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In-silico Study of Certain Phytoestrogen Flavonoids Involved in Breast Cancer by Evaluating their Comparative Binding Interaction with Human Estrogen Receptors (Erα and Erβ) to Identify Better Anti-estrogenic Activity

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Abstract

Breast cancer is the most common cause of cancer deaths among women worldwide. Although estrogen is involved in the development of the mammary gland, hyper-estrogen can be carcinogenic. Phytoestrogens derived from plants exert estrogenic as well as antiestrogenic effects and multiple actions within breast cancer cells. In this molecular docking study, the binding of certain flavonoids and isoflavonoids viz., apigenin, genistein, kaempferol, and quercetin with the human Estrogen Receptor (ER) alpha and beta were analyzed. Apigenin showed high binding efficiency of -7.87 Kcal/mol and -8.06Kcal/mol with ERα and ERβ, respectively, whereas kaempferol showed binding efficiency of -7.44Kcal/mol and -7.46Kcal/mol with ERα and ERβ, respectively. Quercetin showed binding efficiency of -7.57Kcal/mol and -7.89Kcal/mol, while the isoflavonoid genistein showed low binding efficiency of -3.37Kcal/mol and -3.57Kcal/mol towards ERα and ERβ, respectively. Thus, apigenin, kaempferol, and quercetin revealed high binding efficiency with the human estrogen receptors alpha and beta, while genistein showed low binding efficiency towards both receptors. Hence, the consumption of different phytoestrogen-rich foods may help in the prevention and/or treatment of breast cancer, although further scientific investigations are required.

Keywords: Antiestrogenic, Apigenin, Breast Cancer, Docking, Genistein, Kaempferol, Phytoestrogens, Qercetin

1. Introduction

Breast cancer is considered the most prevalent and fatal cancer in women worldwide[1] and is the second leading cause of death by non-skin cancers after lung cancer[2]. Estrogens are steroidal hormones involved in the development maintenance and regulation of mammary glands. It associates with two receptor subtypes in humans, viz., ERα and ERβ, and both the receptor subtypes are expressed in cells. The activation of ERα by estrogens is responsible for the proliferation of breast cells in breast cancers, whereas ERβ possesses an antiproliferative effect[3].

ERα is present mainly in mammary glands, uterus, ovary (thecal cells), bone, male reproductive organs, prostate (stroma), liver, and adipose tissue. By contrast, ERβ is found mainly in the prostate, bladder, ovary

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(granulosa cells), colon, adipose tissue, and immune system. The alpha subtype has a major role in the mammary gland, and uterus, as well as in skeletal homeostasis and metabolism. The beta subtype affects the central nervous and immune systems, and it counteracts the ERα-promoted cell hyperproliferation in the breast and uterus. Their amino acid sequence shows about 59% identity in their respective Ligand-Binding Domains (LBD). The differences within the ligand binding cavities are at only two amino acid positions. In ERα, LEU384 and MET421 are replaced by MET336 and ILE373, respectively, whereas in ERβ, not only are these conservative changes between hydrophobic residues, but they are also reciprocal, with each ER subtype having one methionine residue and then either a leucine or isoleucine residue[3].

Phytoestrogens structurally mimic human estrogen 17β-estradiol which enables them to bind to the Estrogen Receptor (ER) causing estrogenic or antiestrogenic effects. Phytoestrogens are divided into flavanones, flavonoids, isoflavonoids, coumestans, chalcones, stilbenes and lignans. Based on the chemical structure, they are divided into two main subgroups, isoflavonoids and lignans. The isoflavonoids are further divided into isoflavones and coumestans[4].

Flavonoids such as isoflavones and coumestans are polyphenols synthesized by plants and are present in fruits and vegetables[5]. They exhibit a structure similar to estrogens, thereby causing them to show estrogenic or anti-estrogenic effects. Apigenin, a flavone, is found in several dietary plants such as parsley, celery, thyme, onions, lemon balm, and oranges, exhibiting both estrogenic and antiestrogenic effects in breast cancer. It possesses cytotoxic activities against breast cancer cell line MCF7, and is also known to induce apoptosis in human colon cancer cells[6]. Kaempferol is another major flavonoid found in plants such as beans, broccoli, cabbage, and cauliflower. It is known to effectively prevent the growth of breast cancer cells VM7, Luc4E2, MDA- MB-231, and

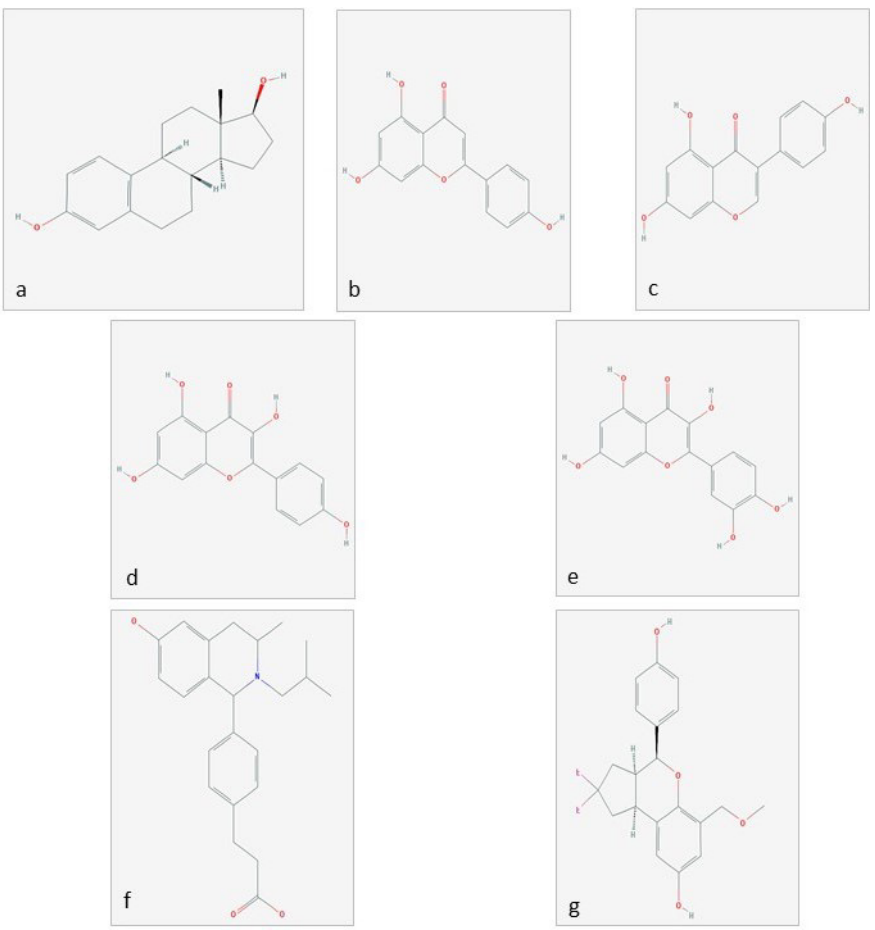

Figure 1. Chemical structures. **(a)** 17β-estradiol. **(b)** Apigenin. **(c)** Genistein. **(d)** Kaempferol. **(e)** Quercetin. **(f)** GQD. **(g)** 3AS (Source- PubChem; https://pubchem.ncbi.nlm.nih.gov).

MCF-7[7], and it also increases the levels of pro-apoptotic enzymes and proteins, such as cleaved caspase-9, -7, -3, p21, p53, Bax, PARP, and p-ATM[8,9]. It also decreases the levels of anti-apoptotic proteins Bcl2, polo-like kinase 1 (PLK-1), pAKT, phosphorylated insulin receptor substrate 1 (pIRS-1), phosphorylated mitogen-activated protein kinase (pMEK)1/2, cyclin-dependent kinase 1 (CDK1), cyclins A, B, D1, and E, and cathepsin D[10–12]. Quercetin is another flavonoid, abundant in onions, apples, legumes, berries, parsley, and citrus fruits[13,14]. In addition to its anti-inflammatory, antioxidant, anti-allergic[13] and anti-platelet aggregation properties, quercetin inhibits the growth and proliferation of cancer cells[15,16]. Genistein, found in soy and beans, also has an antiestrogenic effect in various cell lines. It activates apoptosis, inhibits protein-tyrosine kinase activity, and suppresses angiogenesis[17,18].

Isoflavones can bind to both subtypes of ER, although they have a higher binding affinity to the subtype ERβ. Such specific affinity for ER allows the isoflavones to express estrogenic and antiestrogenic effects, depending on the tissue type and the endogenous estrogen content. Since phytoestrogens are known to protect against breast cancer, four phytoestrogens, namely, apigenin, genistein, kaempferol, and quercetin (Figure 1) were selected for this *in-silico* study to understand their binding efficiency with the estrogen receptors which further describes their anti-estrogenic efficacy.

2. Materials and Methods

2.1 Ligand Retrieval

The ligands were retrieved from the PubChem database (https://pubchem.ncbi.nlm.nih.gov) and the phytoestrogens were searched in the PubChem database individually. The Compound ID was noted down and their 3D structures were downloaded in the SDF (Structure Data File) format (Table 1).

2.2 File Conversion (SDF to PDB)

The SDF files of conformers were converted into PDB file format using Open Babel software (http://openbabel.org). The input and output formats were selected as SDF and PDB, respectively. Later, the SDF files were uploaded and then converted. The name of the files was saved as ligand.pdb and this procedure was repeated for all other ligands thereby making the ligands ready for docking.

2.3 Protein Retrieval and Preparation

The protein names were searched in RCSB PDB (http://www.rcsb.org/pdb/). The best result showing human protein was chosen and its 3D protein structure was downloaded in the PDB format. The two proteins which were retrieved along with their unique ligands are described in Table 2. The co-crystallized human proteins ERα (Figure 2) and ERβ (Figure 3) were analyzed using the BIOVIA Discovery Studio 2021 software for visualizing the 2D interaction between the ligands and the amino acids in the Ligand Binding Domain (LBD) of the proteins. The co-crystallized human proteins were then prepared for docking. The unique ligands (co-crystal ligands) attached to the co-crystallized proteins were removed in UCSF Chimera (https://www.cgl.ucsf.edu/chimera/). The human 3D protein structure was opened and chain A was selected for both the human proteins, while other chains were removed. The unique ligands IOG and estradiol were then removed from chain A of human ERα and ERβ, respectively, thereby making the human proteins ERα and ERβ ready for docking.

Table 1. List of ligands retrieved from PubChem along with their compound ID

Ligands	Type of Ligands	IUPAC Name	PubChem
Apigenin	Test ligand	5,7-dihydroxy-2-(4-hydroxyphenyl)chromen-4-one	5280443
Genistein	Test ligand	5,7-dihydroxy-3-(4-hydroxyphenyl)chromen-4-one	5280961
Kaempferol	Test ligand	3,5,7-trihydroxy-2-(4-hydroxyphenyl)chromen-4-one	5280863
Quercetin	Test ligand	2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxychromen-4-one	5280343
GQD	co-crystal ligand	(E)-3-[4-[(1R,3R)-6-hydroxy-2-isobutyl-3-methyl-3,4-dihydro-1H-isoquinolin-1-yl]phenyl]prop-2-enoic acid	310304205
3AS	co-crystal ligand	(3As,4R,9bR)-2,2-difluoro-4-(4-hydroxyphenyl)-6-(methoxymethyl)-1,2,3,3a,4,9b-hexahydrocyclopenta[c]chromen-8-ol	16758226

Table 2. List of proteins with their unique ligands

Name of proteins	PDB ID	Unique ligand
Human estrogen receptor alpha (ERα)	5FQP	GQD
Human estrogen receptor beta (ERβ)	2QTU	3AS

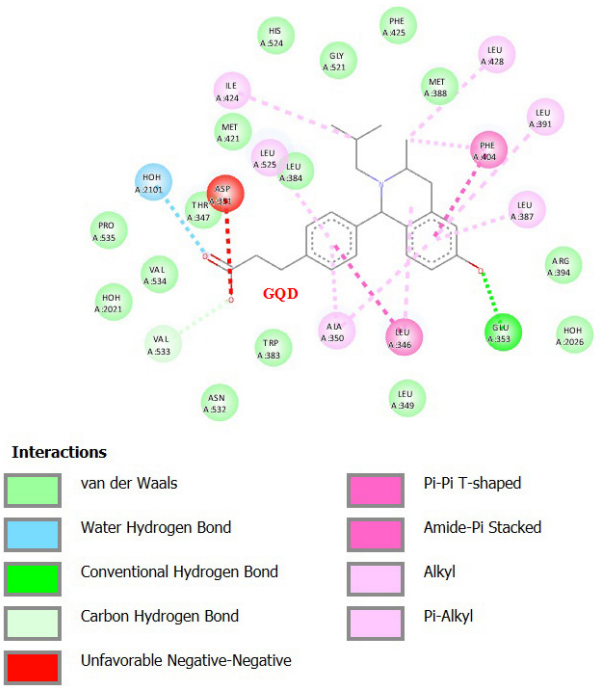

Figure 2. 2D interaction between GQD and the amino acids in the Ligand Binding Domain (LBD) of human Erα.

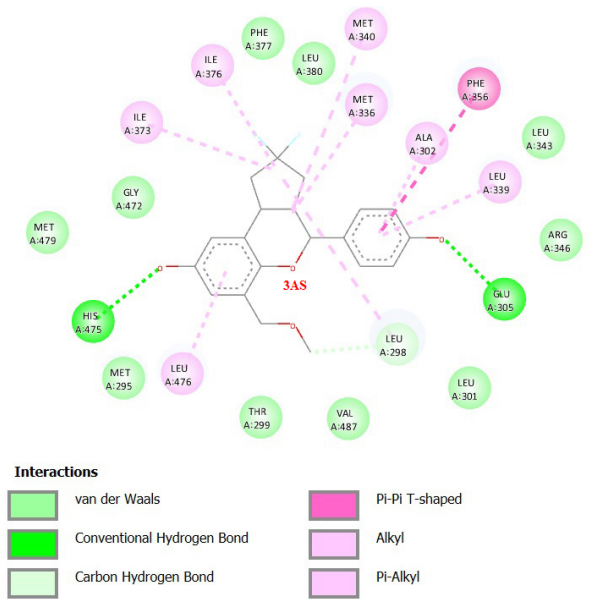

Figure 3. 2D interaction between 3AS and the amino acids in the Ligand Binding Domain (LBD) of human Erβ.

2.4 Docking

Auto Dock Tool 1.5.6 (ADT) software was used for preparing ligands and proteins for docking, which uses Lamarckian Genetic Algorithm (LGA) for the analysis of protein-ligand complexes. First, docking was performed for the template proteins, human ERα and ERβ, and their ligands, IOG and estradiol, respectively. Again, docking was performed for each test ligand (apigenin, genistein, kaempferol, and quercetin) with both human protein ERα and ERβ, respectively. The Auto Dock 1.5.6 program uses free binding energy for scoring the ligand-protein complexes (10 complexes). Then, the 2D interactions between the ligand and the amino acids in the LBD of proteins were visualized in BIOVIA Discovery Studio. Docking was performed for each protein ligand separately and the grid box measurements were applied for each docking as described in Table 3.

3. Results

3.1 Docking of GQD with Human ERα

GQD exhibited ligand-binding interactions with human ERα (Figure 4). The most favourable ligand binding complex obtained after docking had a binding energy (ΔG) of -10.94Kcal/mol. Twenty-three out of 24 amino acids in the LBD were found to interact with GQD. Among them, three amino acids showed conventional H-bond (GLU353, LEU387, and ARG394), thirteen amino acids exhibited Van der Walls interaction (LEU346, THR347, LEU349, LEU384, MET421, ILE424, PHE425, GLY521, HIS524, LEU525, VAL533, VAL534, and LEU383), whereas PRO535, PHE404, and ASP351 showed carbon-hydrogen bond, pi-pi T shaped bond and unfavourable negative-negative bond, respectively, while two amino acids showed alkyl bond (LEU428, and MET388) and pi-alkyl bonds (ALA350, and LEU391) each.

Table 3. Grid box measurements applied for docking between different proteins and their ligands

Protein	Ligand	Grid box details				
		Dimensions	Spacing	Coordinates		
				X	Y	Z
Human ERα	GQD		0.375	13.872	23.806	66.701
Human ERβ	3AS		0.375	14.912	31.962	70.286
Human ERα	Apigenin		0.375	12.976	22.144	66.653
Human ERα	Genistein		0.375	13.285	22.279	66.973
Human ERα	Kaempferol		0.375	15.369	24.299	67.084
Human ERα	Quercetin		0.375	12.672	25.216	66.591
Human ERβ	Apigenin		0.375	75.611	39.399	70.202
Human ERβ	Genistein		0.375	74.912	40.488	70.286
Human ERβ	Kaempferol		0.375	74.912	38.767	70.286
Human ERβ	Quercetin		0.375	75.832	39.244	70.286

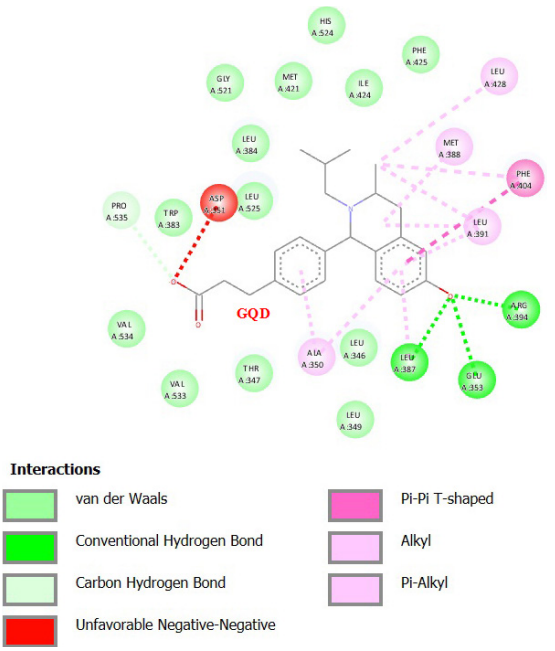

Figure 4. 2D interaction between GQD and human Erα.

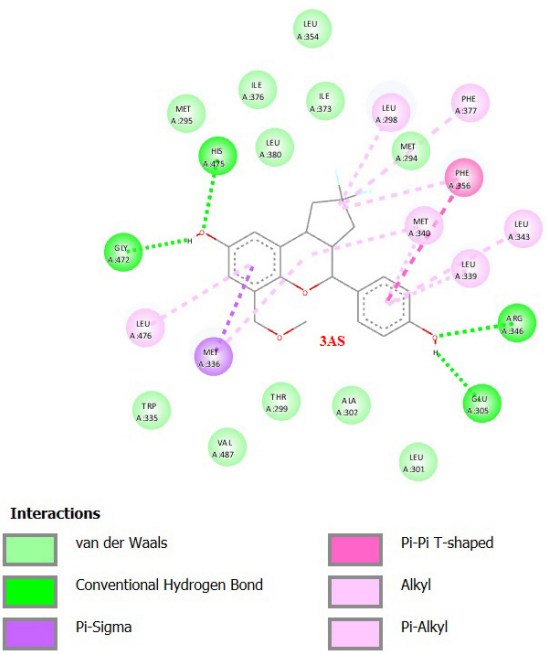

Figure 5. 2D interaction between 3AS and human Erβ.

3.2 Docking of 3AS with Human ERβ

The complex with the lowest binding energy (ΔG) of -10.29Kcal/mol established interaction of 3AS with amino acid residues in LBD of human ERβ (Figure 5). Nineteen out of 20 amino acids were found to interact, among which GLU305, ARG346, GLY472 and HIS475 showed conventional H-bond, eight amino acids showed Van der Walls interactions (MET295, THR299, LEU301, ALA302, ILE373, ILE376, LEU380, and VAL487), three showed alkyl bonds (LEU298, PHE377, and LEU476), three showed pi-alkyl bonds (LEU339, MET340, and LEU343) and one each showed pi-pi T-shaped bond (PHE356) and pi-sigma bond (MET336).

3.3 Docking of Apigenin with Human ERα

The complex with the lowest binding energy (ΔG) of -7.87Kcal/mol (Figure 6) where fifteen out of 24 amino acids were found to interact. Three amino acids showed conventional hydrogen bonds (THR347, GLU353, and ARG394), MET388 showed a carbon-hydrogen bond, MET343 showed a pi-sulfur bond, four amino acids showed Van der Walls interactions (LEU349, TRP383, LEU428, and VAL533), five of them showed pi-alkyl bonds (ALA350, LEU384, LEU387, LEU391, and LEU525), and one each showed pi-pi T-shaped bond (PHE404) and amide-pi stacked bond (LEU346).

3.4 Docking of Apigenin with Human ERβ

The complex with binding energy (ΔG) of -8.06Kcal/mol is the lowest and shows the most favourable interaction (Figure 7). Sixteen out of 20amino acids were found to interact, among which the conventional hydrogen bond was shown by three amino acids (GLU305, ARG346, and GLY472), eight amino acids showed Van der Walls interactions (MET295, LEU298, LEU301, MET336, ILE373, PHE377, LEU380, and LEU476), other four showed pi-alkyl bonds (LEU339, MET340, LEU343, and ILE376), while only one showed pi-pi T-shaped bond (PHE356).

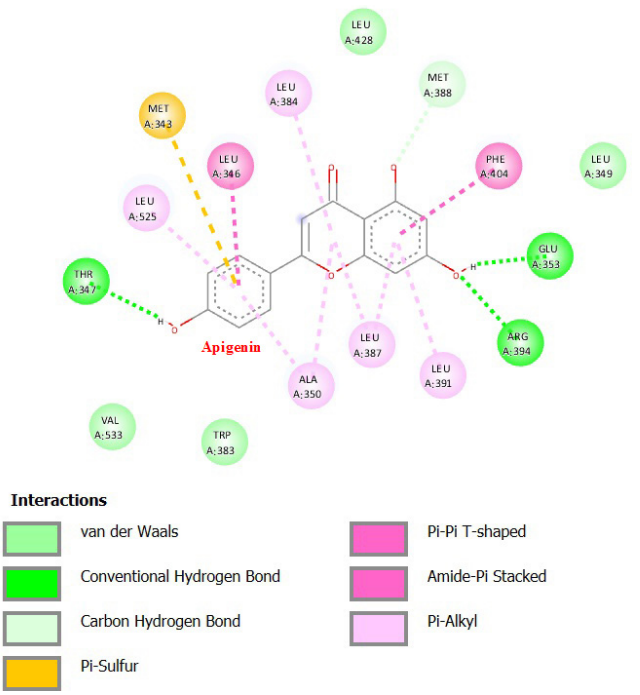

Figure 6. 2D interaction between apigenin and human Erα.

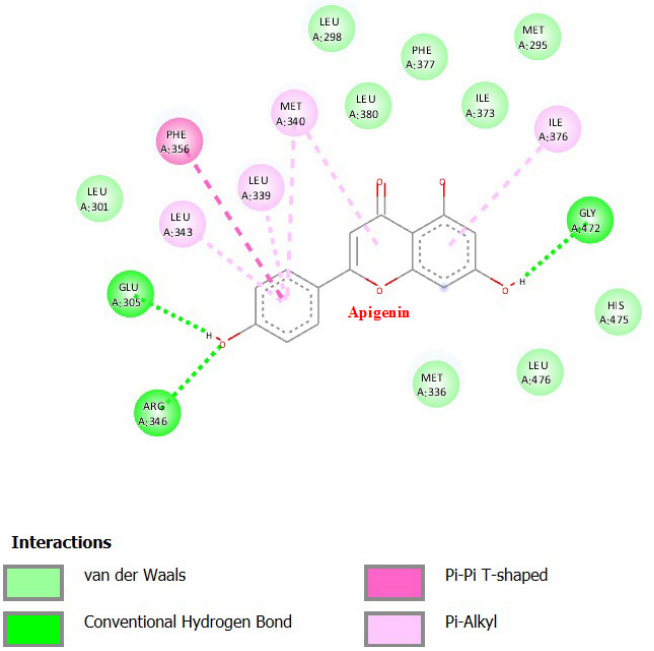

Figure 7. 2D interaction between apigenin and human Erβ.

3.5 Docking of Genistein with Human ERα

The most favourable ligand binding complex obtained after docking had binding energy (ΔG) of -3.6 Kcal/mol (Figure 8). Nine out of 24 amino acids were found to interact with the LBD with four showing Van der Walls interactions (LEU387, MET388, LEU391, and ARG394),

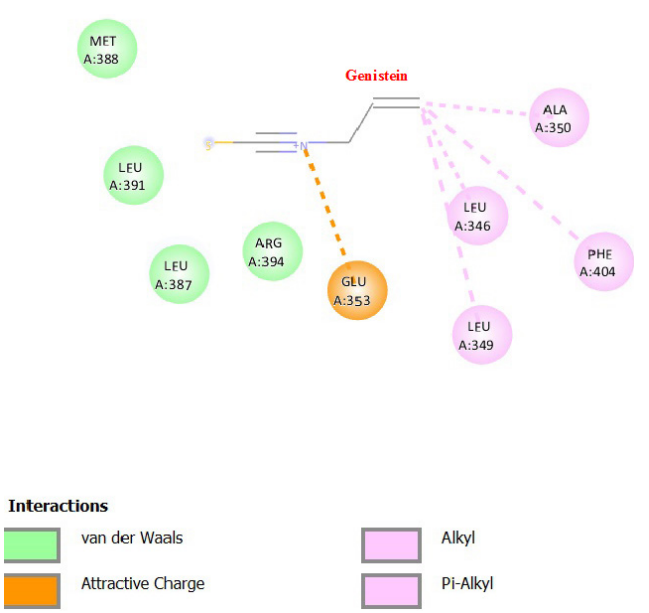

Figure 8. 2D interaction between genistein and human Erα.

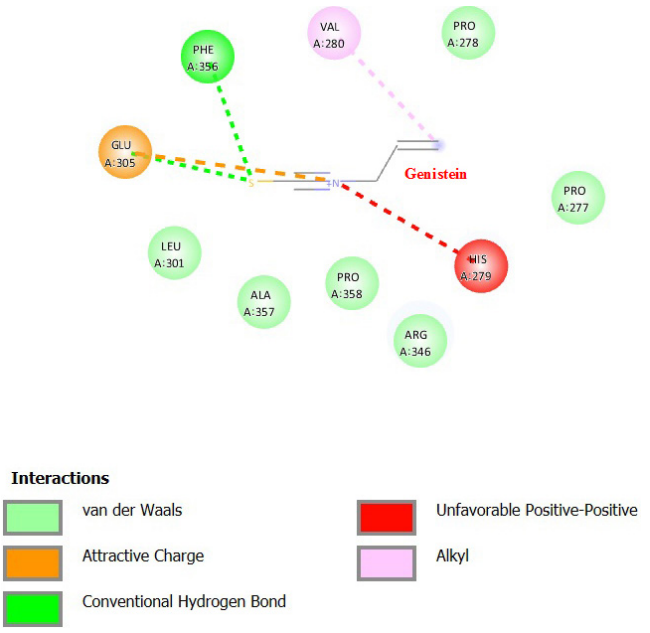

Figure 9. 2D interaction between genistein and human Erβ.

three showing pi-alkyl bonds (LEU346, LEU349, and ALA350) and one showing alkyl bond (GLU353) and one attractive charge (GLU353) each.

3.6 Docking of Genistein with Human ERβ

The complex with binding energy (ΔG) of -3.38Kcal/mol showed the most favourable interaction (Figure 9). Four out of twenty amino acids were found to interact. PHE356 showed conventional H-bond, two showed Van der Walls interaction (LEU301 and ARG346), while GLU305 bond with attractive charge.

3.7 Docking of Kaempferol with Human ERα

The complex with binding energy (ΔG) of -7.43Kcal/mol showed the most favorable interaction (Figure 10). Seventeen out of 24 amino acids were found to interact with the LBD of the protein. Among them, three showed conventional hydrogen bonds (GLU353, ARG394, and THR347), seven were involved in Van der Walls interactions (LEU349, TRP383, MET388, ILE424, LEU428, GLY521, and VAL533), four formed pi-alkyl bonds (ALA350, LEU384, LEU387, and LEU391), two bonds with pi-pi T-shaped bonds (PHE404 and LEU346), while LEU545 formed pi-sigma bond.

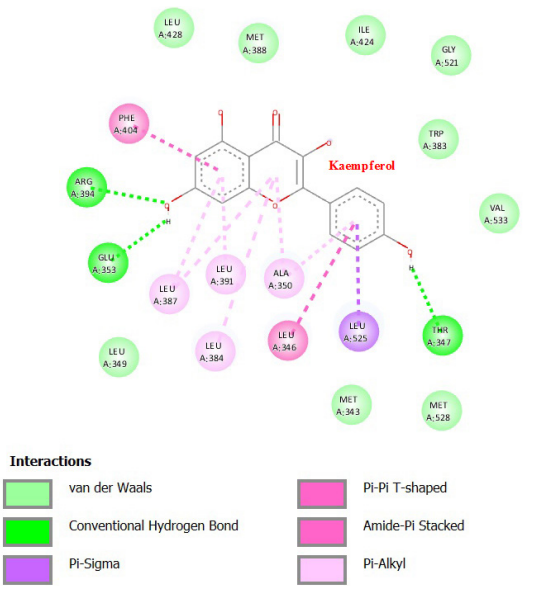

Figure 10. 2D interaction between kaempferol and human Erα.

3.8 Docking of Kaempferol with Human ERβ

The complex with the lowest binding energy (ΔG) of -7.46Kcal/mol (Figure 11) with sixteen out of 20amino acids interacting, has three showing conventional hydrogen bonds (GLU305, ARG346, and GLY472), eight showing Van der Walls interactions (MET295, LEU298, LEU301, MET336, ILE373, PHE377, LEU380, and LEU476), four showing pi-alkyl bonds (LEU339, MET340, LEU343, and ILE376), while pi-pi T-shaped bonds were shown by one amino acid (PHE356).

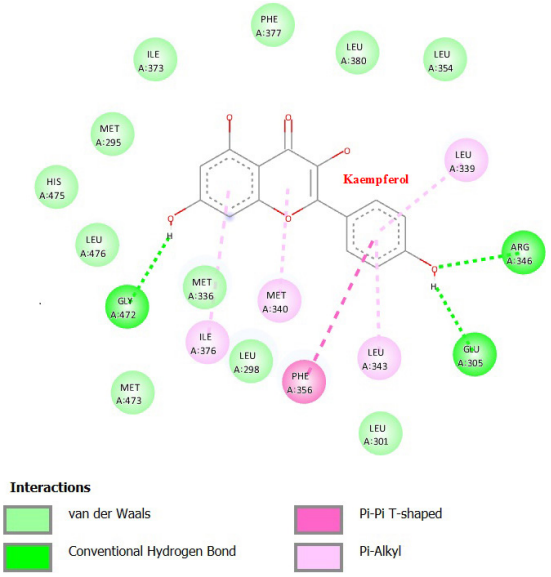

Figure 11. 2D interaction between kaempferol and human Erβ.

3.9 Docking of Quercetin with Human ERα

The complex with binding energy (ΔG) of -7.57Kcal/mol was the lowest and showed most favourable interaction (Figure 12). Fifteen out of 24 amino acids were found to interact with the LBD of the protein. Among them, two showed conventional hydrogen bonds (GLU353 and ARG394), five were involved in Van der Walls interactions (LEU349, TRP383, LEU428, GLY521, and VAL533), while four formed pi-alkyl bonds (ALA350, LEU384, LEU391, and LEU525), two were found to be bonded with pi-pi T-shaped bonds (LEU346 and PHE404), while MET388 showed carbon-hydrogen bond and LEU387 showed pi-sigma bond.

3.10 Docking of Quercetin with Human ERβ

The complex with the lowest binding energy (ΔG) of -7.89Kcal/mol (Figure 13), with seventeen out of 20amino acids interacting, has three amino acids showing conventional hydrogen bonds (GLU305, ARG346, and GLY472), nine showing Van der Walls interactions (MET295, LEU298, LEU301, ALA302, MET336, ILE373, PHE377, LEU380, and LEU476), four showing pi-alkyl bonds (LEU339, MET340, LEU343, and ILE376) while pi-pi T-shaped was shown by one amino acid (PHE356).

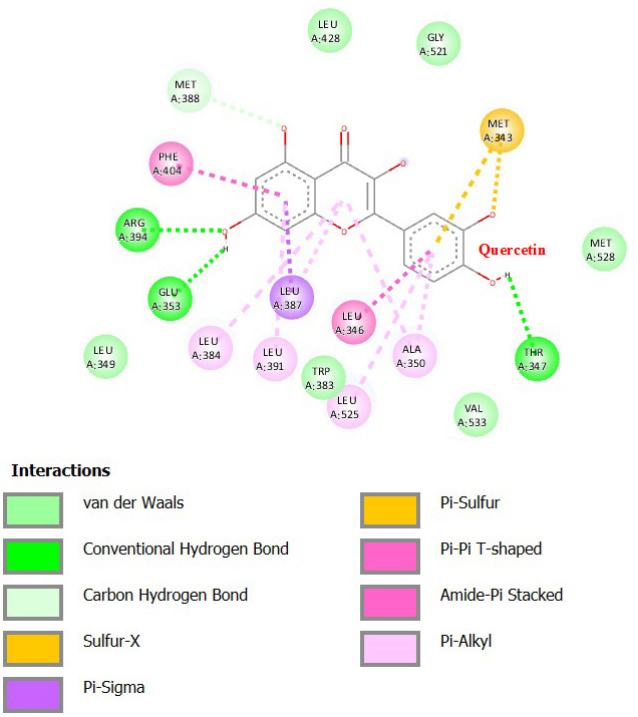

Figure 12. 2D interaction between quercetin and human Erα.

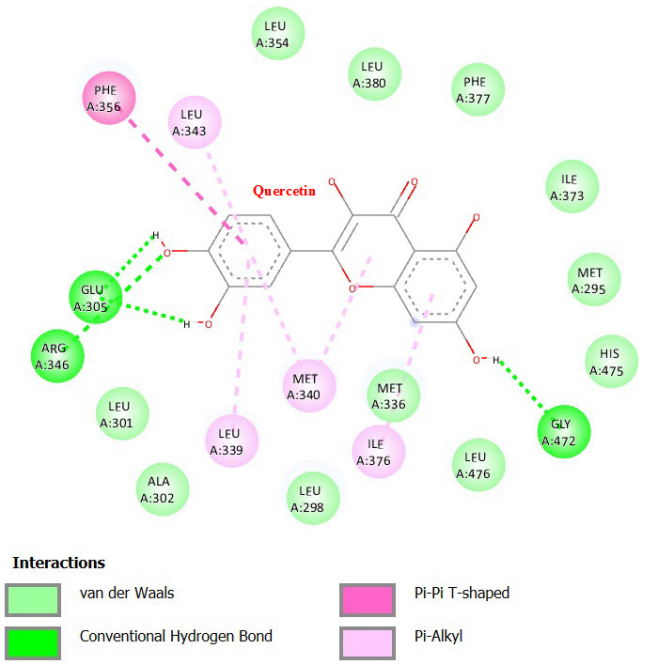

Figure 13. 2D interaction between quercetin and human Erβ.

The binding energy (ΔG) values of the docked co-crystal ligands (GQD and 3AS) with human ERα and human ERβ, respectively, along with the binding energy (ΔG) values of docked phytoestrogens (apigenin, genistein, kaempferol and quercetin) with human ERα and human Erβ are shown in Table 4 and are statistically represented in Figure 14.

Table 4. Values of Binding energy (ΔG) of different docked ligands with respective proteins (human ERα and ERβ)

Proteins	Ligands	Type of ligands	Binding energy(ΔG) kCal/mol
ERα	GQD	Co-crystal	-10.94
ERβ	3AS	Co-crystal	-10.29
ERα	Apigenin	Test ligand	-7.87
ERα	Genistein	Test ligand	-3.37
ERα	Kaempferol	Test ligand	-7.44
ERα	Quercetin	Test ligand	-7.57
ERβ	Apigenin	Test ligand	-8.06
ERβ	Genistein	Test ligand	-3.57
ERβ	Kaempferol	Test ligand	-7.46
ERβ	Quercetin	Test ligand	-7.89

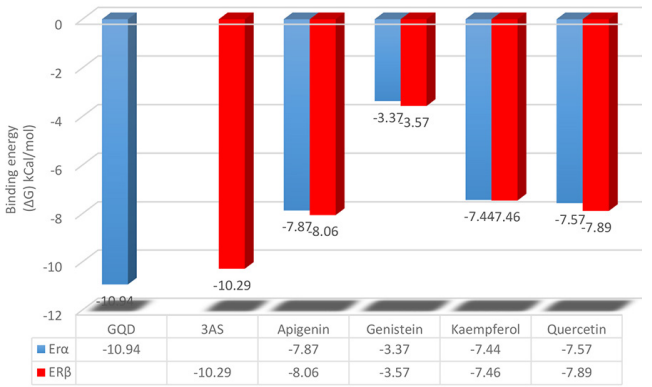

Figure 14. Statistical data interpretation of binding energy (ΔG) of docked ligands with human ERα and human ERβ. Binding energy (ΔG) of co-crystal ligands (GQD and 3AS) with human ERα and human ERβ, respectively, and binding energy (ΔG) values of docked phytoestrogens (apigenin, genistein, kaempferol and quercetin) with human ERα (blue bar) and human Erβ (red bar), where X-axis represents the co-crystal ligands (GQD and 3AS) and phytoestrogens (apigenin, genistein, kaempferol and quercetin) and Y-axis represents the binding energy (ΔG) (kcal/mol).

4. Discussion

Breast cancer is the most common cancer in women. The risk factors include demographic, reproductive, hormonal, heredity, organ-specific, and lifestyle[19]. Also, excess exposure to estrogen increases the risk of breast cancer[20]. Phytoestrogens are diphenolic, non-steroidal substances, secondary plant metabolites that mimic human estrogen, 17β-estradiol, enabling them to bind to the Estrogen Receptor (ER) causing estrogenic or antiestrogenic effects[4]. Molecular dynamics or *in-silico* studies are crucial tools in studying the activity of the mode of action of different compounds or drugs[21]. These studies drastically reduce the time taken by workers to carry out the study. This study has demonstrated the binding of different phytoestrogens with the human estrogen receptors. The binding efficiency of these phytoestrogens was analyzed by the molecular docking method with the help of autodocking tools. Ten different configurations were analyzed for each ligand, out of which the configuration showing the lowest binding energy (ΔG) showed the most favourable interaction between the docked ligand and ligand binding domain of human ERα and ERβ.

The co-crystal ligands of human ERα and ERβ, GQD and 3AS respectively, were also docked. Both GQD and 3AS showed the highest binding efficiency for their respective proteins with binding energy (ΔG) of -10.94 Kcal/mol and -10.29 Kcal/mol, respectively. This study also showed the binding of the flavonoids class of phytoestrogens to human ERα and ERβ. Several dietary flavonoids have been reported to possess anticancer effects[22]. The flavonoid apigenin shows both estrogenic and anti-estrogenic effects. This study showed high binding efficiency with binding energy (ΔG) values of -7.87Kcal/mol and -8.06Kcal/mol, respectively, with human ERα and ERβ, respectively. The isoflavonoid genistein showed a binding affinity several-fold weaker than that of estradiol. Molecular docking studies showed that the binding efficiency of genistein is lowest among the phytoestrogens studied, with a binding energy (ΔG) of -3.37 Kcal/mol and -3.57 Kcal/mol against human ERα and ERβ, respectively. Earlier studies also suggest that genistein is a weak ER agonist[23]. Another flavonoid studied herein, kaempferol, found in many natural products, is known to inhibit the growth of the breast cancer cell MCF-7[7]. It also showed high binding efficiency against both the receptors ERα and ERβ with a binding energy of -7.44 Kcal/mol and -7.46 Kcal/mol, respectively. Likewise, quercetin, a plant-based flavonoid which can be obtained through diet, is known to inhibit the growth and proliferation of breast cancer cell lines[15,16]. It also showed a high binding efficiency towards both the receptors ERα and ERβ with binding energy values of -7.57 Kcal/mol and -7.89 Kcal/mol, respectively.

Although the phytoestrogens have almost similar binding efficiency towards both the receptors, they preferably bind to ERβ and show an anti-estrogenic effect. The binding of phytoestrogens with human estrogen receptors, ERα, shows an estrogenic effect leading to the growth and proliferation of breast cancer cell lines, whereas binding to humans Erβ causes anti-proliferative effects[3].

5. Conclusions

In this molecular docking study, the flavonoid studies showed high binding efficiency towards both the human estrogen receptors. While the flavinoid genistein showed the lowest binding efficiency to both receptors, they were found to inhibit the growth of breast cells

through different mechanisms and pathways. Thus, the consumption of different phytoestrogen-rich vegetables and fruits may help in the prevention and treatment of breast cancer. Since phytoestrogens are also known to exert several adverse effects, this study leaves space for further investigations to evaluate their safety, determine doses, to study gender differences in response to phytoestrogens and the simultaneous effects of phytoestrogen with other drugs and dietary products. Also, *in vivo* studies to determine their anticancer effects are warranted.

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7. References

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