compound</th>
<th>MW</th>
<th>Log P</th>
<th>Log D</th>
<th>Log S W</th>
<th>tPSA</th>
<th>Ro B</th>
<th>RiB</th>
<th>Flx</th>
<th>H B D</th>
<th>H B A</th>
<th>H B D+ HBA</th>
<th>R</th>
<th>MSR</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>17-β estradiol</td>
<td>272.381</td>
<td>4.01</td>
<td>3.74</td>
<td>-4.16</td>
<td>40.46</td>
<td>0</td>
<td>20</td>
<td>0.00</td>
<td>2</td>
<td>2</td>
<td>4</td>
<td>1</td>
<td>17</td>
</tr>
<tr>
<td>2</td>
<td>Estriol</td>
<td>288.381</td>
<td>2.45</td>
<td>2.67</td>
<td>-3.27</td>
<td>60.69</td>
<td>0</td>
<td>20</td>
<td>0.00</td>
<td>3</td>
<td>3</td>
<td>6</td>
<td>1</td>
<td>17</td>
</tr>
<tr>
<td>3</td>
<td>Estrone</td>
<td>270.366</td>
<td>3.13</td>
<td>4.31</td>
<td>-3.59</td>
<td>37.30</td>
<td>0</td>
<td>21</td>
<td>0.00</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>1</td>
<td>17</td>
</tr>
<tr>
<td>4</td>
<td>Genistein</td>
<td>270.236</td>
<td>2.67</td>
<td>0.67</td>
<td>-3.53</td>
<td>93.73</td>
<td>1</td>
<td>18</td>
<td>0.05</td>
<td>3</td>
<td>5</td>
<td>8</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>5</td>
<td>Coumestrol</td>
<td>268.221</td>
<td>2.76</td>
<td>1.93</td>
<td>-3.69</td>
<td>86.64</td>
<td>0</td>
<td>21</td>
<td>0.00</td>
<td>2</td>
<td>5</td>
<td>7</td>
<td>1</td>
<td>17</td>
</tr>
</tbody>
</table>

CO=Compounds; MW=Molecular Weight; RB=Rotatable Bonds; RiB=Rigid Bonds; Flx=Flexibility; HBD=Hydrogen Bond Donors; R=Rings; MSR=Maximum Size Ring; CH=Charge; TCH=Total Charge; HvA=Heavy Atoms; CA=Carbon Atoms; HtA=Hetero Atoms; H/C= Ratio of Heavy Atoms and Carbon Atoms; LV=Lipinski Violations; S=Solubility(mg/l); SFI=Solubility Forecast Index; OBV=Oral Bioavailibility (VEBER); OBE=Oral Bioavailibility (EGAN); SC=Stereocentres; GS=Good Solubility; RS=Reduced Solubility; G=Good; A=Accepted St=Status.

Experiment 3:

To study the activities of the compounds based on their IC₅₀ values using QSAR equation.

Table 8. QSAR Analysis Statistics, Percent contribution of descriptor and QSAR Equations and Inhibitor-Plots for hERα and hERβ

<table>
<thead>
<tr>
<th>STATISTICS OF THE ANALYSIS for hERα</th>
<th>STATISTICS OF THE ANALYSIS for hERβ</th>
</tr>
</thead>
<tbody>
<tr>
<td>SSR</td>
<td>1.03</td>
</tr>
<tr>
<td>SSE</td>
<td>0.29</td>
</tr>
<tr>
<td>SST</td>
<td>1.32</td>
</tr>
<tr>
<td>Rsq</td>
<td>78.13 %</td>
</tr>
<tr>
<td>Adjusted Rsq</td>
<td>70.32 %</td>
</tr>
<tr>
<td>F statistics</td>
<td>10.00</td>
</tr>
<tr>
<td>Critical F</td>
<td>2.74</td>
</tr>
<tr>
<td>SSR</td>
<td>1.89</td>
</tr>
<tr>
<td>SSE</td>
<td>0.27</td>
</tr>
<tr>
<td>SST</td>
<td>2.16</td>
</tr>
<tr>
<td>Rsq</td>
<td>87.30 %</td>
</tr>
<tr>
<td>Adjusted Rsq</td>
<td>82.81 %</td>
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<tr>
<td>F statistics</td>
<td>19.47</td>
</tr>
<tr>
<td>Critical F</td>
<td>2.53</td>
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</tbody>
</table>
Nitu Debnath, 2016/ Studies on in silico estrogenicity and prediction of drug ability

Percentage contribution of each descriptor to activity

<table>
<thead>
<tr>
<th>Descriptor</th>
<th>Percentage Contribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>(LogP)</td>
<td>0.71%</td>
</tr>
<tr>
<td>(MolarVol)</td>
<td>0.68%</td>
</tr>
<tr>
<td>(IndexofRefraction)</td>
<td>0.18%</td>
</tr>
<tr>
<td>(Polarizability)</td>
<td>55.77%</td>
</tr>
<tr>
<td>(NominalMass)</td>
<td>0.18%</td>
</tr>
</tbody>
</table>

Percentage contribution of each descriptor to activity

<table>
<thead>
<tr>
<th>Descriptor</th>
<th>Percentage Contribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>(HBD)</td>
<td>11.29%</td>
</tr>
<tr>
<td>(Parachor)</td>
<td>1.27%</td>
</tr>
<tr>
<td>(SurfaceTension)</td>
<td>4.69%</td>
</tr>
<tr>
<td>(Density)</td>
<td>15.57%</td>
</tr>
<tr>
<td>(Polarizability)</td>
<td>8.66%</td>
</tr>
<tr>
<td>(AverageMass)</td>
<td>5.86%</td>
</tr>
</tbody>
</table>

**GENERATED QSAR EQUATION (hERα)**

Activity

\[ \text{Activity} = -1.718579455403E+000 + -5.482745009635E-002 \times (\log P) + 3.218823158542E-003 \times (\text{MolarVol}) + 1.197801416050E+000 \times (\text{Index of Refraction}) + -6.13307502981E+021 \times (\text{Polarizability}) + -8.265548137499E-004 \times (\text{Nominal Mass}) \]

**GENERATED QSAR EQUATION (hERβ)**

Activity

\[ \text{Activity} = 2.362021105954E+000 + -5.401816959673E-002 \times (\text{HBD}) + -5.918363655963E-003 \times (\text{Parachor}) + 2.725158687081E-002 \times (\text{Surface Tension}) + -3.278776437021E+000 \times (\text{Density}) + -1.744920307356E+022 \times (\text{Polarizability}) + 1.760163429116E-002 \times (\text{Average Mass}) \]

**QSAR Plot for hERα Inhibitors (20)**

**QSAR Plot for hERβ Inhibitors (24)**

Table: 9. Predicted Activity and IC50 (nM) of genistein, coumestrol and endogenous estrogens obtained using Quantitative Structure Activity Relationship (QSAR)

<table>
<thead>
<tr>
<th>Compounds</th>
<th>hER α</th>
<th>hER β</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC50 (nM)</td>
<td>Predicted Activity</td>
</tr>
<tr>
<td>17-beta estradiol</td>
<td>0.5011</td>
<td>0.3</td>
</tr>
<tr>
<td>Estriol</td>
<td>0.4365</td>
<td>0.36</td>
</tr>
<tr>
<td>Estrone</td>
<td>0.512861</td>
<td>0.29</td>
</tr>
<tr>
<td>Genistein</td>
<td>0.416869</td>
<td>0.38</td>
</tr>
<tr>
<td>Coumestrol</td>
<td>0.416869</td>
<td>0.38</td>
</tr>
</tbody>
</table>
Figure 8. Differential predicted activity pattern of phytoestrogens and endogenous reference estrogens for hERα and hERβ, determined through QSAR analysis.

From the result presented above (Fig. 8), it can be seen that all the compounds exhibited relatively higher activity with hERβ than hERα. Genistein has been found to have highest predicted activity with hERβ while both genistein and coumestrol have the same activity with hERα. QSAR Analysis Statistics, Percent contribution of descriptor, QSAR Equations and Inhibitor-plots for hERα and hERβ are shown in Table: 8.

Predicted activity with their derived IC₅₀ values are presented in the Table: 9.

Experiment 4:

To study drug-likeness of genistein and coumestrol.

From the analysis of Drug-likeness scores of the selected phytoestrogens, it is seen that only genistein, posseses the score in the range of 0.5-1.5 along with the endogenous estrogens (Fig. 9). However, both genistein and coumestrol satisfy Lipinski’s rule of five (Table: 7).

Genistein and coumestrol exhibit higher binding affinity than estradiol and binding similarity with estradiol for both hERα & hERβ. But, only Genistein shows acceptable Drug-likeness score.

Table: 10. Drug likeness of genistein and coumestrol compared to Endogenous Estrogens (HBA*=No. of Hydrogen Bond Acceptor ; HBD*-No. of Hydrogen Bond Donor)

<table>
<thead>
<tr>
<th>Sl No.</th>
<th>Compound</th>
<th>Mol wt.</th>
<th>HBA*</th>
<th>HBD*</th>
<th>MolLogP</th>
<th>Drug-likeness Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>17-beta estradiol</td>
<td>272.381</td>
<td>2</td>
<td>2</td>
<td>4.21</td>
<td>0.77</td>
</tr>
<tr>
<td>2</td>
<td>Estriol</td>
<td>288.381</td>
<td>3</td>
<td>3</td>
<td>3.17</td>
<td>0.90</td>
</tr>
<tr>
<td>3</td>
<td>Estrone</td>
<td>270.366</td>
<td>2</td>
<td>1</td>
<td>4.05</td>
<td>0.87</td>
</tr>
<tr>
<td>4</td>
<td>Genistein</td>
<td>270.2369</td>
<td>5</td>
<td>3</td>
<td>2.72</td>
<td>0.71</td>
</tr>
<tr>
<td>5</td>
<td>Coumestrol</td>
<td>268.221</td>
<td>5</td>
<td>2</td>
<td>3.04</td>
<td>-1.07</td>
</tr>
</tbody>
</table>
DISCUSSION

From the findings of the present study it is assumed that the dietary-compounds like genistein and coumestrol have differential affinity for human estrogen receptor alpha (hER\(\alpha\)) and beta (hER\(\beta\)) which is evident from docking analysis. Both compounds exhibited higher docking score for hER\(\beta\). This differential binding ability of phytoestrogens may helps to predict about their tissue-selective activity based on the tissue-specific expression of the two subtypes of estrogen receptor. Similar competitive molecular docking approach has also been utilized for predicting agonists and antagonists of estrogen receptor alpha [33].
In human cells, genistein and coumestrol showed a distinct preference for binding ERβ than for ERα, but only slight preference for transactivation of ERβ compared to ERα [3]. *In vitro* competitive binding assays also revealed that isoflavones, genistein, daidzein and equol have a relatively greater binding affinity for ERβ than for ERα, but are $10^2$ to $10^5$ times less active than steroidal estrogens [2, 5, 6, 7, 34]. Genistein particularly was found to have 20-fold higher binding affinity to ERβ than ERα by solid-phase binding assay [2, 5]. This indicates a significant similarity between *in-vitro* assay and *in-silico* approach to define estrogenicity of plant-derived compounds.

Determination of the potency of genistein and coumestrol through *in vitro* studies using recombinant yeast cells containing both human ERα and ERβ found that coumestrol is more potent than genistein with respect to both receptor subtypes [35]. Coumestrol was found to be 10 times more potent than genistein *in vivo* [36]. The recent investigations by the author also established that coumestrol is estrogenically more potent than genistein in ovariectomized mice [37, 38].

However, predicting the effects of isoflavones *in vivo* is more difficult because the route of administration, chemical form of the phytoestrogen, its metabolism, bioavailability, half-life, timing and level of exposure, intrinsic estrogenic state and non-hormonal secondary mediated actions of isoflavones also have to be considered in the design of clinical studies investigating their effects. Therefore, considering the time and cost involved in the *in vivo* screening methods, *in-silico* studies are extremely essential in screening & predicting toxicity and activities of large number of compounds.

The docking score of genistein for both the receptors also have been found to be higher than the most potent estrogen 17β estradiol. Interestingly, both genistein and coumestrol shared the same binding pockets in the hERα & hERβ with the endogenous estrogen, estradiol. This is probably the most significant outcome of docking study in an attempt to screen the estrogenic potential of the tested compounds. Various *in vitro* competitive binding assays and cell culture bioassays have reported the relative binding affinity of two potent dietary phytoestrogens viz., genistein and coumestrol with ERα & ERβ in the rank order as 17β estradiol> coumestrol>genistein [5, 36, 39, 35, 40]. However, *in-silico* rank order of binding of phytoestrogens with hERα & hERβ presented in the present study (on the basis of docking score) slightly differ from those reported through *in vitro* studies. These differences in results may be explained in terms of differences in the test environments viz. bioassays and computer-aided studies of molecular interaction. Keeping aside this difference, our results invariably supports the recognition of phytoestrogens as Selective Estrogen Receptor Modulators (SERMs).

ERα and ERβ are both expressed in the uterus, ovary, testes, and prostate, but with different cellular localization. In ovary, ERα is mainly expressed in thecal cells and in prostate mainly in the stroma compartment. ERβ, in its turn, was found to be expressed mainly in glandular epithelium of the uterus, granulosa cells of ovary and mainly in the epithelium of testes and prostate. Therefore, tissue and cell-type specific expression of ERα and ERβ are critical in mediating the estrogenic activities of any exogenous compound with differential affinity for these receptor subtypes. Thus, these findings also suggest that the estrogenic effects of these compounds may depend on both
type and the concentration of receptor expressed in a particular tissue. This is also of extreme importance in receptor subtype-specific designing of lead molecules.

The female BERKO mice suffered from reproductive defects due to attenuated response to the ovulatory hormone surge, suggesting the role of ERβ in the maturation of ovarian follicles and ovulation process [41]. In the uteri of ERβ−/− mice, there is an exaggerated responsiveness to E2 resulting in enlargement of the lumen, increase in volume and protein content of uterine secretion, and induction of aberrant epithelial expression of PR after E2 injection [42].

However, the most significant fact which finds a relevance to the present study is that ERβ is expressed in all cell types of uterine tissue and it modulates estrogenic function by inhibiting ERα function [42]. ERα was found to be abundantly expressed in intact mice, but in vehicle-treated ovariectomized mice, ERβ (but not ERα) was expressed in the luminal epithelium and both receptors were expressed in the stroma and glandular epithelium. In the glandular epithelium, the two receptors were colocalized in the same nuclei, but in the stroma, they were in separate cells [43]. Upon E2 treatment, ERβ was lost from the luminal epithelium, whereas the two receptors were colocalized in nuclei of the stroma and glandular epithelium [43]. This same study revealed that, deficiency of ERβ leads to hyperproliferation (due to dysregulation of EGF system) and loss of differentiation in the uterine epithelium, distortion of the shape of luminal epithelial cells due to decreased expression of E-cadherin (required at adherence junctions and essential for anchoring of epithelial cells) and CK-18 (intermediate filament, cellular differentiation marker).

Studies have shown that ERβ via AF-1 domain opposes ERα transactivation on reporter constructs and counteracts physiological effects of ERα on cell proliferation [44]. Indeed, ERβ has been shown to repress c-myc, cyclin D1 and cyclin A and to increase the expression of p21wafl/cip1 and p27kip1 causing a cell cycle arrest of MCF-7 breast cancer cells in G2 phase. It also decreases the contents of proliferation regulators such as cyclin E, cdc25 A, p45skp2 in T47D breast cancer cells.

These evidences in support of anti-proliferative effects of ERβ should provide the most important clue for successful identification and validation of lead molecules from nature which have the potential for preferential binding to the ERβ. The molecular properties of such natural plant-derived chemicals can be exploited to tailor the molecules to develop ERβ-specific leads which may have potential role in cancer chemoprevention. Therefore, genistein with higher binding affinity to hERβ than estradiol which also passed ADME-Tox filtering and showed good drug-likeness score can be of choise for designing herbal drugs against abnormal cellular proliferation. Moreover, genistein has been reported to inhibit aromatase which in turn reduces the endogenous estrogen production thereby reducing the estrogen load and thus helps in prevention of estrogen-dependent cancers [45].

Recent studies on meta-analysis of epidemiological reports also claim that lower endometrial cancer risk is associated with soy intake [46]. Study has also been conducted using molecular docking and 3D-QSAR to assess the potential of isoflavonoids as aromatase inhibitor against estrogen dependent breast cancer [47]. Further, genistein possess multiple mechanisms and
targets which lead to cancer prevention [48]. In silico study also claimed that genistein and daidzein can play important role in preventing breast cancer [49].

CONCLUSION

The present study clearly demonstrates the mode of interaction of dietary compounds like genistein and coumestrol, with human estrogen receptors hERα & hERβ and also establishes their preferential binding affinity to and activity with hERβ. The most exciting outcome of docking study is, in fact, the visualization of the common binding cavities in the receptors shared by genistein, coumestrol and estadiol. Although both compounds passed ADMetox filtering and exhibited considerable receptor-specific activity-IC50 profile, genistein has been found to have positive drug-likeness. Therefore, keeping in view of the multiple health beneficial effects of these natural compounds and their preferential interaction with antiproliferative-receptor subtype hERβ, the results presented here are important, in considering their potential for designing single and combinatorial drugs for cancer chemoprevention (breast and endometrial cancer), alleviation of post-menopausal syndromes, preventing premature puberty, cardiovascular diseases and reproductive toxicity in humans. Moreover, in-silico studies provide a reliable platform for faster and low-cost screening of large numbers of such plant-derived compounds for estrogenicity. However, the studies on the molecular dynamics simulation may provide a better understanding on the binding stability of the compounds with estrogen receptors to predict there in vivo potencies.

REFERENCES


