ORIGINAL PAPER

Molecular Docking for Active Compounds of *Scurrula Atropurpurea* as Anti-inflammatory Candidate in Endometriosis

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ABSTRACT

Introduction: Endometriosis is still a problem for women all over the world. There are no studies that apply herbs, especially *Scurrula atropurpurea* to inhibit the development of inflammation in endometriosis. **Aim:** The purpose of this study was to analyze the docking of active ingredient of *Scurrula atropurpurea* on NFkB-IkB complex with IKK *in silico* way. **Material and methods:** The nine active ingredients of *Scurrula atropurpurea* analyzed here were including aviculin (CID 10391477), caffeine (CID 2519), catechin (CID: 9064), epicatechin (CID: 72276), kaempferol (CID 5280863), quercetin (CID 5280343), quercitrin (CID 5280459)), rutin (CID 5280805), and theobromine (CID 5429). The sequence of study procedures included searching for amino acid sequences and active plant component structures, protein 3D structure modeling, docking and analysis of protein-ligand interaction. **Results:** Regarding the NFkB-IkB complex, it was found that all active ingredients can interact where the strongest interaction sequence was rutin (-314.35 kJ/mol). Regarding the interaction between IKK and NFkB-IkB, the nine active ingredients can reduce bond energy, except rutin. **Conclusions:** the active ingredients of *Scurrula atropurpurea* having the potential effect as anti-inflammatory is rutin so that it can be isolated and used as an alternative ingredient in inhibiting inflammation in endometriosis.

Keywords: anti-inflammatory, endometrium, parasite tea, herbs, in silico.

1. INTRODUCTION

Inflammation is the body's defense response mediated by innate immune system in the terms of cellular homeostasis against foreign pathogenic agents that damage cellular homeostasis. The biological mechanism underlying inflammation consists of three stages, namely initiation, regulation, and resolution. These three mechanisms are strictly regulated in order to maintain cellular and physiological homeostasis. Macrophages as cells located at the infection site will recognize infection and secrete proinflammatory cytokine to attract immune cells, among others, leukocytes and lymphocytes, thus triggering inflammation (1-4). The master regulator from innate immune system is a NF-kB system signal used as immunity defense (5).

Endometriosis is a disease characterized by the growth of endometrial tissue (endometrial and stromal gland cells) outside the uterine cavity. The development of this disease is influenced by estrogen hormone. The estrogen hormone can trigger an inflammatory reaction that may adversely affect the woman's life (6). Endometriosis may occur in all women from adolescent, reproductive age, even menopause, but approximately 20-30% frequently occurs at reproductive age. It is found that one of ten women of reproductive age of 15-49 years may suffer from endometriosis. Endometriosis becomes a scourge for reproductive age women because it is estimated that approximately 50-70% of women may have complaints of chronic pelvic pain and approximately 38% are diagnosed with infertility (7).

Endometriotic lesions locally produce estradiol E2 through aromatase activation for the survival of ectopic endometriosis and stimulation of proinflammatory cytokines (8). Proinflammatory cytokines secreted by macrophages in the peritoneum and ectopic endometrial cells are potentially angiogenic for the development of endometriosis (9). Proinflammatory cytokines (TNF-α) released by peritoneal macro-

phages activate transcription factors such as NF-kB through IkB peptide p50/p65. Active transcription factors may enter the cell nucleus to induce gene transcription and encode the products (10, 11).

Scurrula atropurpurea plants or known by Javanese as tea parasite are parasitic plants for tea (*Thea sinensis*). This plant from generation to generation has been used by the Javanese people as a cancer drug (12, 13). *Scurrula atropurpurea* inhibits cervical cancer cell growth through a mechanism of intrinsic pathway apoptosis (14). *Scurrula atropurpurea* also acts as an antioxidant. Some of active components of this plant are antioxidants of quercetin, quercitrin, and kaempferol (15-19). On the one hand, antioxidant compounds can suppress oxidative stress. On the other hand, moderate oxidative stress activates the inflammatory pathway. Thus, antioxidants of this plant also can potentially inhibit inflammation (20). Until now, the potential of *Scurrula atropurpurea* for endometriosis treatment has not been revealed. If *Scurrula atropurpurea* is an anti-inflammatory, it can potentially inhibit the inflammatory pathways involved in endometriosis.

Interaction	Point Interaction	Category	Binding energy
NFkB, IkB–aviculin	Aviculin – Pro151	Hydrophobic Bond	-311.75 kJ/mol
	Aviculin – Arg152	Hydrophobic Bond	
	Aviculin – Glu111	Hydrophobic Bond	
	Aviculin – Asn105	Hydrophobic Bond	
	Aviculin – Leu179	Hydrophobic Bond	
	Aviculin – Pro154	Hydrophobic Bond	
	Aviculin – Arg155	Hydrophobic Bond	
NFkB, IkB–caffeine	Caffeine – Ile356	Hydrophobic Bond	-170.13 kJ/mol
	Caffeine – Val535	Hydrophobic Bond	
	Caffeine – Leu566	Hydrophobic Bond	
	Caffeine – Arg569	Hydrophobic Bond	
	Caffeine – Ala570	Hydrophobic Bond	
	Caffeine – Pro170	Hydrophobic Bond	
NFkB, IkB–catechin	Catechin – Arg152	Hydrophobic Bond	-239.13 kJ/mol
	Catechin – Pro151	Hydrophobic Bond	
	Catechin – Ala115	Hydrophobic Bond	
	Catechin – Lys112	Hydrophobic Bond	
	Catechin – Ala102	Hydrophobic Bond	
	Catechin – Asp108	Hydrophobic Bond	
NFkB, IkB–epicatechin	Epicatechin – His171	Hydrogen Bond	-232.58 kJ/mol
	Epicatechin – Leu506	Hydrophobic Bond	
	Epicatechin – Val535	Hydrophobic Bond	
	Epicatechin – Ile536	Hydrophobic Bond	
	Epicatechin – Pro170	Hydrophobic Bond	
	Epicatechin – Thr169	Hydrophobic Bond	
NFkB, IkB–kaempferol	Kaempferol – Pro151	Hydrophobic Bond	-238.11 kJ/mol
	Kaempferol – Arg152	Hydrophobic Bond	
	Kaempferol – Asp182	Hydrophobic Bond	
NFkB, IkB–quercetin	Quercetin – Arg152	Hydrophobic Bond	-247.11 kJ/mol
	Quercetin – Glu111	Hydrophobic Bond	
	Quercetin – Ala115	Hydrophobic Bond	
	Quercetin – Lys112	Hydrophobic Bond	
	Quercetin – Pro154	Hydrophobic Bond	
	Quercetin – Pro151	Hydrophobic Bond	
	Quercetin – Asp182	Hydrophobic Bond	
NFkB, IkB–quercitrin	Quercitrin – Gly571	Hydrophobic Bond	-288.36 kJ/mol
	Quercitrin – His171	Hydrophobic Bond	
	Quercitrin – Ala570	Hydrophobic Bond	
	Quercitrin – Ser174	Hydrophobic Bond	
	Quercitrin – His173	Hydrophobic Bond	
	Quercitrin – Arg569	Hydrophobic Bond	
	Quercitrin – His559	Hydrophobic Bond	
	Quercitrin – Pro170	Hydrophobic Bond	
	Quercitrin – Thr169	Hydrophobic Bond	
NFkB, IkB–rutin	Rutin – Leu259	Hydrophobic Bond	-314.35 kJ/mol
	Rutin – Asp255	Hydrophobic Bond	
	Rutin – Arg262	Hydrophobic Bond	
	Rutin – Asp597	Hydrophobic Bond	
	Rutin – Asp208	Hydrophobic Bond	
	Rutin – Glu599	Hydrophobic Bond	
	Rutin – Arg245	Hydrophobic Bond	
	Rutin – Glu258	Hydrophobic Bond	
	Rutin – Gln212	Hydrophobic Bond	
NFkB, IkB–theobromine	Theobromine – Arg569	Hydrogen bond	-162.28 kJ/mol
	Theobromine – Val535	Hydrogen bond	
	Theobromine – Leu577	Hydrophobic Bond	
	Theobromine – Leu566	Hydrophobic Bond	
	Theobromine – Ile536	Hydrophobic Bond	
	Theobromine – Pro170	Hydrophobic Bond	
	Theobromine – Ala570	Hydrophobic Bond	

Table 1. Possible interactions of the Scurrula atropurpurea active compounds and NFkB-IkB complex

2. AIM

Therefore, the purpose of this study was to analyze the anti-inflammatory effects from plant active compounds of *Scurrula atropurpurea* through molecular docking between active compounds and the NFkB-IkB complex with IKK.

3. 3. MATERIAL AND METHODS

3.1 Amino acid sequences and the structure of active components of *Scurrula atropurpurea*

The National Center for Biotechnology Information (NCBI) Database, United States National Library of Medicine (NLM), National Institute of Health (NIH) (http://www.ncbi.nlm.nih.gov) represent a source of amino acid sequences making up protein of NF-kB (GI: 1018443262), IkB kinase-b (IKK-beta) (GI: 4185275), and IkB kinase-a (IKK-alpha) (GI: 4185273). PubChem Open Chemistry Database is a source of 3D structures for components of *Scurrula atropurpurea* active compounds, including Aviculin (CID 10391477), Caffeine (CID 2519), Catechin (CID: 9064), Epicatechin (CID: 72276), Kaempferol (CID 5280863), Quercetin (CID 5280343), quercitrin (CID 5280459), rutin (CID 5280805), and theobromine (CID 5429). The 3D structures of the *Scurrula atropurpurea* active compound were obtained in the form of *.sdf file format. This format was converted to a *.pdb file using OpenBabel software (21).

3.2 3D protein structure modeling

The 3D structure of the target protein was predicted using the SWISS-MODEL web server with the homology modeling method. The 3D protein structures were then validated using Ramachandran plot (22, 23).

3.3 Docking and visualization between protein-ligand

Molecular docking modeling between *Scurrula atropurpurea* active components and target proteins was carried out using HEX 8.0 software (24). The docking procedure consisted of three stages of visualization, namely rigid-body energy minimization, semi-flexible repair, and finishing refinement in explicit solvent. The docking results were then visualized with Chimera 1.6.2 and Discovery Studio 4.1 softwares.

3.4 Analysis for bond interactions between protein and ligand

Molecular docking results were then visualized using Discovery Studio 4.1, LigPlot + and LigandScout 3.1 softwares (25, 26). Analysis of interactions between protein and ligand was made to see the number and type of chemical bonds formed.

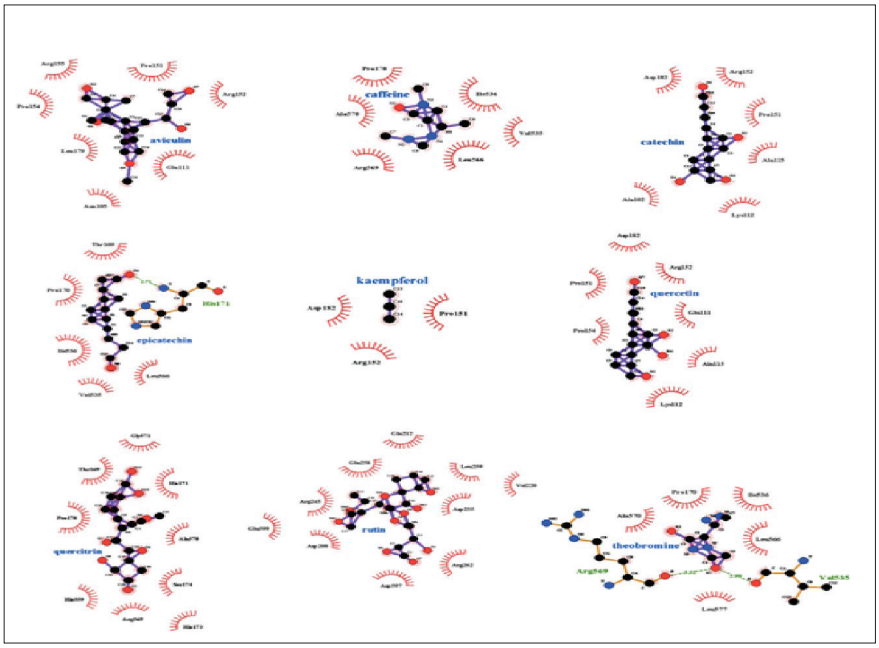

Figure 1. Interaction between active compounds of Scurrula atropurpurea and NFkB-IkB complex

4. RESULTS

The docking between nine active compounds of *Scurrula atropurpurea* has been carried out against NFkB-IkB complex. The compounds which are most easily to form a docking with NFkB-IkB complex in sequence are rutin (-314.35 kJ/mol), aviculin (-311.75 kJ/mol), quercetin (-247.11 kJ/mol), quercitrine (-288.36 kJ/mole), catechin (-239.13 kJ/mol), kaempferol (-238.11 kJ/mol), epicatechin (-232.58 kJ/mol), caffeine (-170.13 kJ/mol), and theobromine (-162.28 kJ/mol). The point of interaction, type of bond, and the amount of energy needed by each compound to interact with the NFkB-IkB complex in the interaction process can be seen in Table 1.

Table 2 shows the energy of interaction between NFkB-IkB complex and IKK. The results of this study indicate the energy needed for IKK to interact with NFkB-IkB complex under normal condition (without S. atropurpurea active compound) is -211.95 kJ/mol. The results of *in silico* analysis showed that all active compounds can potentially support the interaction between NFkB-IkB and IKK where by the energy needed to interact is smaller when there is an active compound. The sequences of interactions are including kaempferol (-226.88 kJ/mol), aviculin (-223.17 kJ/mol), caffeine (-219.11 kJ/mol), catechin (-219.04 kJ/mol), epicatechin (-216.75 kJ/mol), quercetin (-220.20 kJ/mol), and quercitrine (-215.16 kJ/mol). For rutin (-185.88 kJ/mol) the bonding energy is greater than without the active compound so the interaction is slower than normal condition (-211.95 kJ/mol).

Molecule	Binding energy
NFkB/IkB–IKK	-211.95 kJ/mol
NFkB/IkB, aviculin–IKK	-223.17 kJ/mol
NFkB/IkB, caffeine–IKK	-219.11 kJ/mol
NFkB/IkB, catechin–IKK	-219.04 kJ/mol
NFkB/IkB, epicatechin–IKK	-216.75 kJ/mol
NFkB/IkB, kaempferol–IKK	-226.88 kJ/mol
NFkB/IkB, quercetin–IKK	-220.20 kJ/mol
NFkB/IkB, quercitrin–IKK	-215.16 kJ/mol
NFkB/IkB, rutin–IKK	-185.88 kJ/mol
NFkB/IkB, theobromine–IKK	-221.40 kJ/mol

Table 2. Interactions between IKK and NFkB-IkB complex with or without the presence of the active compounds of Scurrula atropurpurea

5. DISCUSSION

Some previous studies have proven an involvement of inflammation in endometriosis, which is characterized by an increase in up-regulation of proinflammatory cytokines, TNF-a, IL-1, IL-11, and interferon-g (27). This increase occurs through activation of transcription factors such as NF-kB which enter the cell nucleus to induce gene transcription and encode the proinflammatory cytokine products (10, 11).

In this study, we analyze how the role of *Scurrula atropurpurea* active compounds on the classic NFkB signaling pathway, which involves the complex activity of IkB kinase (IKK) in phosphorylation of NFkB (IkB) inhibitor, so causing IkB to be degraded through the ubiquitination process. Furthermore, NFkB will translocate to nucleus and activate transcription from target genes. The results of this study revealed that various active ingredients of *Scurrula atropurpurea* can interact with NFkB-IkB complex. Of the nine active ingredients of *Scurrula atropurpurea*, the ingredients which are most easily to make interaction (which is characterized by low bond energy) in sequence are rutin (-314.35 kJ/mol), Aviculin (-311.75 kJ/mol), quercetin (-247.11 kJ/mol), quercitrine (-288.36 kJ/mol), catechin (-239.13 kJ/mol), kaempferol (-238.11 kJ/mol), epicatechin (-232.58 kJ/mol), caffeine (-170.13 kJ/mol), and theobromine (-162.28 kJ/mol). This indicates that nine active ingredients of *Scurrula atropurpurea* can form the complexes with NFkB-IkB in the cytoplasm. Previous studies have proved the docking between piperine and NFkB, the interaction energy of (-24.685 kcal/mol) and have hydrophobic and hydrogen bonds, indicating NFkB inhibitors (28). Interestingly, almost all interactions between the active ingredients of *Scurrula atropurpurea* and NFkB-IkB complex will facilitate its interaction with the IKK. This indicates that the active ingredient cannot inhibit NFkB activation. For rutin, the energy interaction is greater than in

normal condition, so it can be an NFkB activation inhibitor. This finding is consistent with previous studies, stating that rutin is capable to suppress phosphorylation and IkB degradation (29, 30). This finding is contrary to previous findings that catechin, theobromine, quercitrin, and caffeine have been proven capable to inhibit NFkB activation (31-34).

6. CONCLUSION

Thus it is concluded that one of the active ingredients of *Scurrula atropurpurea* which can potentially act as anti-inflammatory substance is rutin thereby it can be isolated and used as an alternative ingredient for inhibiting inflammation in endometriosis.

- **Author's contribution:** Study concept and design: C.Y; N.R; E.P; N.R; Y.Y; N.N; A.M; I.L; A.A; M.M. Acquisition of data: N.N; A.M; I.L; A.A; M.M. Analysis and interpretation of data: C.Y; N.R; E.P; N.R; Y.Y; N.N. Drafting of the manuscript: A.M; I.L; A.A; M.M. Critical revision of the manuscript for intellectual content: C.Y; N.R; E.P; N.R; Y.Y; N.N; A.M; I.L; A.A; M.M.
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REFERENCES

1. Petersen HJ, Smith AM. The role of the innate immune system in granulomatous disorders. Front Immunol 2013; 4: 120.
2. Chovatiya R, Medzhitov R. Stress, inflammation, and defense of homeostasis. Mol Cell 2014; 54: 281-288.
3. Arango Dugue G, Descoteaux A. Macrophage cytokines: involvement in immunity and infectious diseases. Front Immunol 2014; 5: 491.
4. Turner MD, Nedjai B, Hurst T, Pennington DJ. Cytokines and chemokines: at the crossroads of cell signaling and inflammatory disease. Biochim Biophys Acta 2014; 1843: 2563-2582.
5. Salminen A, Huuskonen J, Ojala J, Kaupplinen A, Kaarniranta K, Suuronen T. Activation of innate immunity system during aging: NF-kB signaling is the molecular culprit of inflamm-aging. Ageing Res Rev 2008; 7: 83-105.
6. Wu MH, Lu CW, Chang FM, Tsai SJ. Estrogen receptor expression affected by hypoxia inducible factor-1a in stromal cells from patients with endometriosis. Taiwanese J Obstet & Gynecol 2012; 51: 50-51.
7. Farrell E, Garad R. Endometriosis. Australian Nursing Journal. 2012; 20(5): 37-39.
8. Bulun SE. Mechanisms of disease endometriosis. N Eng J Med. 2009; 360: 268-279.
9. Stratton P, Merino MJ, Winkel CA, Zimmer C, Sinaii N, Nieman LK. Location, color, size, depth, and volume may predict endometriosis in lesions resected at surgery. Fertil & Steril. 2002; 78(4): 743-746.
10. Reinaldo GR, Lousse JC, Langendonck AV, Colette S, Ere SD, Devoto L, Donnez J. Involvement of the nuclear factor-kB pathway in the pathogenesis of endometriosis. Fertil & Steril. 2010; 94(6): 1986-1987.
11. Soares S, Martínez-Varea A, Hidalgo-Mora J, Pellicer A. Pharmacologic therapies in endometriosis: systematic review. Fertil & Steril. 2012; 98(3): 529-555.
12. Ohashi K, Winarno H, Mukai M, Shibuya H. Preparation and cancer cell invasion inhibitory effects of C16-alkynic fatty acids. Chem Pharm Bull. 2003; 51(4): 463-466.
13. Ohashi K, Winarno H, Mukai M, Inoue M, Prana MS, Simanjuntak P, Shibuya H. Indonesian Medicinal Plants. XXV. Cancer cell invasion inhibitory effects of chemical constituents in the parasitic plant Scurrula atropurpurea (Loranthaceae). Chem Pharm Bull. 2003; 51(3): 343-345.
14. Parwati NWM, Lindayani IK, Ratnawati R, Winarsih S, Nurseta T. Possible effect of tea plant parasite, *Scurrula atropurpurea* (Blume) Danser, on growth inhibition of culture HeLa cells *in vitro* through DNA repair and apoptosis intrinsic pathways mechanism. Asian Pacific Journal of Tropical Disease. 2015; 5(9): 743-746.
15. Athiroh N, Permatasari N, Sargowo D, Widodo MA. Antioxidative and blood pressure-lowering effects of Scurrula atropurpurea on deoxycorticosterone acetate-salt hypertensive rats. Biomarkers and Genomic Medicine. 2014; 6(1): 32-36.
16. Afanasev IB, Dorozhko AI, Brodskii AV, Kostyuk VA, Potapovitch AI. Chelating and free radical scavenging mechanisms of inhibitory action of rutin and quercetin in lipid peroxidation. Biochem. Pharmacol. 1989; 38: 1763-1769.
17. Van Acker SA, van Balen GP, van den Berg DJ, Bast A, van der Vijgh WJ. Influence of iron chelation on the antioxidant activity of flavonoids. Biochem. Pharmacol. 1998; 56: 935-943.
18. Saija A, Scalese M, Lanza M, Marzullo D, Bonina F, Castelli F. Flavonoids as antioxidant agents: Importance of their interaction with biomembranes. Free Radic Biol Med. 1995; 19: 481-486.
19. Jung HA, Jung MJ, Kim JY, Chung HY, Choi JS. Inhibitory activity of flavonoids from Prunus davidiana and other flavonoids on total ROS and hydroxyl radical generation. Arch Pharm. Res. 2003; 26:809–815.
20. Glorie G, Legrand-Poels S, Piette J. NF-kB activation by reactive oxygen species: fifteen years later. Biochem Pharmacol 2006; 72:1493-1505.
21. O'Boyle N, Banck M, James CA, Morley C, Vandermeersch T, Hutchison GR. Open Babel: An open chemical toolbox. J Cheminform 2011; 3:33.
22. Arnold K, Bordoli L, Kopp J, Schwede T. The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling. Bioinformatics 2006; 22(2):195-201.
23. Kiefer F, Arnold K, Kunzli M, Bordoli L, Schwede T. The SWISS-MODEL repository and associated resources. Nucleic Acids Res 2009. 37(Database issue): 387-392.
24. Macindoe G, Mavridis L, Venkatraman V, Devignes MD, Ritchie DW. HexServer: an FFT-based protein docking server powered by graphics processors. Nucleic Acids Res. 2010; 38(Web server issue): 445-449.
25. Laskowski RA, Swindells MB. LigPlot+: multiple ligand-protein interaction diagrams for drug discovery. J Chem Inf Model. 2011; 51(10): 2778-2786.
26. Wolber G, Langer T. LigandScout: 3-D pharmacophores derived from protein-bound ligands and their use as virtual screening filters. J Chem Inf Model. 2005; 45(1): 160-169.
27. Mruk DD, Xiao X, Lydka M, Li MWM. Bilinska B, Cheng CY. Intercellular adhesion molecule 1: Recent findings and new concepts involved in mammalian spermatogenesis. Semin Cell & Dev Biol. 2014; 29: 43-54.
28. Verma A, Kushwaha HN, Srivastava AK, Srivastava S, Jamal N, Srivastava K, Ray RS. Piperine attenuates UV-R induced cell damage in human keratinocytes via NF-kB, Bax/Bcl-2 pathway: An application for photoprotection. J Photochem Photobiol. 2017; 172: 139-148.
29. Yeh CH, Yang JJ, Yang ML, Li YC, Kuan YH. Rutin decreases lipopolysaccharide-induced acute lung injury via inhibition of oxidative stress and the MAPK-NF-κB pathway. Free Radic Biol Med. 2014; 69: 249-257.
30. Jantrawut P, Phongpradist R, Muller M, Viernstein H. Enhancement of anti-inflammatory activity of polyphenolic flavonoid rutin by encapsulation. Pak J Pharm Sci. 2017; 30(5): 1521-1527.
31. Camuesco D, Comalada M, Rodríguez-Cabezas ME, Nieto A, Lorente MD, Concha A, Gálvez J. The intestinal anti-inflammatory effect of quercitrin is associated with an inhibition in iNOS expression. Brit J Pharmacol. 2004; 143: 908-918.
32. Li XY, Xu L, Lin GS, Li XY, Jiang XJ, Wang T, Lu JJ, Zeng B. Protective effect of caffeine administration on myocardial ischemia/reperfusion injury in rats. Shock. 2011; 36: 289-294.
33. Fernandez-Murga L, Tarin JJ, Garcia-Perez MA, Cano A. The impact of chocolate on cardiovascular health. Maturitas. 2011; 69: 312-321.
34. Rahayu B, Baktiyani SCW, Nurdiana N. Theobroma cacao increases cells viability and reduces IL-6 and sVCAM-1 level in endothelial cells induced by plasma from preeclamptic patients. Pregnancy Hypertension: An International Journal of Women's Cardiovascular Health. 2016; 6: 42-46.