Section A-Research paper

In Silico Analysis of Fenugreek Bioactive Compounds Potential for Residual Ridge Resorption Inhibitors

Running title: ***Fenugreek as Residual Ridge Resorption Inhibitor***

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ABSTRACT

Background: The phrase "residual ridge" refers to the morphology of the clinical alveolar ridge after the restoration of bone and soft tissue following tooth extraction. Residual ridge resorption (RRR) is a common and frequently disabling condition, especially in those with edentulous mandibles. There may be a link between osteoporosis and ridge resorption, according to previous studies. We propose a traditional strategy of using Fenugreek bioactive compounds to treat RRR by suppressing inflammation-associated pathways. **Objective:** This study aimed to evaluate the potential of Fenugreek bioactive compounds as RRR inhibitors. **Methods:** We conducted an in silico investigation by collecting Fenugreek bioactive compounds using the PubChem database and determining their biological activities using Quantitative Structure-Activity Relationship (QSAR) analysis. The initial target proteins of Fenugreek bioactive compounds were predicted using the Comparative Toxicogenomics Database (CTD). Protein-protein interactions (PPI) were investigated using network analysis in the STRING database and Cytoscape. **Results:** The findings indicated that Fenugreek bioactive compounds had antioxidant, anti-inflammatory, heme oxygenase 1 (HMOX1) expression enhancing, and tumor necrosis factor (TNF) expression inhibiting properties. Moreover, the PPI network suggested that four bioactive compounds, quercetin, kaempferol, naringenin, and luteolin, interacted with estrogen receptor (ESR)-α/β. Molecular docking studies showed that kaempferol interacted with ESRα (-9.1 kcal/mol) and naringenin bound to ESRβ (-9 kcal/mol). **Conclusion:** It is concluded that Fenugreek bioactive compounds may be useful for RRR patients. To validate these in silico results, in vivo and in vitro studies need to be performed.

KEYWORDS: Anti-inflammatory, Antioxidant, HMOX1 enhancer, Protein target interaction, TNF inhibitor

1. BACKGROUND

Gradual tooth loss has traditionally been viewed as a normal component of aging[1]. Tooth loss, known as "dental mortality," is one of the most important measures of oral health status in older persons; it represents the lifelong cumulative impact of both illness and social variables[2-3]. About 57% of persons aged 65 and over had poor or fair dental health, according to the National Health and Nutrition Examination Survei[4]. Following tooth extraction, the quantity and quality of the residual ridge gradually decrease, a complex biophysical process known as residual ridge resorption (RRR). One of the most significant variables affecting the degree of bone loss is the length of time a person has been without teeth[5-9].

Following tooth extraction, the remaining alveolar bone's buccolingual width is drastically decreased in comparison to its height. The percentage of horizontal linear decrease across the gap between the buccal and lingual borders of the alveolar bone prior to tooth extraction is characterized as the rate of buccolingual width reduction. Meanwhile, the rate of height reduction is defined as the percentage of vertical linear decrease over the space between the base of the socket and the crest of the alveolar bone prior to tooth extraction. Previous studies revealed that the rates of buccolingual width reduction were 32% and 29-63%, while the rates of height reduction were 15% and 11-22%, at three- and six-months post-extraction, respectively[10]. Changes in the crestal bone height, however, differ by the site[10-11]. These clinical observational findings imply that the structural changes in the edentulous jawbone are unidirectional and are predominantly due to osteoclasts resorbing the remaining alveolar bone[12].

Estrogen mostly inhibits bone remodeling, probably via osteocytes. Inhibition of bone resorption by estrogen occurs primarily through direct effects on osteoclasts, although effects on osteoblast/osteocyte and T-cell regulation of osteoclasts are also likely to be important. A gap between bone resorption and bone production is related to estrogen insufficiency. This may be due to the effect of estrogen in reducing osteoblast apoptosis, oxidative stress, osteoblastic nuclear factor (NF)-κB activity, and additionally, due to loss of processes that have not yet been identified[13]. The ratio of pro-inflammatory and anti-inflammatory mediators is modified by estrogen. If this homeostasis is disturbed, persistent inflammation is likely to occur, leading to increased tissue damage and impaired angiogenesis. When angiogenesis is disrupted, both wound healing and new bone development slow down. As a result, scar tissue and resorption, which occur more rapidly than new bone formation, dominate the newly produced tissue and bone[14].

Fenugreek (*Trigonella foenum-gaecum*), also known as klabat plant in Indonesia, has been used traditionally as medicine throughout the world, particularly in Asia and the Mediterranean, using its seeds and leaves to promote breast milk production and relieve joint and bone pain. Numerous bioactive compounds, including alkaloids (trigonelline), steroidal sapogenins (yamogenin and diosgenin), polysaccharides (galactomannan), amino acids (4-hydroxy isoleucine), and flavonoids (quercetin), have been identified in Fenugreek seeds and leaves. Those compounds have the potential to be developed into drugs for the treatment of diabetes, cancer, Alzheimer's disease, inflammation, and other diseases. However, because more bioactive compounds were extracted from the seeds than from the leaves in some studies, more components of the seeds were utilized[15-18]. Previous in silico studies on Fenugreek showed that galactomannan, quercetin, diosgenin, and trigonelline have the potential to bind to the estrogen receptor, similar to estrogen. With this knowledge, Fenugreek was developed as a phytopharmaceutical estrogen replacement agent[17-21].

In silico studies are expected to support the hypothesis regarding the advantages of Fenugreek as a potent phytoestrogen and provide an initial assessment and basis for in vivo studies. Apart from reducing wasted time and money, this strategy is undertaken to minimize in vivo study failure. In this study, in silico method was carried out to demonstrate whether the administration of bioactive compounds contained in Fenugreek seeds can inhibit residual ridge resorption by inhibiting inflammatory processes and bone remodeling in hypoestrogenic conditions after tooth extraction. This in silico study was performed by docking the Fenugreek bioactive compounds with target proteins to prove their potential as estrogen agonists.

2. MATERIALS AND METHODS

Sample collection of bioactive compounds and proteins

Twenty-eight bioactive compounds contained in Fenugreek were obtained through the KANAYA KnapSack database (http://www.knapsackfamily.com/KNApSAcK/). Each compound was then searched for SMILE (simplified molecular-input line-entry system) and 3D structure through the PubChem database (https://pubchem.ncbi.nlm.nih.gov/). SMILE has a unique code to describe the structure of each

compound. Target proteins, estrogen receptor (ESR)-α and β, were retrieved from the Protein Data Bank (PDB) database, with IDs 1X7R and 5TOA, respectively. The proteins were prepared by removing their water molecules using the PyMOL 2.5.2 software, whereas the ligands were optimized by minimizing the energy of the Universal Force Field using the Open Babel software in PyRx v.0.9.9.

Docking analysis of Fenugreek bioactive compounds and target proteins

To determine the binding affinity between bioactive compounds and target proteins, a docking analysis was performed using Autodock Vina integrated into PyRx v.0.9.9[22]. Docking was carried out using the targeted docking method with an exhaust parameter of 25. The size of the grid box was adjusted to the position of the protein amino acid residues bound to the experimental control. For ESRα, the grid box was set as follows: Center X: 15.1459833174, Y: 31.1794651394, Z: 21.8784262334, and dimensions (Å) X: 20, Y: 20, Z: 20. While the grid box settings for ESRβ were as follows: Center X: 19.7414220736, Y: 42.8083137782, Z: 15.8294390702, and dimensions (Å) X: 23.5813501083, Y: 22.8862644419, and Z: 21.6996053098. Furthermore, the interactions between the compounds and the docked proteins were visualized using the BioVia Discovery Studio 2019 software.

Biological activity analysis

Each SMILE data of bioactive compounds was then predicted for their bioactive properties using the Quantitative Structure-Activity Relationship (QSAR) analysis. QSAR analysis can predict the role of compounds by comparing the similarity of the input structure with the structure of compounds whose role is known. The more similar the structure, the higher the prediction confidence value. QSAR analysis was performed using PASS Online (http://www.way2drug.com/PASSOnline/). The probability to be active (Pa) score (range 0 – 1) is a prediction score from PASS Online to describe the activity potential of a compound. The closer to value 1, the stronger the prediction. The cut-off used in this study was Pa > 0.5[23].

Prediction of target proteins of Fenugreek bioactive compounds

Compounds acting as estrogen agonists in the QSAR analysis were then predicted for their target proteins using the Comparative Toxicogenomics Database (CTD, http://ctdbase.org/). The threshold used was having ≥ 5 interactions to be visualized in a Venn diagram.

Networking analysis

Protein targets by all selected bioactive compounds were analyzed for protein-protein interactions (PPI) using STRING database v.11.5 (https://string-db.org/). The inputs used were 17 proteins at the target intersection of selected compounds, the proteins of interest (transforming growth factor-β1 (TGFB1), tumor necrosis factor superfamily member 11 (TNFSF11, RANK), and osteoclast stimulatory transmembrane protein (OCSTAMP)), with a confidence level of 0.4 in Homo sapiens. Data from STRING were then processed with Cytoscape v.9.1 to be visualized.

3. RESULT AND DISCUSSION

Biological activity of twenty-eight Fenugreek active compounds

Twenty-eight Fenugreek bioactive compounds (Table 1) retrieved from the database were collected and analyzed for their biological activities. Based on QSAR data, the compounds in Fenugreek had the potential as free radical scavengers (0.55), anti-inflammatory (0.57), and HMOX1 expression enhancers (0.66) (Fig. 1). Heme oxygenase (HO)-1 encoded by HMOX1 is downstream of the nuclear factor E2-related factor 2 (Nrf2). As an essential cytoprotective pathway, the transcription factor Nrf2 regulates substances that detoxify the body and fight inflammation, such as HO-1[24]. Heme oxygenase-1 is considered a negative regulator of osteoclasts (OCL). A study conducted by Florczyk-Soluch et al (2018) confirmed the effect of HO-1 on osteoclastogenesis and revealed that the plasma of HO-1-/- mice had higher tartrate-resistant acid phosphatase (TRAP) levels, leading to an increased amount of bone-resorbing OCL[25]. The in vivo studies revealed that HO-1 can inhibit the formation of OCL.

Fig. 2 shows the four best compounds in Fenugreek that had activity as estrogen agonists, namely quercetin (0.514), naringenin (0.733), luteolin (0.555), and kaempferol (0.505). Interestingly, these compounds also had high activity as anti-inflammatories, HMOX1 expression enhancers, and TNF expression inhibitors. Evidence that HMOX1 up-regulation acts as a stress-related adaptation mechanism

to protect cells from oxidative damage has been provided by the results of previous in vitro and in vivo studies[26].

Table 1. Fenugreek metabolites (molecular weight < 500 Dalton) retrieved from Kanaya database

C_ID	CAS ID	Metabolite	Molecular formula	Molecular weight	Organism
C00004631	117-39-5	Quercetin	C15H10O7	302.04265	*Trigonella foenum-graecum*
C00003597	512-06-1	Yamogenin	C27H42O3	414.3134	*Trigonella foenum-graecum*
C00001110	3681-93-4	Vitexin	C21H20O10	432.10565	*Trigonella foenum-graecum*
C00001555	535-83-1	Trigonelline	C7H7NO2	137.04768	*Trigonella foenum-graceum*
C00013329	520-32-1	Tricin	C17H14O7	330.07395	*Trigonella foenum-graecum*
C00003593	77-60-1	Tigogenin	C27H44O3	416.32905	*Trigonella foenum-graecum*
C00003592	126-18-1	Smilagenin	C27H44O3	416.32905	*Trigonella foenum-graecum*
C00002499	92-61-5	Scopoletin	C10H8O4	192.04226	*Trigonella foenum-graecum*
C00034219	7432-28-2	Schisandrol A	C24H32O7	432.2148	*Trigonella foenum-graecum*
C00002904	155-58-8	Rhaponticin	C21H24O9	420.14203	*Trigonella foenum-graecum*
C00005374	522-12-3	Quercetin 3-O-L-rhamnoside	C21H20O11	448.10056	*Trigonella foenum-graceum*
C00001078	28608-75-5	Orientin	C21H20O11	448.10056	*Trigonella foenum-graecum*
C00057400	470-01-9	Neotigogenin	C27H44O3	416.32905	*Trigonella foenum-graecum*
C00000982	480-41-1	Naringenin	C15H12O5	272.06847	*Trigonella foenum-graecum*
C00000674	491-70-3	Luteolin	C15H10O6	286.04774	*Trigonella foenum-graecum*
C00004565	520-18-3	Kaempferol	C15H10O6	286.04774	*Trigonella foenum-graceum*
C00001059	38953-85-4	Isovitexin	C21H20O10	432.10565	*Trigonella foenum-graecum*
C00004635	480-19-3	Isorhamnetin	C16H12O7	316.0583	*Trigonella foenum-graecum*
C00001055	4261-42-1	Isoorientin	C21H20O11	448.10056	*Trigonella foenum-graecum*
C00002647	149-91-7	Gallic acid	C7H6O5	170.02152	*Trigonella foenum-graecum*
C00002525	485-72-3	Formononetin	C16H12O4	268.07356	*Trigonella foenum-graecum*
C00001017	578-74-5	Apigenin 7-O-beta-D-glucopyranoside	C21H20O10	432.10565	*Trigonella foenum-graecum*
C00013291	38070-97-2	7-Hydroxy-6-methoxy flavone	C16H12O4	268.07356	*Trigonella foenum-graecum*
C00003800	2196-14-7	7,4'-Dihydroxyflavone	C15H10O4	254.05791	*Trigonella foenum-graecum*
C00053459	14417-51-7	4-O-beta-D-Mannopyranosyl-D-mannose	C12H22O11	342.11621	*Trigonella foenum-graecum*
C00057876	14002-93-8	3,4,7-Trimethylcoumarin	C12H12O2	188.08373	*Trigonella foenum-graecum*
C00003825	2150-11-0	3',4',7-Trihydroxyflavone	C15H10O5	270.05282	*Trigonella foenum-graecum*
C00003672	83-46-5	(-)-beta-Sitosterol	C29H50O	414.38617	*Trigonella foenum-graceum*

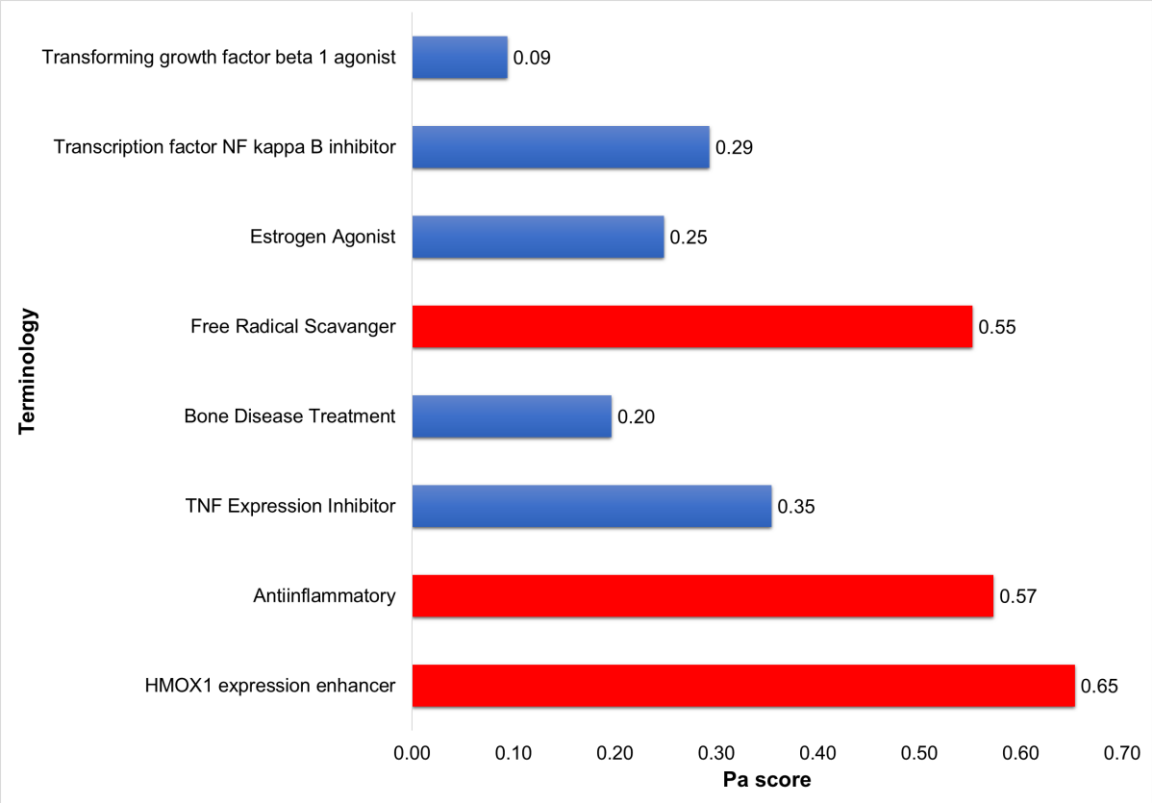


Figure 1. Predicted potential activity (Pa) score of Fenugreek by QSAR analysis

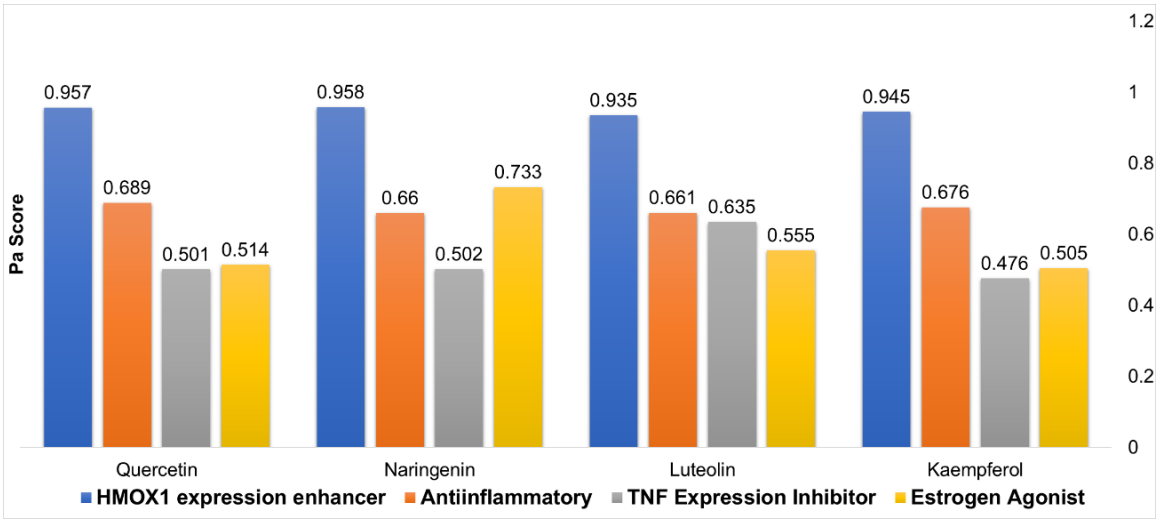


Figure 2. Predicted potential activity (Pa) score of Fenugreek as estrogen agonist by QSAR analysis

Prediction of target proteins of Fenugreek bioactive compounds

To narrow down the target protein studies of Fenugreek bioactive compounds, we performed target protein analysis using CTD. Data showed 17 target proteins of quercetin, kaempferol, luteolin, and naringenin, namely B-cell lymphoma 2 (BCL2), Bcl-2 associated X-protein (BAX), caspase 3 (CASP3), CASP9, catalase (CAT), cyclin-dependent kinase inhibitor 1A (CDKN1A), cytochrome P450 family 1 subfamily A member 1 (CYP1A1), estrogen receptor 1 (ESR1), ESR2, HMOX1, interleukin-6 (IL6), matrix metallopeptidase 9 (MMP9), mitogen-activated protein kinase 1 (MAPK1), MAPK3, nitric oxide

synthase 2 (NOS2), nuclear factor erythroid 2-related factor 2 (NFE2L2), and TNF (Fig. 3). Based on metadata retrieved from CTD, administration of quercetin, luteolin, and kaempferol had the potential to reduce IL-6 protein secretion. According to previous studies, osteoporotic bone marrow stromal cells (BMSCs) secrete IL-6 which is higher when compared to healthy controls. The findings indicate that excessive IL-6 release prevents osteogenesis in osteoporotic BMSCs. Furthermore, it was discovered that excessive IL-6 secretion inhibits β-catenin function. More significantly, it was found that in vivo treatment of an IL-6 neutralizing antibody is effective in reversing the mouse vertebral osteoporotic phenotype. Consequently, osteoporosis therapy has identified IL-6 as a possible target[27].

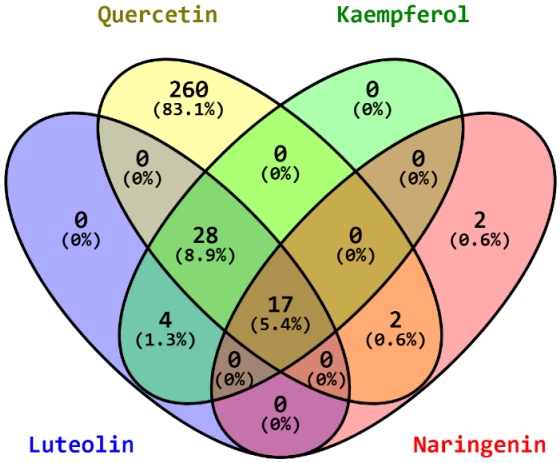


Figure 3. Predicted targets of quercetin, luteolin, naringenin, and kaempferol based on CTD

Protein-protein interaction analysis of Fenugreek bioactive compounds and target proteins

Protein-protein interaction (PPI) analysis was performed to link the target proteins of each Fenugreek bioactive compound. Seventeen proteins targeted by quercetin, kaempferol, naringenin, and luteolin were then analyzed for their interactions. TNFSF11 is a cytokine that binds to TNFRSF11B/OPG and TNFRSF11A/RANK. OCSTAMP is a possible cell surface receptor that plays a role in cellular fusion and cell differentiation. OCSTAMP cooperates with dendrocyte-expressed seven transmembrane protein (DCSTAMP) in modulating cell-cell fusion in both osteoclasts and foreign body giant cells (FBGCs).

Proteins that can interact with ESRα are visualized with the green lines, while the blue lines represent ESRβ interactions. Proteins targeted by ESRα and ESRβ are visualized with green nodes, namely CASP3, CYP1A1, IL6, MAPK1, MAPK2, MMP9, TGFB1, TNF, and TNFSF11 (Fig. 4). ESRα is considered a higher degree value than other proteins. The larger the circle diameter, the higher the degree score. The degree of centrality describes the number of proteins interacting with the nodes (Fig. 5).

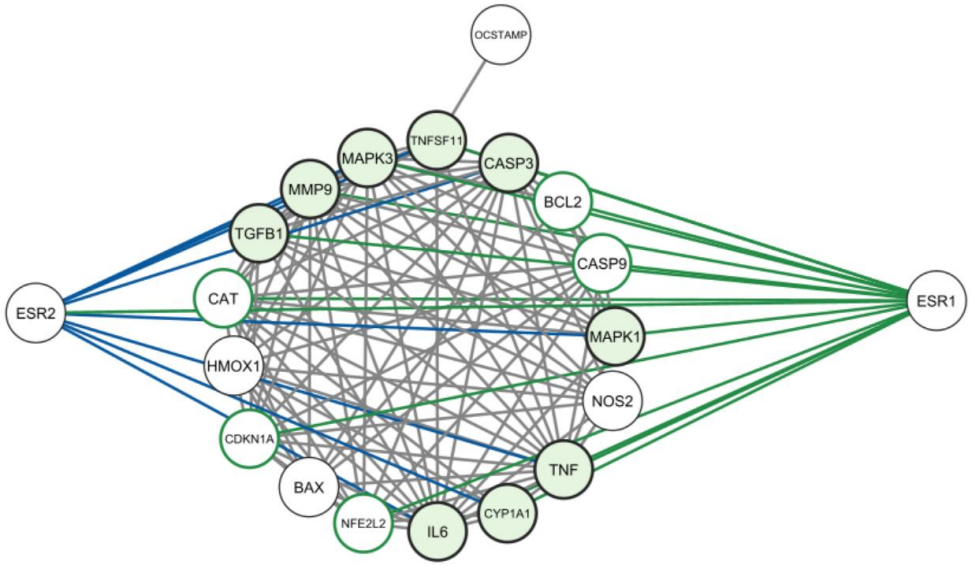
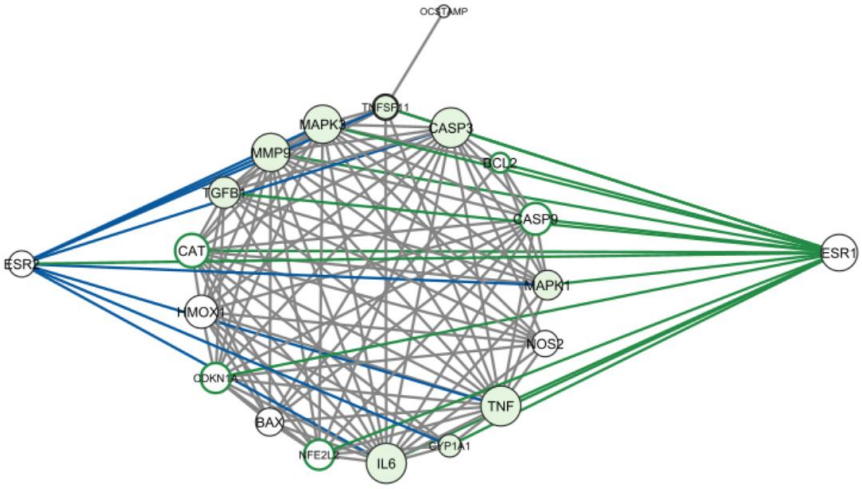


Figure 4. Interaction of estrogen agonist compounds with several target proteins
Green lines: ESRα target line, blue lines: ESRβ target line, green nodes: protein targeted by ESR1 and ESR2.



Name	Degree
CASP3	17
TNF	17
IL6	17
MAPK3	16
MMP9	16
ESR1	15
CAT	14
CASP9	13
HMOX1	13
TGFB1	12

Figure 5. Interaction of estrogen agonist compounds with target proteins
Green lines: ESRα target line, blue lines: ESRβ target line, green nodes: protein targeted by ESR1 and ESR2. The wider the circle, the higher the degree value.

Molecular docking analysis

A molecular docking analysis was performed to determine the binding affinity of Fenugreek bioactive compounds to ESRα/β, which supports the above initial data. Here, we carried out several validation methods: (1) Re-docking of bioactive compounds and target protein results must be compared with the experimental structure in the Protein Data Bank (PDB) database. The root mean standard deviation (RMSD) between the re-docked ligand and the native ligand control must be <2 Å. Values below 2 Å indicated that the re-docked ligand and the native ligand had the same coordinates. (2) There must be the same amino acid residues between the control and the comparison ligand. (3) The binding position between the target protein and the control (database of experimental results) or the potential bioactive compound must be similar. The docking findings must meet at least all three of these requirements to be applicable.

The docking results showed that four bioactive compounds, quercetin, naringenin, luteolin, and kaempferol, had a strong binding affinity towards ESRα (Fig. 6–7) and/or ESRβ (Fig. 8–9), although their scores were not higher than the control ligands (genistein and estradiol). Naringenin was the best ligand to bind to ESRα (score -9 kcal/mol), while kaempferol was the best ligand to interact with ESRβ (score -9.1 kcal/mol) (Table 2). The stronger the binding strength between the protein and the ligand, the more negative the binding affinity score. Structurally, genistein (control ligand) and the ESRα complex compared to the genistein crystallographic structure showed an RMSD score of 0.562 Å. Genistein on PDB 1X7R had the same coordinates as estradiol on ESRα. Unfortunately, there were no experimental results of ESRα (wildtype) protein with estradiol. On the other hand, the results of estradiol and ESRβ redocking compared to estradiol crystallography showed an RMSD score of 0.415 Å, allowing verification of docking findings based on the RMSD value.

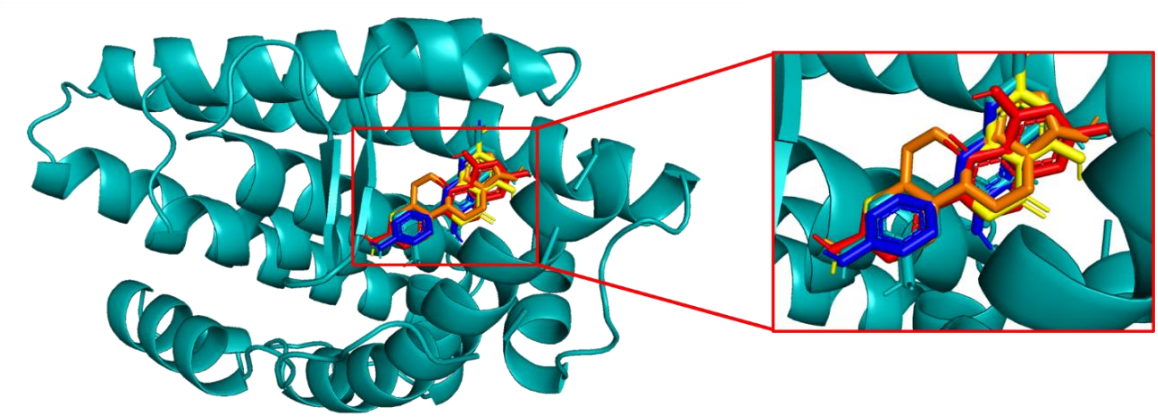


Figure 6. Visualization of the ESRα complex docking and Fenugreek bioactive compounds
ESRα (teal, cartoon), naringenin (yellow, stick), kaempferol (blue, stick), luteolin (teal, stick), quercetin (cyan, stick), genistein (red, stick), estradiol (orange, stick).

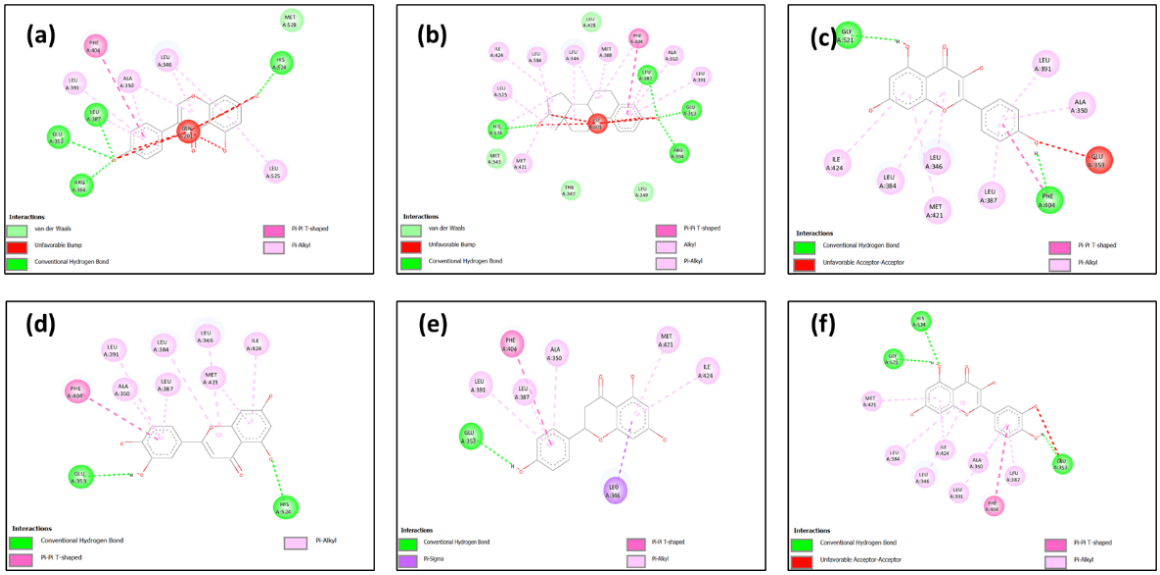


Figure 7. Amino acid interactions between ESRα and Fenugreek bioactive compounds
(a) Genistein, (b) estradiol, (c) kaempferol, (d) luteolin, (e) naringenin, (f) quercetin.

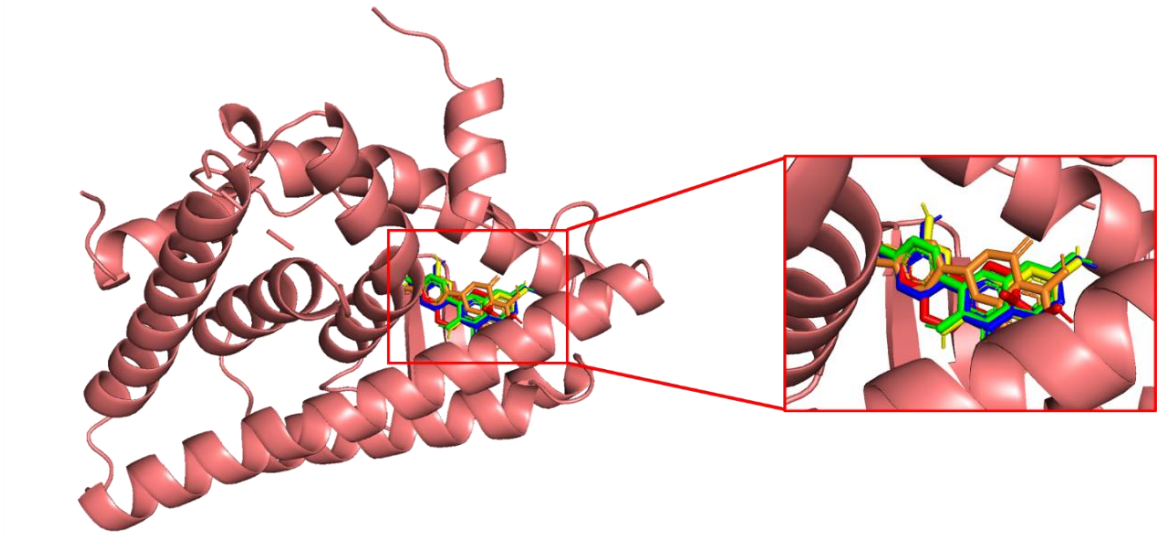


Figure 8. Visualization of the ESRβ complex docking and Fenugreek bioactive compounds
ESRβ (salmon, cartoon), naringenin (orange, stick), kaempferol (green, stick), luteolin (blue, stick), quercetin (yellow, stick), estradiol (red, stick).

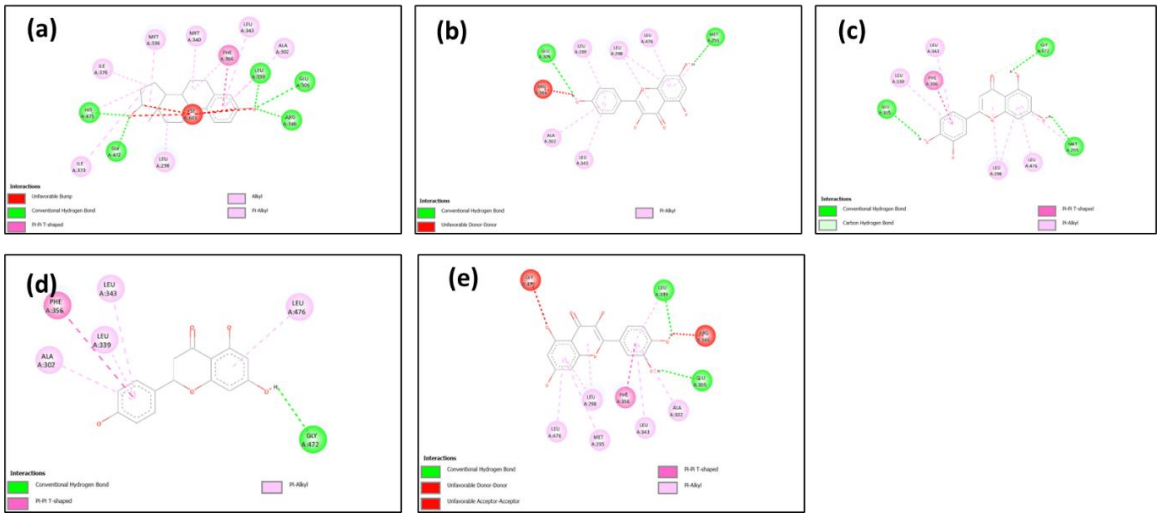


Figure 7. Amino acid interactions between ESRβ and Fenugreek bioactive compounds
(a) Estradiol, (b) kaempferol, (c) luteolin, (d) naringenin, (e) quercetin.

Table 2. Results of molecular docking of the four Fenugreek bioactive compounds and ESRα/ESRβ target proteins

Ligand	Binding Affinity (kcal/mol)	
	ESRα	ESRβ
Quercetin	-8.8	-8.9
Naringenin	-9.0	-9.0
Luteolin	-8.9	-8.9
Kaempferol	-8.8	-9.1
Genistein (Control)	-9.0	N/A
Estradiol (Control)	-10.7	-11

N/A: not available

Because of its affinity for binding and comparable residues to genistein, quercetin was the ligand that most closely resembles the control in terms of profile. However, the interaction between ESR and estradiol outperformed the binding affinity score (Table 2). The interactions were strengthened by hydrogen bonds and stabilized by hydrophobic bonds (Table 3–4).

Table 3. Detailed interactions of the binding between ESRα and Fenugreek bioactive compounds

Ligand	Binding Affinity (kcal/mol)	Hydrogen Bond	Hydrophobic Bond	Unfavorable	Van der Waals
Genistein	-9.0	GLU353 LEU387 ARG394 HIS524	LEU391 ALA350 PHE404 LEU525		MET528
Estradiol	-10.7	**HIS524 LEU387 GLU353 ARG394**	ILE424 LEU384 LEU346 MET388 **ALA350 LEU391** MET421		LEU428 MET343 THRR347 LEU349
Kaempferol	-8.8	GLY521 PHE404	ILE424 LEU384 LEU346 MET421 **LEU387 LEU391** ALA350	**GLU353**	
Luteolin	-8.9	GLU353 HIS524	**LEU391 ALA350 LEU387** LEU384 LEU346 MET421 ILE424		
Naringenin	-8.8	**GLU353**	**LEU391 LEU387 ALA350** MET421 ILE424 PHE404 LEU346		
Quercetin	-9.0	HIS524 GLY521 **GLU353**	MET421 LEU384 LEU346 ILE424 **ALA350 LEU391 LEU387 PHE404**		

Bold letters represent amino acid interactions similar with the control (estradiol).

Table 4. Detailed interactions of the binding between ESRβ and Fenugreek bioactive compounds

Ligand	Binding Affinity (kcal/mol)	Hydrogen Bond	Hydrophobic Bond	Unfavorable bond
Estradiol	-11.0	HIS475 GLY472 LEU339 GLU305 ARG346	ILE376 MET336 MET340 PHE356 LEU343 ALA302 ILE373 LEU298	
Kaempferol	-9.1	MET295 **GLU305**	LEU339 LEU298 LEU476 **LEU343 ALA302**	**ARG346**
Luteolin	-8.9	**GLY472** MET295 **GLU305**	LEU339 **LEU343 PHE356 LEU298** LEU476	
Naringenin	-9.0	**GLY472**	**PHE356 LEU343** LEU339 **ALA302** LEU476	
Quercetin	-8.9	**LEU339 GLU305**	LEU476 LEU298 MET295 **PHE356 LEU343 ALA302**	GLY472 **ARG346**

Bold letters represent amino acid interactions similar with the control (estradiol).

4. CONCLUSION

The bioactive compounds contained in Fenugreek, quercetin, naringenin, luteolin, and kaempferol, have the potential as anti-inflammatories and free radical scavengers. Molecular docking data showed that the four bioactive compounds also have the potential as estrogen agonists and HMOX1 expression enhancers. In addition, the interaction profile of the quercetin and amino acid residues of ESR is identical to that of the control ESR agonist. Thus, Fenugreek bioactive compounds may be useful for RRR patients. To validate these in silico results, in vivo and in vitro studies need to be performed.

ACKNOWLEDGMENTS: None

AUTHOR CONTRIBUTION

E.H.L.: Conceptualization, data collection, and writing-draft; M.C.E. and N.P.: Conceptualization, writing-review, and editing; R.S.D.: Conceptualization, supervision, writing-review, and editing.

CONFLICT OF INTEREST

The authors have no conflicts of interest regarding this investigation**.**

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