Manuscript HT2AR & PRLR

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Active Compounds of *Polyscias scutellaria* Stimulate Breast Milk Production: In Silico Study on Serotonergic 5-HT2A Receptors and Prolactin Receptor

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Abstract. Mother's Milk has an important impact on infant nutrition. Exclusive breastfeeding can reduce the risk of various pathogens infection in infants. Colostrum in mother's milk contains various immune systems, especially immunoglobulin A (IgA). Some cases are related to the lack of milk production due to stress and poor nutrition on mothers. Serotonin plays a role in stimulating prolactin secretion by the pituitary gland. Shield Aralia (*Polyscias scutellaria*), in Java called *daun mangkokan*, has various active compounds, including afzelin, kaempferol, quercetin, quercitrin, and rutin. The aim of this study was to analyze the potential of the active compounds of *P. scutellaria* leaves to enhance breast milk production based on in silico studies on protein 5-HT$_{2A}$ receptors (5-HT2AR) and prolactin receptor (PRLR). The results showed that the active compound afzelin (-7.7 kcal/mol) has a better binding affinity than risperidone (-6.3 kcal/mol) as a control for breast-enhancing drugs. The result showed that the active compounds in the *P. scutellaria* leaves could be used as an alternative medicine to increase milk production.

Keywords: breast milk, *Polyscias scutellaria,* prolactin, Serotonin 5-HT2AR, PRLR

INTRODUCTION

Exclusive breastfeeding for the first six months continued as essential infant nutrition until the age of 1-2 years [1,2]. Breast milk provides all the nutritional components required by infants particularly during the first six months of newborn birth. Human milk contains protein, fat, starch,

minerals, vitamins, and water [2,3]. The protein content of breast milk is less than that of animal milk. Children might have immature kidneys; thus, they will face difficulty for nitrogen waste product excretion. Breast milk has no β-lactoglobulin, which causes allergies to infants [4]. Breast milk also contains numerous bioactive factors relating to the immune system, defense against various infections, and other ingredients that help digest and absorb food [3,4]. Colostrum is one of the components that were detected after giving birth in the first breast milk fluids. Colostrum contains high immunoglobulin A (IgA) levels, lactoferrin, leukocytes, and various growth factors. Lactoferrin is the most effective antibacterial, anti-viral, and anti-fungal portion [1,5].

However, low milk production is still issued for infants who cannot be completing their exclusive breastfeeding. Low milk production caused by lifestyle, especially in terms of high-fat diet, environment that affects in mother emotional condition [6], nutrition shortages [7] and stress [8]. Several antipsychotic drugs are used to increase milk production, for example, risperidone [9]. However, consumption of antipsychotic drugs has limitations due to safety reasons and decreases side effects for both mother and infants [10,11]. The utilization of galactagogues herbal plants is an alternative in order to replace antipsychotics drugs. Indonesian society traditionally used fenugreek (*Trigonella foenum-graecum*) [12] and shield aralia leaves (*Polyscias scutellaria*) or *daun mangkokan* in local name to help increase milk production. Fenugreek is commonly used in by society globally and has been scientifically reported to increase breast milk production. However, there is limited scientific research related to the use of *P. scutellaria* leaves to increase breast milk production.

This study aimed to analyze active compounds in *P. scutellaria* leaf through in vitro and in silico studies and its function in increasing breast milk production. Antioxidant activity, phenolic content, and flavonoid content were analyzed as in vitro study. In Silico is a computer-aided drug design (CADD), a computational method of identifying, designing, and discovering drugs (drug design and discovery). The potential of a compound for a particular drug candidate using published bank data can be predicted using in silico techniques [13].

METHODS

Preparation Structure of Protein and Compounds

The compounds used as ligands in this study were selected from the results of LCMS shield aralia leaf aqueous extract, namely afzelin, kaempferol, quercetin, quercitrin, and rutin. The 3D

chemical structure of ligands was obtained in .sdf format from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/). Accession numbers for ligands were CID 5316673 for afzelin, CID 5280863 for kaempferol, CID 5280343 for quercetin, CID 5280459 for quercitrin, CID 5280805 rutin, and CID 5073 for risperidone as a drug control. All ligands then converted into Protein Data Bank (PDB) using PyMoL software. The 3D structure of the target protein was obtained from PDB (https://www.rcsb.org/) with accession ID 6A93 for protein 5-Hydroxytryptamine 2A receptors (5-HT2AR) and ID 3D48 for prolactin receptor (PRLR). Removing water molecules dan ligands from obtained target protein used PyMoL software.

Molecular Docking Analysis

Molecular docking between ligands and target protein was analyzed using PyRx 0.8 software [14,15]. Visualization and interaction of ligands and target protein binding were using PyMoL software and BIOVIA Discovery Studio 2016. Binding affinity values were analyzed to determine the strength of the interaction between ligands and target protein and compared with control drugs [16].

Protein-ligand interaction and network

Protein-protein interactions were analyzed by BioGRID Database (https://biogrid.org/) [17], then analysis for interaction network for certain pathway used STRING (https://string-db.org/) [18]. Protein and ligand interaction networks were examined using the STITCH database (http://stitch.embl.de/) [19].

Pathway Analysis

Pathway analysis for 5-HT2AR and PRLR was examined using Kyoto Encyclopedia Gene and Genome (KEGG) (http://www.genome.jp/kegg/) [20]. The role of 5-HT2AR and PRLR on lactogenesis in various molecular pathways over breast milk production was identified using the STRING database (https://string-db.org/), meanwhile interaction of active compounds in various protein that involved in breast milk production was analyzed using STITCH database (http://stitch.embl.de/) [19].

RESULTS AND DISCUSSION

Molecular Docking Analysis

TABLE 1. Binding affinity value from Shield aralia active compounds with 5-Hydroxytryptamine 2A receptors (5-HT2AR) and Prolactin receptor (PRLR)

Ligands	Binding Affinity (kcal/mol)	
	5-HT2AR	PRLR
Risperidone (control)	-11.8	-7.6
Afzelin	-7.7	-6.3
Kaempferol	-8.9	-6.7
Quercetin	-8.9	-6.7
Quercitrin	-7.1	-6.2
Rutin	-8.6	-6.7

Molecular docking results on 5-HT2AR showed binding affinity value of risperidone as a drug control was -11.8 kcal/mol. The active compounds in *P. scutellaria* leaves extract, kaempferol, and quercetin have less binding affinity value than the control drug (-8.9 kcal/mol). Gibbs free energy is a measurement of the amount of free energy derived from interactions between ligands and proteins. Binding affinity is negative, so the greater and more stable the relationship between ligand and protein, the result is more negative [21,22]. Meanwhile, docking on PRLR showed the binding affinity value of risperidone was -7.6 kcal/mol, and it is greater than *P. scutellaria* active compounds. However, three compounds found in P. scutellaria namely kaempferol, quercetin, and rutin, have binding affinity value close to risperidone (-6.7 kcal/mol). These results showed that active compounds found in *P. scutellaria* leaves could be candidate to bonding with 5-HT2AR and PRLR.

Visualization of molecular docking and ligands interactions (Figure 2 & 3) showed the interactions between ligand-proteins. Docking of 5-HT2AR showed all ligands attached into the same amino acid residues, namely Leu229, Phe234, Ser159, Asp155, Val366, Leu228, and Phe339 (Table 2). Furthermore, docking of PRLR showed that all ligands were binding into the same amino acid residues, namely Tyr94, Tyr99, Tyr190, Ile100, and Lys11 (Table 2).

TABLE 2. Amino acid residues from molecular docking of 5-HT2AR and PRLR

5-HT2AR		PRLR	
Interactions	Residues	Interactions	Residues
Risperidone		Risperidone	
Van der walls	Cys227, Leu362, Asn343, Ile152, Leu229, Ile163, Phe332, Phe234,	Van der walls	Thr74, Asp96, Val95, Cys12

5-HT2AR		PRLR	
Interactions	Residues	Interactions	Residues
	Thr134, Tyr139, Asn363		
Halogen	Ser159	Halogen	Gln102
CHB	Thr160, Tyr370	CHB	Tyr94
Carbon HB	Ser131, Asp155, Ser242	Carbon HB	-
Pi-Anion	-	Pi-Anion	-
Pi-sigma	Val366	Pi-sigma	-
Pi-Pi T-shaped	Trp336, Phe340	Pi-Pi T-shaped	Tyr99, Tyr190
Pi-Alkyl	Trp151, Leu228, Phe339	Pi-Alkyl	Ile100, Arg13, Lys11
Afzelin		Afzelin	
Van der walls	Ser159, Ile152, Val366, Asn363, Ser242, Gly238, Ile210, Phe234, Ser239, Trp336	Van der walls	Phe10, Lys11, Ile100, Val95
Halogen	-	Halogen	-
CHB	Asp155, Leu229, Asn343, Val235	CHB	Ile9, Tyr190
Carbon HB	Gly288	Carbon HB	-
Pi-Anion	Asp155	Pi-Anion	-
Pi-sigma	-	Pi-sigma	-
Pi-Pi T-shaped	Phe339	Pi-Pi T-shaped	Tyr99, Tyr190
Pi-Alkyl	Val156, Leu228, Leu362, Phe340	Pi-Alkyl	Ile100, Arg12, Lys11
Kaempferol		Kaempferol	
Van der walls	Trp336, Val366, Trp151, Leu228, Asn343, Phe234, Gly238, Ser242, Thr160, Ser159	Van der walls	Tyr99, Arg13, Phe10, Lys11, Leu 93, Val95, Tyr94
Halogen	-	Halogen	-
CHB	Cys227, Val235, Ser239	CHB	Tyr190, Gln102
Carbon HB	Val156	Pi-sigma	Ile100
Pi-Anion	Asp155	Pi-Pi T-shaped	-
Pi-sigma	Leu229		
Pi-Pi T-shaped	Phe339, Phe340		
PI-Alkyl	Ile152	Pi-Alkyl	-
Quercetin		Quercetin	
Van der walls	Trp336, Thr160, Ser242, Gly238, Phe234, Leu229,	Van der walls	Tyr99, Val95, Leu93, Lys11, Arg13, Gln 102

5-HT2AR		PRLR	
Interactions	Residues	Interactions	Residues
	Ile152, Leu228, Trp151, Tyr370		
Halogen	-	Halogen	-
CHB	Val235, Ser239	CHB	Tyr94, Cys12
Carbon HB	Val156, Ser159	Carbon HB	-
Pi-Anion	Asp155	Pi-Anion	-
Pi-sigma	Val156	Pi-sigma	Ile100
Pi-Pi T-shaped	Phe339, Phe340	Pi-Pi T-stacked	Tyr190
Pi-Alkyl	Val366	Pi-Alkyl	-
Quercitrin		Quercitrin	
Van der walls	Trp336, Thr160, Ser242, Ser239, Phe234, Cys227, Leu362, Phe339, Ile152	Van der walls	Arg13, Lys11, Val95, Tyr99
Halogen	-	Halogen	-
CHB	Tyr370, Leu229, Asn343, Val235, Gly238	CHB	Tyr190, Ile9
Carbon HB	-	Carbon HB	-
Pi-Anion	Asp155	Pi-Anion	-
Pi-sigma	Leu229	Pi-sigma	-
Pi-Pi T-shaped	Trp151, Phe340	Pi-Pi T-sulfur	Cys12
Pi-Alkyl	Val366, Val156, Leu228	Pi-Alkyl	Arg13, Ile100
Rutin		Rutin	
Van der walls	Ser226, Cys227, Val235, Trp151, Val156, Tyr370, Ser159, Thr160, Ser242, Gly238, Ile210, Trp367, Phe234, Ser239, The134, Tyr139	Van der walls	Leu93, Val95, Thr74, Tyr94, Lys11, Gln102
Halogen	-	Halogen	-
CHB	Ser131, Asn343, Ile152	CHB	Tyr190, Asp96
Carbon HB	Asp155	Carbon HB	-
Pi-Anion	-	Pi-Anion	-
Pi-sigma	-	Pi-sigma	-
Pi-Pi T-shaped	Phe339	Pi-Pi T-shaped	Tyr99
Pi-Alkyl	Val366, Leu228, Leu229, Leu362, Phe340	Pi-Alkyl	Arg12, Ile100

CHB: Conventional Hydrogen Bond; HB: Hydrogen Bond

Risperidone is an antipsychotic drug widely used to treat schizophrenia. Risperidone has a high binding affinity to 5-HT2AR [23,24]. The 5-HT2AR binding increases serotonin production as a dopamine antagonist. Decreasing dopamine levels will increase lactocyte prolactin secretion [25]. Further analysis evaluated the position of the *P. scutellaria* active compounds when binding with 5-HT2AR has similar site with risperidone as control drug.

On the other hand, the orientation of docking analysis on PRLR active site showed that *P. scutellaria* active compounds were binding to PRLR in a similar position with risperidone as well. The comparison related to the binding affinity value of risperidone and P. scutellaria active compound demonstrated that risperidone binds to PRLR stronger than P. scutellaria active compound, but the binding affinity value was still close to the risperidone. The data suggested that active compounds found in *P. scutellaria* have the potential to increase breast milk production by binding to 5-HT2AR and PRLR.

Figure 1. Interaction and amino acid residues between 5-HT2AR and *P.scutellaria* active compounds. (A) risperidone (control); (B) afzelin; (C) kaempferol; (D) quercetin; (E) quercitrin; and (F) rutin.

Figure 2. Interaction and amino acid residues between PRLR and *P. scuttelaria* active compounds. (A) risperidone (control); (B) afzelin; (C) kaempferol; (D) quercetin; (E) quercitrin; and (F) rutin.

Molecular docking results showed active compounds from aqueous leaf extract of *P. scutellaria* have the ability to bind to the target protein 5-HT2AR. Kaempferol and quercetin in *P. scutellaria* leaf extract showed a lowest binding affinity than other compounds (except Risperidone). The previous study reported that Asp155 and Leu229 are critical residues in the binding pocket of HT2AR [26]. The four factor which strongly contributed between the protein target and ligands are hydrophobic, H-bond, electronic, and π-π stacking. Further, the hydrophobic residues play major roles in initiating the active conformation between ligands and protein targets.

Interestingly, our result demonstrated that Leu228 and Val366 are found in all ligands. These two amino acid residues are taken place in the hydrophobic pocket [27]. Meanwhile, other studies reported that other amino acid residue, Phe339 involved in allosteric communication and 5HT2AR activation, then produced second messenger as an intracellular signaling pathway [28].

On the other hand, our present work demonstrated that kaempferol, quercetin, and rutin showed the lowest binding affinity than other compounds (except risperidone) in PRLR interaction. Interestingly, the bioactive compounds of *P. scutellaria* have the same binding site with risperidone at Tyr94, Tyr99, Tyr190, Ile100, and Lys11. The previous study demonstrated that tyrosine phosphorylation by prolactin is required for maximum STAT5 activation [29]. Further, Tyr473, 479, and 580 are considered the essential amino acid residues necessary for activating STAT5 and STAT5-dependent gene transcription. Meanwhile, our study showed that all ligands bond at tyrosine residues, although it is in different tyrosine sites. The tyrosine phosphorylation in JAK2 will further activate STAT, including STAT5, to produce a lactogenic response [30]. However, further evaluation is still required about the potential role of Tyr90, Tyr94, and Tyr190 to bind with PRLR. Based on the docking result, we hypothesize that the bioactive compounds from *P. scutellaria* may involve in PRLR activation, which makes the possible response to induces JAK/STAT signaling pathway and induce a lactogenic response.

Protein interaction and pathway analysis

Breast milk production involves a variety of complex physiological processes, emotional factors, and the interaction of various hormones. Prolactin is the main hormone involved in lactation [25]. Serotonin mediates prolactin secretion indirectly through 5-hydroxytryptamine 2A receptors (5-HT2AR) [31]. Lactogenesis pathways were investigated to investigate proteins that involved in breast milk production using the STRING database. This analysis provided the map of protein network in pathway and false discovery rate for each pathway. The proteins are participated in two pathways related to breast milk production, including lactation and receptor signaling pathway via JAK-STAT (Figure 3-5). Lactation pathway is closely related with signaling pathway via JAK-STAT in order for mammary gland development and differentiation process [32].

Figure 3. Protein-protein interaction. (A) The network of protein interaction by STRING database involved lactation pathways (violet) and growth hormone receptor signaling pathway via JAK-STAT (red). (B) Involved genes in lactation and growth hormone receptor signaling pathway via JAK-STAT following with false discovery rates. The thickness for each line indicated the amount of evidence that has been found.

Figure 4. The proposed mechanism of active compounds binds with 5-HT2AR in tuberoinfundibular (TIDA) pathway as an agonist to increase prolactin secretion by lactotroph cell in the anterior pituitary.

Figure 5. The proposed mechanism of active compounds found in *P. scutellaria* involved in mammary gland development along the lactation stage in breastfeeding mothers. PRLR simultaneously activated the JAK-STAT signaling pathway plays a role in adequate breast milk production. PRLR, prolactin receptor; JAK, Janus kinase; STAT, signal transducer and activator of transcription.

Under normal conditions, the blood level of prolactin is low. This case due to an inhibitory mechanism by neurons tuberoinfundibular dopamine (TIDA) (Figure 4). Inhibition of TIDA activity, especially dopamine D2 receptors (D2R) will remove inhibition in lactotroph cells and the subsequent of prolactin release [33]. Liang (2000) reported that 5-HT2AR showed an inhibitory effect on TIDA activity which increased prolactin production in female mice [33,34]. The current study results reported that 5 compounds chosen from *P. scutellaria* were predicted to increase breast milk production by binding to 5-HT2AR and PRLR. Analysis from the STITCH database reported that risperidone has been reported to bind to D2R, 5-HT2AR, and PRLR, while kaempferol and quercetin are found in *P. scutellaria* bound to STAT3 and STAT1 in the JAK-STAT cascade involved in the growth hormone signaling pathway (Figure 6). Furthermore, the results also predicted that prolactin and prolactin receptors are involved in the signaling pathway as well. It might be suggested that the binding result of these compounds either to 5-HT2AR or PRLR has not been reported before.

Figure 6. Protein and ligand interaction in JAK-STAT cascade involved in growth hormone signaling pathway via (red circle). *P. scutellaria* active compound as the ligands (afzelin, kaempferol, quercetin, quercitrin, and rutin) and risperidone as drug control. Red line indicated a relation between ligands. The green line indicated the interaction between the ligands into the target protein. The grey line indicated the interaction between proteins. The thickness for each line indicated the amount of evidence that has been found for certain interactions.

Human milk has a dynamic and varies composition depends on the time of lactation, changing needs of growing infants, and food consumed by the mother [35]. The dietary phytochemicals alter the flavonoids content in human milk 12 h after ingestion [36,37].

Interestingly, numerous studies reported that some phytochemicals, such as kaempferol and quercetin, are found in the mother's milk [36,38]. These phytochemicals are known to prevent oxidative stress and intestinal diseases in infants [39]. The utilization of natural products to increase milk production have believed and traditionally used in some society [40]. In this case, based on our molecular docking and pathway analysis, the bioactive compounds of *P. scutellaria* was identified as a promising candidate as the galactagogues plant through 5-HT2AR and PRLR signaling pathway. The dietary of phytochemicals, such as from *P. scutellaria* may protect infants from oxidative damages and related diseases, and mainly could boost the mother's milk production during lactation.

CONCLUSION

The present study suggested that the bioactive compounds from *P. scutellaria* might have potential as a natural candidate involved in 5-HT2AR and PRLR complex. Kaempferol and quercetin were predicted to elevate prolactin secretion by lactotroph cells via the 5-HT2AR signaling pathway. Meanwhile, kaempferol, quercetin, and rutin were predicted to enhance the lactogenesis via JAK/STAT signaling pathway. The bioactive compound may be expected to regulate proliferation, differentiation, and lactogenesis during breastfeeding. Besides, *P. scutellaria* may become an alternative for substituting synthetic drugs to overcome low milk production during breastfeeding. Further in vitro and in vivo experiments should be performed to validity the bioactive compounds of *P. scutellaria* to be used as breast milk booster.

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