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Original Research Article

Prediction of Antiosteoporosis Activity of Thirty-Nine Phytoestrogen Compounds in Estrogen Receptor-Dependent Manner Through *In Silico* Approach

Burhan Ma'arif[1]*, Muhammad Aminullah[1], Nisfatul L. Saidah[1], Faisal A. Muslikh[2], Ana Rahmawati[3], Yen Y. A. Indrawijaya[1], Dewi P. Sari[4], Maximus M. Taek[5]

[1]*Department of Pharmacy, Faculty of Medical and Health Science, Maulana Malik Ibrahim State Islamic University, Malang 65151, Indonesia*
[2]*Faculty of Pharmacy, Airlangga University, Surabaya 60115, Indonesia*
[3]*Department of Medicine, Faculty of Medical and Health Science, Maulana Malik Ibrahim State Islamic University, Malang 65151, Indonesia*
[4]*Department of Pharmacy, Faculty of Science and Health, PGRI Adi Buana University, Surabaya 60245, Indonesia*
[5]*Department of Chemistry, Faculty of Mathematics and Natural Sciences, Widya Mandira Catholic University, Kupang 85225, Indonesia*

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ABSTRACT

Osteoporosis is one of the health problems in postmenopausal women due to estrogen deficiency. Phytoestrogen compounds can be used as an alternative osteoporosis treatment because of their similarity in structure and activity to estrogen. This research was conducted to predict the antiosteoporosis activity of thirty-nine phytoestrogen compounds and raloxifene, a modern antiosteoporosis drug *in silico*. The first step of the study involved the analysis of physicochemical properties of thirty-nine compounds and raloxifene using the SwissADME web tool. Compounds that met the criteria of the physicochemical properties were then subjected to molecular docking using PyRx 0.8 software with the AutoDock Vina method. The results were analyzed using Biovia Discovery Studio Visualizer 2016 software to find one or more compounds that predicted ER*β* agonists. Finally, a toxicity test using the pkCSM web tool on the predicted agonist compounds was conducted to determine the values of hepatoxicity, skin sensitization, and Ames toxicity. AdmetSAR2 web tool was also used to predict the LD_{50} class of toxicity. The results of this *in silico* study revealed that raloxifene and 23 compounds displayed agonist interaction toward ER*β*, and two of these compounds, namely catechin and epicatechin, were predicted agonist to ER*β* with binding values of -5.6 and -5.9 kcal/mol, respectively. These two compounds also showed the lowest toxicity. The finding from this research indicated that catechin and epicatechin are the most potent and non-toxic antiosteoporosis compounds among the 39 phytoestrogens.

***Keywords*:** Antiosteoporosis, Catechin, Epicatechin, ER*β*, *In silico*, Phytoestrogen.

Introduction

Postmenopause is one of the phases experienced by women due to the aging process. Postmenopausal women are characterized by cessation of menstruation and estrogen deficiency.[1] An estrogen deficiency condition causes women to experience various health problems, one of such is osteoporosis.[2] The disease is a condition of decreased bone density as a result of damage to the bone micro-architecture, which may cause bone brittleness.[3] Osteoporosis due to estrogen deficiency can be treated with hormone replacement therapy (HRT) or selective estrogen receptor modulators (SERMs).[4] Raloxifene is one of the SERMs, that have been shown to effectively reduce vertebral fracture in postmenopausal women.[5] However, long-term use of HRT or SERMs can cause various side effects, such as an increased risk of cardiovascular disorders, coronary events, venous thromboembolism, stroke, breast cancer, and dementia. These problems necessitated the search for an alternative treatment, such as phytoestrogen compounds.[2,6]

*Corresponding author. E mail: burhan.maarif@farmasi.uin-malang.ac.id
Tel: +62 81335555725

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A phytoestrogen is a group of compounds derived from plants that have a structure similar to estrogen. It can replace the function of estrogen in maintaining organ homeostasis, either by binding to the estrogen receptor (ER-dependent) or not (ER-independent). Phytoestrogen is easy to obtain and relatively has no side effects in application. Studies reported that phytoestrogen is effective to decrease complaints of diseases that arise due to estrogen deficiency,[8,9] thus, it can be a potential alternative for the treatment of osteoporosis due to estrogen deficiency.[9] Phytoestrogen can be found in several plants, such as *Glycine max*, *Marsilea crenata*, *Pueraria montana*, *Humulus lupulus*, *Glycyrrhiza glabra*, *Ipomoea batatas*, *Rheum rhabarbarum*, and *Vitex agnus-castus*.[10] According to literature studies, there are at least 39 compounds that can be classified as phytoestrogen, including catechin, epicatechin, genistein, kaempferol, luteolin, myricetin, naringenin, and quercetin.[11-17]

In silico study is a type of drug discovery approach, in which the activity of a drug is determined by evaluating the interaction between a ligand (drug) and target (protein) using computer programs.[18] The role of *in silico* studies in the discovery of new drugs is quite important as they help to visualize the mechanism of the drug against its target and optimize the compound form of the drug.[19] Visuals from *in silico* studies are in the form of anchoring ligands or drug compounds to targets in the form of macromolecules to obtain physical and chemical properties from the most optimal to the worst.[20] This research was performed to predict the antiosteoporosis activity of thirty-nine phytoestrogen compounds and a modern antiosteoporosis drug, raloxifene through an *in silico* study.

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Materials and Methods

Materials

The three-dimensional structure of the thirty-nine compounds and raloxifene was prepared using ChemDraw Ultra 12.0 software. The compounds were: apigenin, arbutin, baicalein, biochanin A, catechin, chalconaringenin, chrysin, coumestrol, cyanidin, daidzein, delphinidin, epicatechin, fisetin, formononetin, gallocatechin, genistein, glycitein, hesperidin, kaempferol, lariciresinol, luteolin, malvidin, matairesinol, medioresinol, morin, myricetin, naringenin, pelargonidin, peonidin, phloretin, phloridzin, puerarin, quercetin, resveratrol, rutin, secoisolariciresinol, sesamolin, syringaresinol, tangeretin, and one of SERMs, raloxifene.

Besides the 39 tested compounds and raloxifene mentioned above, the structure of the native ligand, 17*β*-estradiol, and the protein (receptor) was also prepared. The three-dimensional crystal structure of the phosphorylated ER*β* ligand-binding domain (ID 3OLL) was obtained from a protein data bank (www.rcsb.org). This protein was chosen because it has a native ligand in the form of estradiol. In addition, it is a phosphorylated ER*β* structure, so its efficiency as a receptor is high.[21]

Analysis of physicochemical properties

The first step was changing all the 39 phytoestrogen compounds as well as raloxifene to a simplified molecular-input line-entry system (SMILES) format from ChemDraw Ultra 12.0 software. Then, the SMILES format from each compound was copied and entered into the SwissADME web tool (http://www.swissadme.ch) to discover physicochemical properties based on its location in the Boiled-Egg diagram, Topological Polar Surface Area (TPSA) value, and Lipinski's rule of five.[22,23] Compounds that met physicochemical properties criteria were then subjected to molecular docking.

Molecular docking

The compounds that passed the selection of physicochemical analysis were prepared using Avogadro 1.0.1 software for geometry optimization to obtain a stable structure.[24,25] The ER*β* protein structure downloaded from PDB was then separated into native ligand and its protein using Biovia Discovery Studio Visualizer 2016 software.[26] After that, an internal validation was done using ER*β* protein re-docking and native ligand 17*β*-estradiol to determine the validation of the AutoDock Vina method toward ER*β* protein.[27,28] Root Mean Square Deviation (RMSD) value of <2Å of the internal validation showed that the AutoDock Vina method is valid for molecular docking toward ER*β*.[28,29] The process of molecular docking using software PyRx 0.8 with the AutoDock Vina method on each compound toward ER*β* protein (Figure 1), and the result was analyzed using Biovia Discovery Studio Visualizer 2016 software. The results of the molecular docking of each compound compared its similarity parameters with the native ligand to predict similar activity.

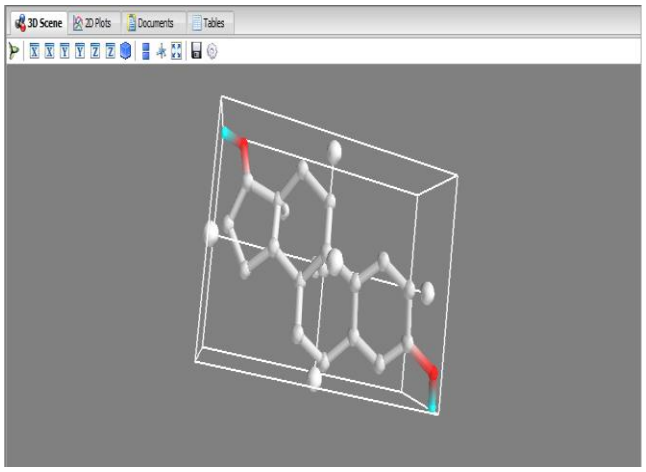

Figure 1: Grid box 17*β*-estradiol as native ligand

Toxicity test

This was conducted by inserting the compound's SMILES format on the pkCSM web tool (http://biosig.unimelb.edu.au/pkcsm) to predict the values of Ames toxicity, skin sensitization, and hepatotoxicity. Meanwhile, the admetSAR2 web tool was used to predict the toxicity class of compounds' lethal dose 50 (LD_{50}).

Results and Discussion

Phytoestrogen compounds can be used as an alternative to treat health problems due to estrogen deficiency, such as osteoporosis.[8] The use of alternative phytoestrogens becomes necessary due to the serious side effects of HRT or SERMs. Raloxifene is one example of SERMs and became the most widely studied compound within the second generation. It acts as an estrogen agonist in some tissues, while it functions as an estrogen antagonist in others. For example, when binding to the ER in osteoclast, osteoblast, and vascular endothelial cells, raloxifene can inhibit bone resorption mediated by the osteoclast and depress the serum cholesterol and low-density lipoprotein like the estrogen. While binding to the ER in mammary and endometria cells, raloxifene inhibits the hyperplasia of the two kinds of cells as the anti-estrogen. Therefore, long-term consumption of raloxifene can cause serious effects due to its agonist properties in other tissues.[5,30] In this research, an evaluation of the antiosteoporosis activity of 39 phytoestrogen compounds was conducted through an *in silico* study. This kind of study can help to predict the simple structure of a compound that has potency as medicine, using computer software.[31]

The results of the analysis of physicochemical properties of the compounds through the Boiled-Egg diagram, Lipinski's Rule of Five, and TPSA value are represented in Figure 2 and Table 1. The Boiled-Egg diagram and TPSA value indicate the ability of the compound to penetrate cell membranes. Compounds that can penetrate well are indicated by the TPSA value of ≤140 $Å^2$, and the position of the compound in yellow and white spots in the Boiled-Egg diagram.[22,24] The white spot indicates a high probability of gastrointestinal absorption, and a yellow spot indicates a high probability of brain penetration. Boiled-Egg diagrams can show the bonding of compounds with P-glycoprotein (P-gp), namely P-gp "yes" or plus sign with blue color (P-gp substrate), and P-gp "no" or minus sign with red color (P-gp non-substrate).[22] The other parameters from the physicochemical analysis are Lipinski's rule of five, including molecular weight ≤500 g/mol, log P ≤5, HBD ≤ 5, HBA ≤ 10. Lipinski's rule of five is stated with "Yes, 0 violation" in the SwissADME web tool. If a compound met the criteria of Lipinski's rule of five, thus, that compound can be used orally and accepted by the body.[23] The result of the physicochemical analysis showed that 32 of the 39 phytoestrogen compounds and raloxifene met the parameter requirements. The result obtained from the molecular docking process was the bond affinity value of each compound that showed an affinity degree when it binds to ER*β*.

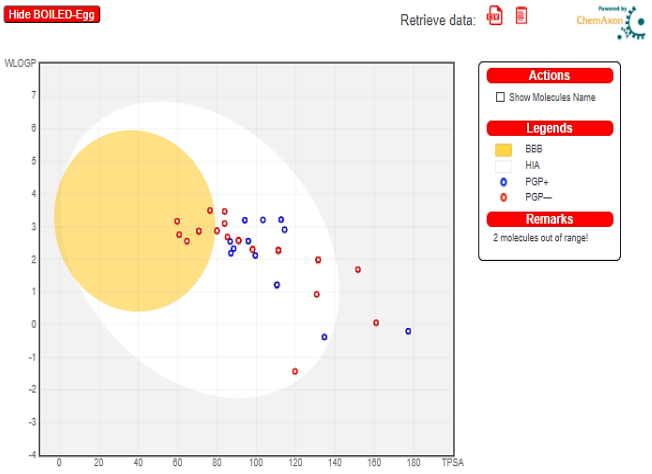

Figure 2: Physicochemical analysis of the phytoestrogen compounds through the Boiled-Egg diagram

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Table 1: Physicochemical properties of phytoestrogen compounds based on Lipinski's Rule of Five and TPSA value

No	Compounds	Parameters of Lipinski's Rule of Five				Lipinski's Rule of Five	TPSA (Å^2)
		BM ≤500 g/mol	Log P ≤ 5	HBA ≤ 5	HBD ≤ 5		
1	17*β*-estradiol	272.38	3.53	2	2	Yes	40.46
2	Raloxifene	473.58	3.21	5	2	Yes	98.24
3	Apigenin	270.24	2.11	5	3	Yes	90.90
4	Arbutin	272.25	-0.77	7	5	Yes	119.61
5	Baicalein	270.24	2.24	5	3	Yes	90.90
6	Biochanin A	284.26	2.44	5	1	Yes	79.90
7	Catechin	290.27	0.83	6	5	Yes	110.38
8	Chalconaringenin	272.25	1.83	5	4	Yes	97.99
9	Chrysin	254.24	2.55	4	2	Yes	70.67
10	Coumestrol	268.22	2.46	5	2	Yes	83.81
11	Cyanidin	287.24	0.56	6	5	Yes	114.29
12	Daidzein	254.24	2.24	4	2	Yes	70.67
13	Delphinidin	338.70	-0.79	7	6*	No*	134.52
14	Epicatechin	290.27	0.85	6	5	Yes	110.38
15	Fisetin	286.24	1.55	6	4	Yes	111.13
16	Formononetin	268.26	2.66	4	1	Yes	59.67
17	Gallocatechin	306.27	0.52	7	6*	No*	130.61
18	Genistein	270.24	2.04	5	3	Yes	90.90
19	Glycitein	284.26	2.30	5	2	Yes	79.90
20	Hesperidin	610.56*	-1.06	15*	8*	No*	234.29*
21	Kaempferol	286.24	1.58	6	4	Yes	111.13
22	Lariciresinol	360.40	2.38	6	2	Yes	85.22
23	Luteolin	286.24	1.73	6	4	Yes	111.13
24	Malvidin	331.30	0.71	7	4	Yes	112.52
25	Matairesinol	358.39	2.76	7	0	Yes	64.61
26	Medioresinol	388.41	2.33	3	3	Yes	60.69
27	Morin	302.24	1.2	7	5	Yes	131.36
28	Myricetin	318.24	0.79	8	6*	No*	151.59*
29	Naringenin	272.25	1.84	5	3	Yes	86.99
30	Pelargonidin	271.24	0.73	5	4	Yes	94.06
31	Peonidin	301.27	0.76	6	4	Yes	103.29
32	Phloretin	274.27	1.93	5	4	Yes	97.99
33	Phloridzin	436.41	0.06	10	7*	No*	177.14*
34	Puerarin	416.38	0.23	9	6*	No*	160.82*
35	Quercetin	302.24	1.23	7	5	Yes	131.36
36	Resveratrol	228.24	2.48	6	3	Yes	88.38
37	Rutin	610.52*	-1.51	16*	10*	No*	269.43*
38	Secoisolariciresinol	362.42	2.46	6	4	Yes	99.38
39	Sesamolin	370.35	2.74	8	2	Yes	95.84
40	Syringaresinol	418.44	2.33	7	2	Yes	86.61
41	Tangeretin	372.37	3.02	7	0	Yes	76.36

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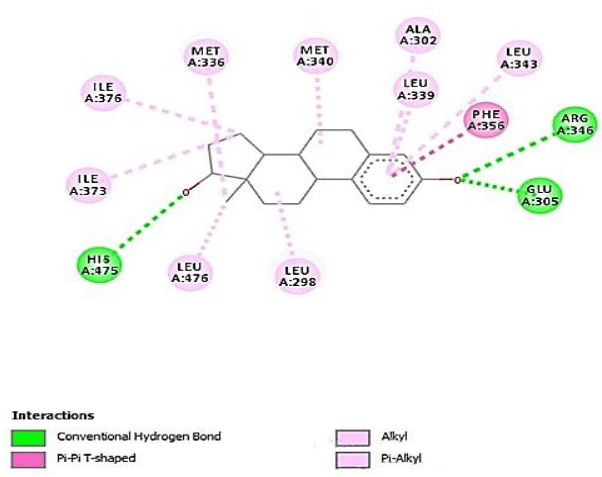
The bond affinity value is used to describe bond energy in the compound-receptor complex. The more negative bond affinity value or the smaller bond energy, the more stable conformation of compound and receptor.[32,33]

The result of the molecular docking process was analyzed using Biovia Discovery Studio Visualizer 2016 to discover which amino acid was bonded, types of amino acid bonds, and to map the pharmacophore distance of each compound when it binds to ER*β*. Amino acids form a protein, so it is important to see the similarity of the results of the amino acid residues that are bound from the molecular docking process between proteins and ligands. Compounds that have agonist interaction are shown by binding to amino acids, His 475, and Glu 305 or Arg 346. The relatively similar types of amino acids with the native ligand show the same interaction pattern, while compounds bind at least two of the same amino acids. However, the more similarities, the stronger the predictions of the similarity of activity.[34] The type of amino acid bond shows the stability of the bond as well. A hydrogen bond is the most stable and strong bond.[35] Meanwhile, the pharmacophore distance is the minimum range that is required by the molecule to bind with the receptor and produce activity. A compound is called an ER*β* agonist if it had a pharmacophore distance of about 11,126 Å. The similarity of the pharmacophore distance has a deviation of 1.0 from the native ligand; however, pharmacophore distance that slightly exceeds the tolerance limit can still be predicted to have similar activity if looking at the other parameters, for instance, amino acid bond.[26,36] The result of the molecular docking also revealed that raloxifene and the 23 compounds showed agonist interaction to ER*β*. The parameters of the native ligand and the results of molecular docking of compounds that were agonist can be seen in Figure 3 and Table 2.

Toxicity tests were performed on compounds that have agonist interaction to ER*β*, using pkCSM web tool to predict the values of hepatoxicity, skin sensitization, and Ames toxicity, whereas prediction of toxicity class of compound LD_{50} used admetSAR2 web tool. Hepatotoxicity is one of the kinds of toxicity used to identify compounds that are toxic to the liver.[37] Skin sensitization is a hypersensitivity reaction caused by reactive chemicals that penetrate the stratum corneum layer of the skin.[38] Ames toxicity is one of the methods used to discover the mutagenic and carcinogenic activity of several compounds.[39] Toxicity tests are divided into various classes. The toxicity class of LD_{50} is used to predict the toxicity level of the compounds. There are four toxicity classes of LD_{50}, namely class I, II, III, and IV. Class I contains the most toxic compound with a value of LD_{50} ≤50 mg/kg. Class II contains the quite toxic compound with a value of LD_{50} 50 mg/kg, but less than 500 mg/kg. Class III contains the slightly toxic compound with a value of LD_{50} over 500 mg/kg, but less than 5000 mg/kg; and Class IV contains the safe compound (non-toxic) with a value of LD_{50} >5000 mg/kg.[40,41] The result of the toxicity tests showed that raloxifene is quite toxic in Class II with LD_{50} of 400 mg/kg, indicated "yes" to hepatoxicity and Ames toxicity. Meanwhile, of the 23 agonist compounds, there were 16 non-toxic phytoestrogens, where the values of hepatoxicity, skin sensitization, and Ames toxicity were shown by “no” on the pkCSM web tool. On the level of toxicity class, each compound was shown by grade I until IV on the admetSAR2 web tool (Table 3). Two of the 16 phytoestrogen compounds had the lowest toxicity. It was observed that the best toxicity class of LD_{50}, such as catechin and epicatechin, which are in Class IV had a value of LD_{50} >5000 mg/kg and are considered safe (non-toxic).[40,41] As a result of the physicochemical analysis, molecular docking, and toxicity tests in this research showed that raloxifene is an agonist ER*β* and can be used to overcome osteoporosis in postmenopausal women. It has been reported in the literature that raloxifene acts as estrogen receptor (ER) agonist in bone. It prevents bone fracture by decreasing bone turnover and increasing bone mineral density.[30] However, the results (Figure 4) obtained revealed that the pharmacophore distance and binding affinity values are far from the native ligand. In addition, one of the bound amino acid residues has a type of bond that is not strong enough. This indicates that some phytoestrogen compounds have molecular docking results that are more similar to native ligand than raloxifene. The molecular docking results (Figure 5 and Figure 6) of catechin and epicatechin were predicted to have the most potential to be developed into antiosteoporosis agent, not only because it has low toxicity, but also due to the similarity with 17*β*-estradiol. There are bonds between catechin and the amino acid residues His 475, Glu 305, and Arg 346, with a pharmacophore distance of 10,798 Å. As for epicatechin, there are bonds with amino acid residues His 475 and Glu 305, with a pharmacophore distance of 10,627 Å. These results indicated that both compounds are agonists to ER*β*.

Phytoestrogen compounds that have agonist interaction with ER*β* and are non-toxic can be predicted to have the ability to bind with ER*β*. These compounds bind to ER*β* to produce *osteoblastogenesis* cytokines such as TGF-*β*, IGF-1, and IGF-2. Production of these cytokines may cause the occurrence of the process of the osteoblast differentiation to become mature osteoblasts in the process of bone formation. In contrast, phytoestrogens that can bind to ER*β* will reduce the production of *osteoclastogenic* cytokines including TNF-α, IL-1, and IL-6. If the production of cytokines decreased, it can inhibit the occurrence of osteoclast differentiation process and become mature osteoclast, resulting in the inhibition of the bone resorption process. On the other hand, the bonding phytoestrogen with ER*β* can increase the production of OPG, which may inhibit the occurrence of RANKL-RANK bonds.



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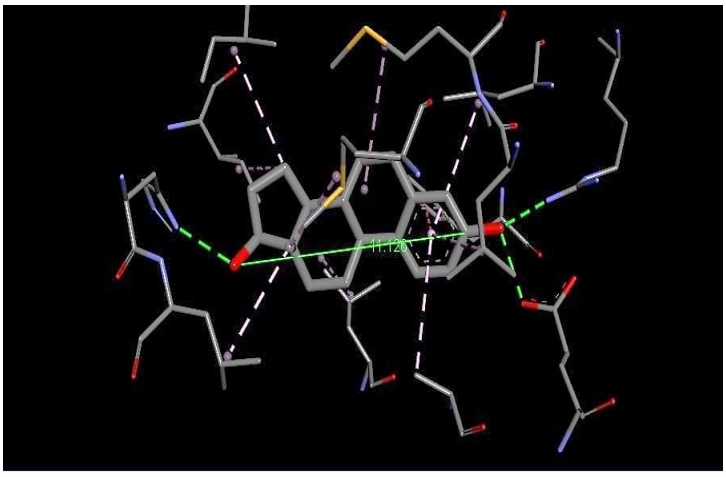

B

Figure 3: Visualization of molecular docking of 17*β*-estradiol as native ligand against ER*β*. **A:** 2D; **B:** 3D

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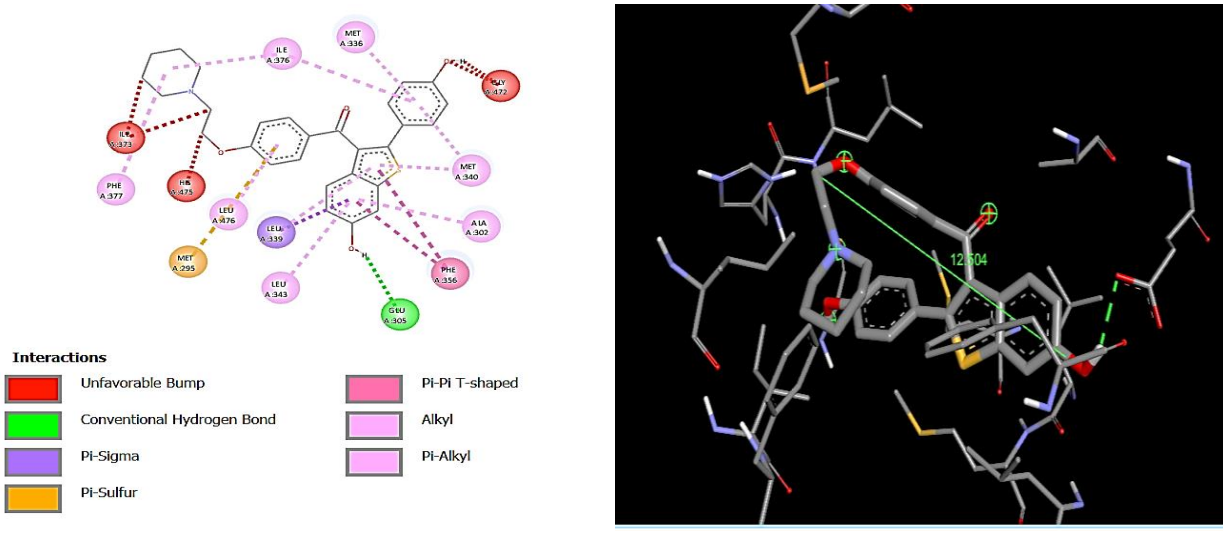
Table 2: Phytoestrogen compounds that have agonist interaction with ER*β*

No	Compounds	Binding Affinity (kcal/mol)	Amino Acid (Type of Bond)	Pharmacophore Distance (Å)
1	17*β*-estradiol	-10.2	His475(Hidrogen) Glu305(Hidrogen) Arg346(Hidrogen)	11.126
2	Raloxifene	5.3	His475(unfavorable) Glu305(Hidrogen)	12.504
3	Apigenin	-6.3	His475(Hidrogen) Glu305(Hidrogen) Arg346(Hidrogen)	10.772
4	Arbutin	-6.2	His475(Hidrogen) Glu305(Hidrogen)	9.913
5	Catechin	-5.6	His475(Hidrogen) Glu305(Hidrogen) Arg346(Hidrogen)	10.798
6	Chalconaringenin	-7.1	His475(Hidrogen) Glu305(Hidrogen)	11.647
7	Coumestrol	-8.7	His475(Hidrogen) Glu305(Hidrogen) Arg346(Hidrogen)	11.293
8	Cyanidin	-7.4	His475(Hidrogen) Glu305(Hidrogen)	10.800
9	Daidzein	-8.2	His475(Hidrogen) Glu305(Hidrogen) Arg346(Hidrogen)	12.133
10	Epicatechin	-5.9	His475(Hidrogen) Glu305(Hidrogen)	10.627
11	Fisetin	-7.5	His475(Hidrogen) Glu305(Hidrogen) Arg346(Hidrogen)	10.727
12	Genistein	-8.2	His475(Hidrogen) Glu305(Hidrogen) Arg346(Hidrogen)	12.138
13	Glycitein	-5.0	His475(Hidrogen) Glu305(Hidrogen) Arg346(Hidrogen)	12.103
14	Kaempferol	-7.6	His475(Hidrogen) Glu305(Hidrogen)	10.871
15	Lariciresinol	-3.6	His475(Hidrogen) Glu305(Hidrogen) Arg346(Hidrogen)	12.162
16	Luteolin	-5.3	His475(Hidrogen) Glu305(Hidrogen)	9.660
17	Malvidin	-3.9	His475(Hidrogen) Arg346(Hidrogen)	10.750
18	Morin	-7.5	His475(Hidrogen) Glu305(Hidrogen) Arg346(Hidrogen)	10.923
19	Naringenin	-6.8	His475(Hidrogen) Glu305(Hidrogen)	10.708
20	Pelargonidin	-7.0	His475(Hidrogen) Glu305(Hidrogen)	10.237
21	Peonidin	-6.9	His475(Hidrogen) Arg346(Hidrogen)	10.742
22	Phloretin	-7.3	His475(Hidrogen) Glu305(Hidrogen)	11.361
23	Quercetin	-5.2	His475(Hidrogen) Glu305(Hidrogen)	10.590
24	Resveratrol	-6.6	His475(Hidrogen) Glu305(Hidrogen) Arg346(Hidrogen)	11.335
25	Secoisolariciresinol	-4.8	His475(Hidrogen) Glu305(Hidrogen)	10.183



A **B**

Figure 4: Visualization of the molecular docking of Raloxifene against ER*β*. **A:** 2D; **B:** 3D

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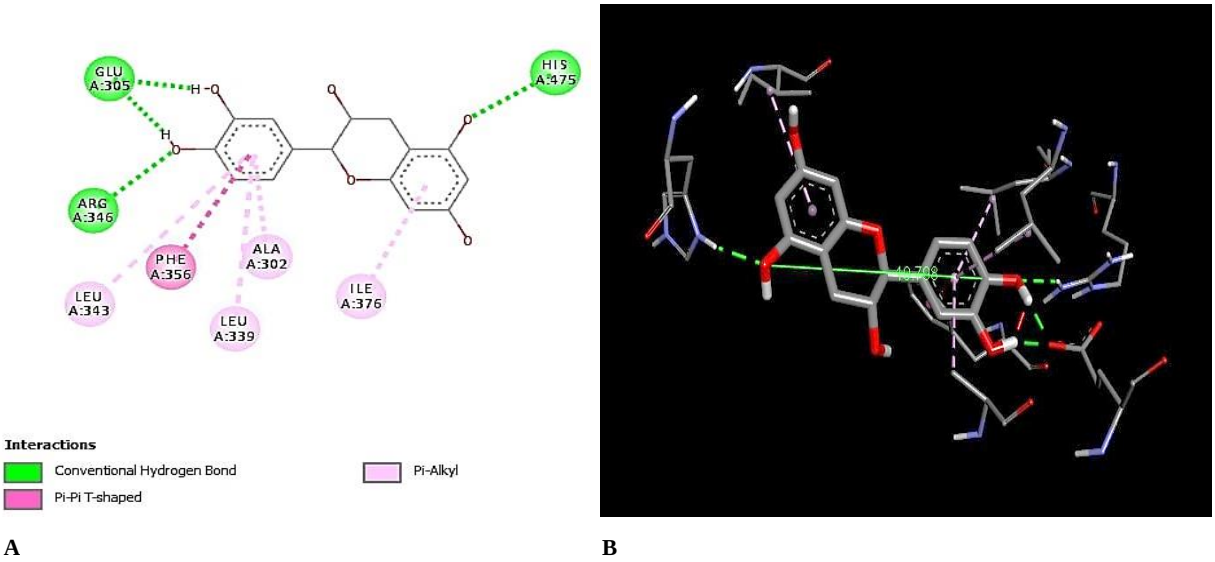

Figure 5: Visualization of the molecular docking of catechin against ER*β*. **A:** 2D; **B:** 3D

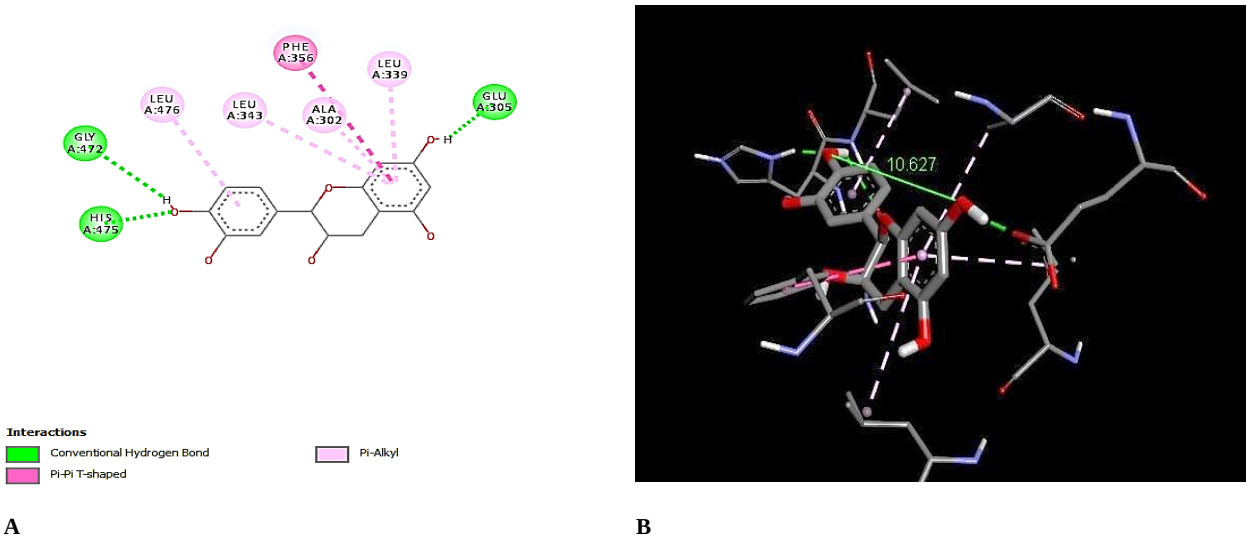

Figure 6: Visualization of the molecular docking of epicatechin against ER*β*. **A:** 2D; **B:** 3D

This can inhibit the occurrence of bone loss.[42] Catechin and epicatechin have potency as antiosteoporosis with ER-dependent mechanisms. It is indicated by agonist interactions of both compounds while binding with ER*β*, which are also in the low toxicity class. Some literature showed that catechin and epicatechin have anti-resorptive properties and, hence, can increase osteoclast apoptosis and inhibit osteoclastogenesis. This is discovered by the ability of both compounds to inhibit the secretion of TNF-α and IL-6 in osteoblast cells. Decreased TNF-α and IL-6 resulted in increased bone mass and, decreased bone resorption.[43] Furthermore, catechin and epicatechin can provide activity as well by ER-independent pathway mechanism. They can provide antioxidant activity by reducing NF-κB, TNF-*α*, nitric oxide (NO), and reactive oxygen species (ROS) activities. Because these cytokines induce oxidative stress, which leads to bone loss, reducing oxidative stress with antioxidants may be a possible strategy for osteoporosis prevention.[43,44] Therefore, catechin and epicatechin can inhibit osteoporosis and may be developed into antiosteoporosis medicine for oral use.

Conclusion

Catechin and epicatechin are the most potent and non-toxic antiosteoporosis compounds among the 39 phytoestrogens that act through an ER-dependent mechanism.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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