

Molecular Pharmacology Study of Andrographolide Extracted from *Andrographis Paniculata* on Atherosclerosis Preventive Effect

Ni Kadek Warditiani¹, Pande Made Nova Armitha Sari^{1,2}, Yan Ramona³, Made Agus Gelgel Wirasuta^{1,2,*}

¹Pharmacy Department, Faculty of Mathematic and Natural Science, Udayana University, Badung 80361, Bali, Indonesia; kadektia@unud.ac.id.

²Forensic Science and Criminology Laboratory of Udayana University, Badung 80361, Bali, Indonesia; nova.armita@unud.ac.id

³Biology Department, Faculty of Mathematic and Natural Science, Udayana University, Badung 80361, Bali, Indonesia; yan_ramona@unud.ac.id

*Corresponding author; gelgel.wirasuta@unud.ac.id

ABSTRACT

Diet pattern resulting in hyperlipidemia is likely the main factor of atherosclerosis development. Balinese have been using *Andrographis paniculata* (*Sambiloto*) traditionally to maintain their lipid blood level and stamina. Andrographolide (AND) is its active compound. Therefore, molecular pharmacology study of AND on atherosclerosis-related diseases is urgently needed. The study aimed to develop a Thin Layer Chromatography (TLC) fingerprint method for *Sambiloto* standardization and to carry out the pharmacological effect of this compound on atherosclerosis prevention. This study comprised of several stages: development of the TLC fingerprint, pre-clinical research including atherosclerosis induction in an animal model, experimental treatment and prevention of atherosclerosis development in the animal model using AND, comparison of the results with those for atorvastatin treatment, and *in-silico* docking simulation of AND's molecular pharmacological effect. TLC with a ratio of 9:1 (v/v) chloroform: ethanol mobile phase was found to be a suitable method for *Sambiloto* standardization. Rat diets composition of 5% duck yolk, 10% pork fat, Vit D3 20,000 IU, and 1% calcium induced atherosclerosis development. Daily administration of AND at 1.5 mg/kg rat body weight with atherogenic food prevented atherosclerosis development. The docking simulation showed that AND interacted well with NF-kB, ICAM-1, VCAM-1, TNF α , IFN- γ and Cyt MAP kinase P32 proteins responsible for the anti-atherosclerotic effect. *Sambiloto* is a potential traditional herbal medicine for use in treating and preventing hyperlipidemia from triggering atherosclerosis-related disease development. AND inhibited the ox-LDL formation and interacted well with the atherosclerosis-protein receptor targets.

Keywords: Andrographolide; dyslipidemia; atherosclerosis; *in silico* docking simulation

Correspondence:

Made Agus Gelgel Wirasuta

Pharmacy Department, Faculty of Mathematic and Natural Science, Udayana University, Badung 80361, Bali, Indonesia

Email: gelgel.wirasuta@unud.ac.id, kadektia@unud.ac.id

Introduction

Atherosclerosis epidemic increases under diet patterns causing hyperlipidemia. *Sambiloto* is a traditional herbal medicine that significantly decreases cholesterol, triglyceride, and LDL cholesterol content in the blood ⁽¹⁾. AND is the main active compound of *Sambiloto*.

Atherosclerosis is initially induced by high LDL content in the blood, which leads to ox-LDL formation. The ox-LDL infiltration into tunica intima endothelial cells induces ICAM-1 and VCAM-1 expression. These released VCAM-1 and ICAM-1 migrate into tunica intima endothelial cells and induce inflammation. The electron scavenging effect of AND potentially suppresses LDL oxidation into ox-LDL ⁽²⁾. *In vitro* experiments with AND using human platelets found that it has an anti-inflammation effect by inhibiting NF-kB activation ⁽³⁾. AND also decreases both ICAM-1 and VCAM-1 expression on human umbilical endothelial cells ⁽⁴⁾. Therefore, AND could potentially hinder the atherosclerotic process.

This study reports quality control of herbal medicine raw materials by applying TLC fingerprint to control AND content, AND isolation, and pre-clinical tests to elucidate AND's ability to prevent atherosclerosis development. The molecular mechanism by which this compound works *in vivo* (in animals) was simulated using *in silico* testing.

Material and Method

Materials

Materials used included *Sambiloto*, methanol technical 96% ethanol, technical ethyl acetate, technical hexane, water, 98% AND (Sigma-Aldrich), atorvastatin, fat-rich feeds termed as "atherogenic feed" (standard feed: duck-yolk: pork fat = 85:5:10), calcium and vitamin D3 20.000 IU per week. Male Wistar rats aged 6-7 weeks, weighing of between 180-200 grams were used for *in vivo* experiments.

Isolation and TLC fingerprint of *Sambiloto* extract

The *Sambiloto* was obtained from Kulonprogo-Yogyakarta, Indonesia and was determined by Biology Pharmacy Department, Faculty of Pharmacy, Gajah Mada University, with certificate number of BF/08/Ident/Det/VII/2013. One kilogram of the dry sample was macerated in 5 L 96% ethanol for 24 hours, filtered, re-macerated twice using the same solvent and evaporated in a rotary evaporator at 40 °C until a concentrated extract was obtained⁽¹⁾. The concentrated extract was then dissolved in n-hexane; this was conducted several times until clear hexane was obtained. The insoluble fraction was washed with ethyl acetate and boiling water. The isolate was dissolved in hot ethanol and recrystallized in a container with ice cubes in it. Andrographolide crystals appeared after 24 hours incubation.

TLC plates were prepared according to Wirasuta et al. 2012⁽⁵⁾. The *Sambiloto* extract was spotted on a TLC plate

at various volumes of 3, 5, 10, 15, 20 and 25 µL with a Linomat 5 Camag, and then eluted using a mixture of chloroform: methanol 9:1 (v/v) in a twin chamber previously saturated with this mixture for 30 minutes. After elution, the plate was dried at 60 °C for 5 minutes on a hotplate (6). Sample spots following elution were scanned with a TLC-Scanner 3 Camag at a wavelength of 230 nm, and the peak spectrum was scanned at wavelengths of 200–400 nm.

Pre-clinic test

Male Wistar rats (six rats for group treatment) were acclimatized for seven days before treatment. Ethical clearance was obtained from the Faculty of Veterinary Science, Udayana University, with certificate number of 0143/KE-PH/IX/2013. The rats were grouped, as follows (1,7):

- (A) Healthy group: rats fed with standard feed and sufficient water
- (B) Atherogenic group: rats fed with atherogenic feed and sufficient water
- (C) Athorvastatin group: rats fed with atherogenic feed, athorvastatin 7.2 mg/kg bodyweight and sufficient water
- (D) AND 1.5 mg group: rats fed with atherogenic feed, isolate AND 1.5 mg/kg bodyweight and sufficient water
- (E) AND 4.5 mg group: rats fed with atherogenic feed, isolate AND 4.5 mg/kg bodyweight and sufficient water

induction of atherosclerosis

Fifteen rats were fed with atherogenic feed. Three rats were sacrificed for morphology aorta alteration on day 0 before being fed with atherogenic feed and at 30, 40, 50 and 60 days after feeding with atherogenic feed.

Anti-hyperlipidemia test

The rats were grouped as above, but only one dose of AND was administered. Six rats each from groups (C) and (E) were induced with atherogenic feed for 30 days, before administrated daily dose of atorvastatin or isolate AND for 30 days. The rats of both (C) and (E) groups were

simultaneously fed with atherogenic feed for 30 days (7,8). Rats' blood lipid levels were measured before and after atorvastatin or AND administration. The results were statistically analysed to determine aorta alteration because of treatments.

Prevention of atherosclerosis

Rats in groups (B-E) were simultaneously fed with atherogenic and active compounds (atorvastatin or AND) for 60 days. On day 60, all rats were sacrificed, and their aortas were collected and stained with hematoxylin-eosin dyes. These stained aorta preparations were observed under a light microscope, and their anatomy was scored in the range of 0-4. Scores 0, 1, 2, 3 and 4 were given if the aorta conditions were normal, cells comprising aorta tunica intima were dilated, elastic and some foams were fragmented, smooth muscle increased, and plaque ulceration or lipid calcification were found, respectively(1).

2.4 Simulation of molecular pharmacological in silico

Protein targets (NF-kB, ICAM-1, VCAM-1, TNF-α, IFN-γ, Cyt MAP kinase P32) were downloaded from PDB (Protein Data Bank). The ligand was eliminated from the protein using Discovery Studio and the compound was docked with target proteins by applying Autodock Vina. The results obtained were in the form of an energy bond between the target protein and compounds. The energy bond of AND was compared with Dexamethasone (as a reference).

Result

TLC-fingerprint and AND identification

Fig. 1 (a and b) shows the TLC fingerprint of *Sambiloto* leaf ethanol extract, while Table 1 presents the chromatogram peaks. P1–P8 peaks have a positive slope with r values of > 0.900. Fig. 1 (c, d, e, and f) shows the densitogram and spectra of AND reference, fraction, isolated AND crystal, and extracted AND. Based on their similarity in measured spectra, which were compared with their TLC fingerprint peaks, P5 was identified as AND. Both isolated and reference AND presented one peak on TLC chromatogram and belonged both identical spectra and Rf-value. This indicated that the isolated AND possessed purity equal to reference AND.

Table 1: The correlation between spotted volume extract and the area under the curve of detected peaks

Peaks	regression equation	reg. Coef.
N1	$y = -30.18x + 2238.2$	0.089
P1	$y = 50.06x + 184.9$	0.988
N2	$y = 2.09x + 274.0$	0.750
P2	$y = 84.55x + 348.9$	0.993
P3	$y = 64.64x + 224.8$	0.991
P4	$y = 98.31x + 211.1$	0.958
P5	$y = 825.91x + 12123.0$	0.917
P6	$y = 285.73x - 551.9$	0.972
P7	$y = 776.79x + 4296.0$	0.966
P8	$y = 285.73x - 551.9$	0.937
N3	$y = 853.17x + 4992.9$	0.891

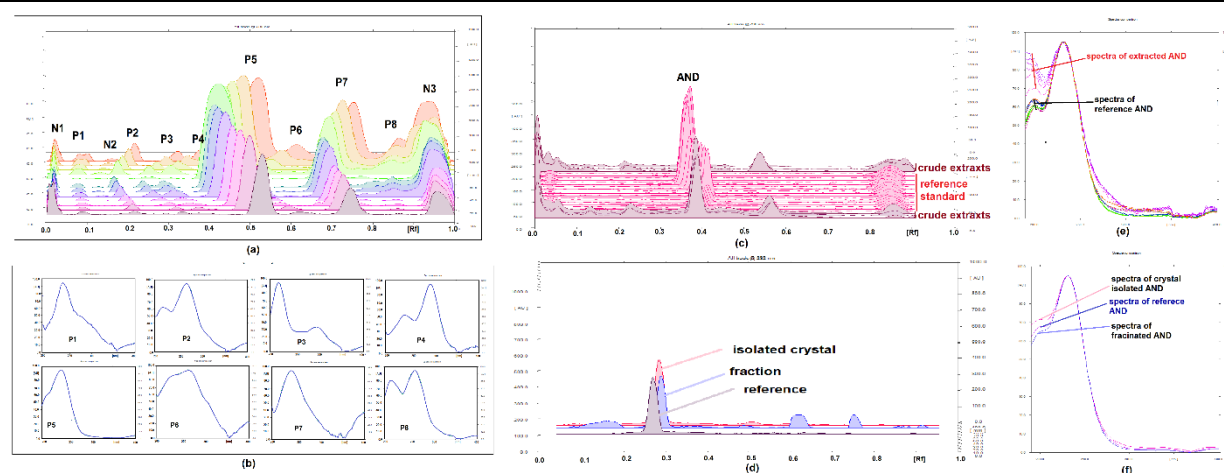


Figure 1: TLC-Fingerprint of the Sambilito herb ethanol extract. (a) densitogram of the extract, (b) spectra of each identified peak (P1-P8), (c and d) densitogram comparison between reference AND, the extract, fraction, and isolated AND crystal, and (e and f) spectra comparison between AND peaks.

Pre clinic test

Administration of the atherogenic feed for 30 days resulted in dilation of cells comprising the aorta tunica intima (Fig.2, A-ii; score=1). Extension of feeding to 40, 50 and 60 days resulted in foamy cell formation (Fig. 2, A-iii; score=2), smooth muscle cell proliferation on the aorta tunica intima (Fig. 2, A-iv; score=3), and aorta calcification in all animals (Fig. 2, A-v; score=4), respectively.

Fig. 2, (B-i) shows the rats' lipid profiles following various treatments. Lipid levels (TC, TG, LDL) of rat blood treated with atherogenic feed increased by 2.3 to 8.8 times when compared to controls, and this was statistically significant ($p < 0.05$). Interestingly, 0.36 times decrease in the HDL level in the control group was observed. Lipid levels (TC, TG, LDL) in rats treated with AND and atorvastatin decreased but remained higher than those of control rats ($p < 0.05$). The HDL level of rats treated with AND and atorvastatin increased, although its value was lower than control ($p < 0.05$).

Fig. 2, (B-ii and B-iii) presents scoring of morphology the rat aorta morphology in the five treatment groups (fed with atherogenic feed for 60 days followed by administration of AND, atorvastatin, and atherogenic feed for 30 days which was started from day 61) was observed

and scored regularly every week. Of rats in the healthy group, 87% and 13% had scores of 0 and 1, respectively. Of those treated with AND, 25%, 50%, and 25% had scores of 0, 1 and 2, respectively. Of those fed with atorvastatin, 12.5%, 17.5%, 25% and 25% had scores of 0, 1, 2, and 3 respectively. The results showed that a change in aorta morphology occurred in rats treated with AND and atorvastatin for 30 days. The percentage of rats with score 0 was higher in the AND-treatment group than that for atorvastatin.

Aorta cells in the healthy group were tight and healthy of healthy rats, 85% and 15% scored 0 and 1, respectively. Rats treated with atherogenic feed showed smooth muscle cell proliferation on the tunica intima. Of these rats, 33% and 67% scored 3 and 4, respectively. Cells comprising rat aorta appeared healthy in the preventive atorvastatin group. Of these rats, 67% and 33% scored 0 and 2, apiece. AND administration at doses of 1.5 and 4.5 mg/kg body weight produced similar scores; 82% and 18% scored 0 and 1, respectively. These results suggest that AND administration simultaneously with high atherogenic food consumption could prevent atherosclerosis (AND had an anti-atherosclerotic effect).

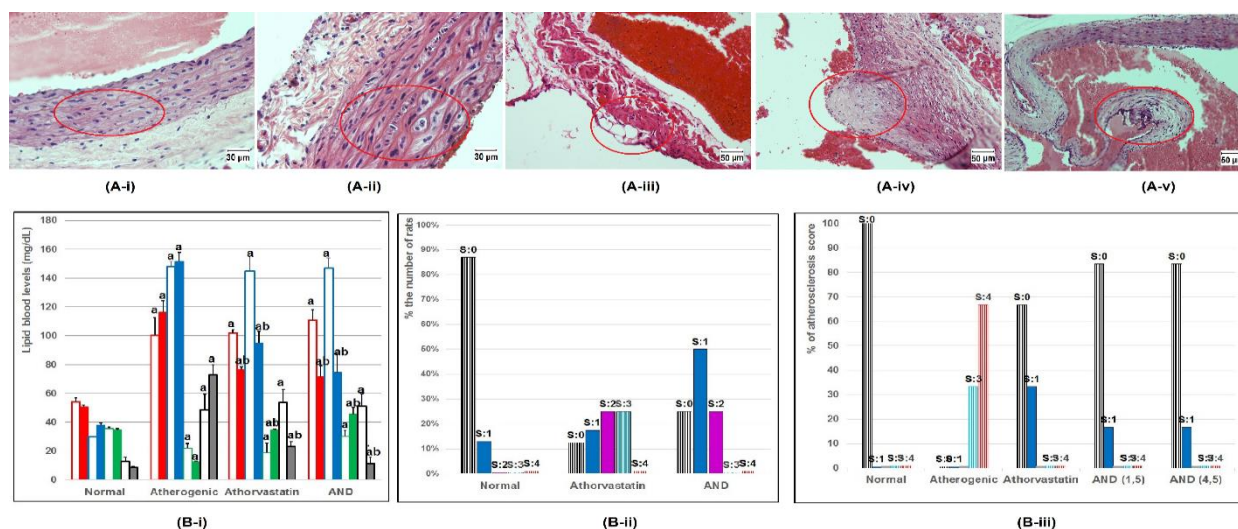


Figure 2: The process of atherosclerosis on rat's aorta (A), the blood lipid profile of rat treated with anti-hyperlipid (B-i) and aorta scoring morphology of rat's aorta on treated with anti-hyperlipid or preventive atherosclerosis (B-ii, iii). (B-i): Blood lipid profile; ■ ■ = cholesterol total (no filled: pre-treatment; red filled: post-treatment); ■ ■ = triglyceride (no filled: pre-treatment; blue filled: post-treatment); ■ ■ = HDL (no filled: pre-treatment; green filled: post treatment); ■ ■ = LDL (no filled: pre-treatment; gray filled: post -treatment) a = significantly different to normal control; b = significantly different to atherogenic control, AND = isolate andrographolide; (B-ii) aorta scoring morphology treated with anti-hyperlipid test; (B-iii): aorta scoring morphology of rat's aorta preventive atherosclerosis; S:= score.

In silico molecular pharmacological simulation.

Fig. 3 presents molecular binding between AND to atherosclerosis target proteins. The best affinity energy bond (kcal/ mol) between AND and target proteins (NF-kB, ICAM-1, VCAM-1, TNF- α , IFN- γ , Cyt MAP kinase P32) were -7.9, -7.3, -6.5, -7.8, -6.2, and -7.7, respectively. Meanwhile, the best affinity energy bond (kcal/ mol) between the reference substance and target proteins were -8.5, -7.9, -7.2, -8.5, -6.9, and -7.6 respectively. The energy

bonds of Dexa with NF-kB, ICAM-1, VCAM-1, IFN- γ , and TNF- α were more negative than those with AND. The bond energy of Dexa on cytokine MAP kinase P32 was more harmful than that of AND. The values of AND's bond energy with those target proteins were not significantly different from those of Dexa. These indicated that AND binds with the target proteins and prevents induction of atherosclerosis.

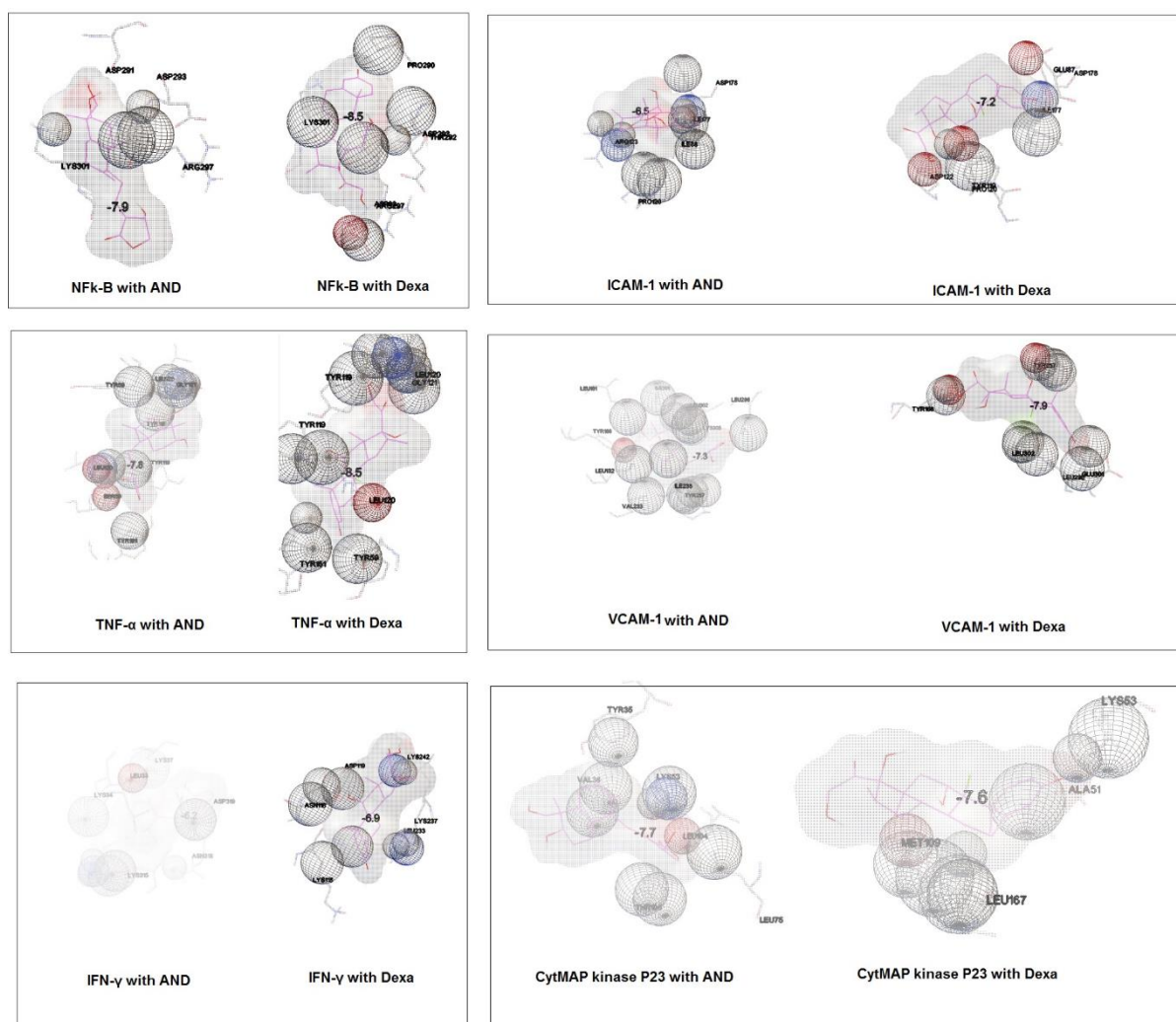


Figure 3: Bounds of AND and Dexa on targeted proteins

Discussion

AND was identified on P5 peak. The P1-8 peaks were detected in all tracks and their area under the curve presented a positive linear correlation to the increasing spotted volume. This was used to determine marker of the Sambiloto extract fingerprint ⁽⁹⁾. This TLC fingerprint

method could be used to control of the quality of the Sambiloto extract.

Atherogenic feed (1% cholesterol, 3% pork lipid and pro-Vit-D) without calcium need longer time to induce an atherosclerosis ⁽¹⁰⁾, incompere to our study. High doses of Vit D3 increased the absorption of calcium in the lumen intestine and also macrophage activity ⁽¹¹⁾. This induced

the activity of TNF- α and so increased promotion of vascular calcification⁽¹²⁾. Excessive supply of Vit D3 caused a substantial accumulation of calcium deposits in the intima- and media-tunica, degradation of elastin, increased the rigidity of vessel cell walls and hypertrophy. Atherosclerosis occurred because of a series of inflammation reactions. AND has antioxidant activity⁽²⁾ and help reduce the ox-LDL level in rats. In macrophages, ox-LDL bound with CD36 and induces pro-inflammatory cytokine release, so that it stimulates monocyte penetration into the tunica intima endothelial cells⁽¹³⁾. Macrophages also activate T lymphocytes to enter endothelial cells. NF- κ B is a transcription factor that regulates E-selection and VCAM-1 expression. An increase in E-selection and VCAM-1 expression results in leucocyte deposit in the arterial intima. In such a situation, the T lymphocytes will secrete IFN- γ , TNF- α , and β , which affects the inflammatory response on the endothelial cells. Macrophages and foamy cells continuously release cytokine, which activates monocytes and T lymphocytes. In our study, this condition was marked with a score of 2 (Fig. 2, A-iii). Fibroblast growth factor and platelet-derived growth factor are secreted by endothelial cells and foamy cells. Fibroblast growth factor and platelet-derived growth factor stimulate smooth muscle cells to migrate into the tunica media and cause differentiation and proliferation⁽¹⁴⁾. The smooth muscle cells synthesize collagen, which results in cap fibrosis formation. The proliferation of smooth muscle cells occurred on the tunica intima of the aorta, and this proliferation has a score of 3 (Fig. 2, B-iv). The calcification of aorta is a manifestation of spotty calcification plaques. This unstable plaque could develop into a stable plaque to form macrocalcifications. This condition was marked as score 4 (Fig. 2, B-v). Score 0 and 1 indicated healthy aorta cells. Score 2 was the inflammatory cells, which signifies the beginning of the induction of foamy cell formation. Score 3 was the initial phase of atherosclerosis.

AND inhibit NF- κ B activity so that proinflammatory genes are not expressed⁽³⁾. AND binds with ICAM-1 and VCAM-1 proteins to block the inflammation⁽⁴⁾. AND decreased TNF- α production, which is associated with inflammatory⁽¹⁵⁾. AND also suppresses TNF- γ signalling *in vitro* on monocytes⁽¹⁶⁾. NF- κ B regulates expression of E-selection, VCAM-1, and TF (tissue factor), which synergistically respond to the inflammation and excessive thrombosis. This process occurs in hyperplasia neointima and vasculitis thrombosis. Arterial disturbances such as restenosis can be overcome by administering NF- κ B antagonist compounds such as AND⁽¹⁷⁾. In our research, molecular docking of AND indicated that this compound binds with NF- κ B, VCAM-1, and ICAM-1 (Fig. 3). These AND-molecular bindings presumed induced a releasing plaque on the anti-hyperlipidemia test (Fig. 2, B-ii) and prevent inflammation and foamy cell formation on prevention of atherosclerosis preclinical study (Fig. 2, B-iii).

Conclusion

In vivo, AND extract prevented atherosclerosis because of its ability to reduce blood lipid level and function as an antioxidant. In the *in-silico* analysis, AND could bind with proteins, involved in the atherosclerotic process, to prevent inflammatory cells and foam cell formation.

Conflict of interest

We wish to confirm that there are no known conflicts of interest associated with this publication, and there has been no significant financial support for this work that could have influenced its outcome.

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