RESEARCH ARTICLE

Butterfly Pea and Roselle Combination Extracts Reduce V-CAM, ICAM, and IL-6 Levels in High Fat Atherogenic Diet Rats

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Abstract

BACKGROUND: Atherosclerosis, driven by inflammation and oxidative stress, increases the risk of coronary heart disease (CHD). Flavonoids in butterfly pea and roselle are known for their potent antioxidant and anti-inflammatory properties. While their individual effects on cardiovascular health have been studied, no studies have explored the combined impact on atherosclerosis biomarkers, including vascular cell adhesion molecules (VCAM)-1, intercellular adhesion molecule (ICAM)-1, and interleukin (IL)-6. Therefore, this study was performed to evaluate the synergistic effects of butterfly pea and roselle combination extracts (BPRCE) on these biomarkers.

METHODS: A study with a post-test control group design using 36 male white rats was performed. The rats were randomly assigned to 6 groups; 1 group was fed with standard feed, while 5 groups were fed with a high-fat atherogenic diet (HFAD) to create atherosclerosis rat models. The HFAD rats were given either no treatment, sodium carboxymethylcellulose (Na-CMC), 300, 400, or 500 mg/kgBW BPRCE. Serum levels of VCAM-1, ICAM-1, and IL-6 of rats were measured using enzyme-linked immunosorbent assay (ELISA) methods.

RESULTS: Increasing doses of BPRCE resulted in a significant reduction in VCAM-1, ICAM-1, and IL-6 compared to the other groups. The group with the highest dose, 500 mg/kgBW BPRCE, showed the greatest reduction of VCAM-1 level (32.73±3.57 pg/mL), ICAM-1 level (5.68±1.17 ng/mL), and IL-6 levels (21.49±4.62 pg/mL).

CONCLUSION: Administration of BPRCE in atherosclerosis rats model reduces VCAM-1, ICAM-1, and IL-6 in a dose-dependent manner. This study showed that using BPRCE as a traditional remedy for preventing and treating CHD at an optimal dose of 500 mg/kgBW might be a potential future application in reducing atherosclerosis biomarkers.

KEYWORDS: VCAM-1, ICAM-1, IL-6, butterfly pea, rosella, atherosclerosis

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Introduction

Atherosclerosis is a chronic inflammatory disease caused by the accumulation of fat on arterial walls and is characterized by increased levels of molecules such as vascular cell adhesion molecules (VCAM)-1, intercellular adhesion molecule (ICAM)-1, and interleukin (IL)-6. This condition can lead to the narrowing and hardening of blood vessels, thereby increasing the risk of coronary heart disease (CHD), stroke, and peripheral artery disease. VCAM-1 is a glycoprotein endothelial cells produce in response to cytokines. This molecule facilitates the adhesion of leukocytes, including lymphocytes and monocytes, to the endothelial surface, intensifying inflammation in the arterial wall. Elevated VCAM-1 expression is associated with the development of CHD. ICAM-1 is another glycoprotein found on the surface of endothelial cells and leukocytes, with increased expression playing a crucial role in the pathogenesis of atherosclerosis and CHD. IL-6, an

inflammatory biomarker in atherosclerosis, can reduce nitric oxide (NO) production and inhibit endothelial nitric oxide synthase (eNOS) activity, leading to endothelial cell damage and contributing to CHD.(1–3) Data from the World Health Organization (WHO) indicate that the prevalence of CHD worldwide continues to rise each year. In 2019, there were 6.7 million cases with a mortality rate of 1.8 million deaths. In Indonesia, it is estimated that there are approximately 1.36 million cases of CHD, with a mortality rate of 245,343 deaths.(4–6)

Inflammation and oxidative stress are involved in the mechanism of atherosclerosis.(7) Atherosclerosis begins with increased free radicals due to low-density lipoprotein (LDL) buildup, leading to oxidative stress and oxidized LDL (Ox-LDL) formation. This damages endothelial cells and triggers pro-inflammatory cytokines like VCAM-1, IL-6, and ICAM-1, attracting monocytes that transform into macrophages. These macrophages absorb LDL, forming foam cells and promoting fatty streaks in the coronary arteries, which narrows the arteries and raises the risk of CHD.(8–11) Simvastatin treats CHD by reducing LDL cholesterol and preventing arterial blockage by inhibiting 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase. However, long-term use can cause side effects such as liver and kidney damage, muscle pain, and digestive issues.(12–16)

An alternative approach to treating coronary heart disease (CHD) involves non-pharmacological therapy using traditional herbal remedies like butterfly pea and roselle. These plants could act as substitutes for simvastatin in CHD treatment due to their rich flavonoid content, which possesses potent antioxidant and anti-inflammatory properties. The flavonoids in these plants play a crucial role in inhibiting pro-inflammatory enzymes such as cyclooxygenase-2 (COX-2) and lipoxygenase, safeguarding cells from damage caused by free radicals and preventing oxidative stress contributing to CHD progression. This reassures us of the potential effectiveness of this treatment.(17–19) Several studies have explored using butterfly pea and roselle as traditional CHD remedies. Research on butterfly pea extract (BPE) has shown its potential to treat cardiovascular issues in hypertensive rats with nitric oxide deficiency.(20,21) Additionally, BPE has demonstrated the ability to reduce total cholesterol levels in *Rattus norvegicus* and prevent cardiovascular disease.(22,23)

Likewise, 500 mg/kgBW roselle extract (RE) effectively reduces blood cholesterol.(24) Further support comes from another study that suggests RE's ability to lower cholesterol and aid CHD recovery.(25) The bioactive compounds from butterfly pea and roselle are extracted using the ethanol maceration method, which is cost-effective, simple, and operates at low temperatures. Despite this, no studies have explored the combined effects of butterfly pea and roselle combination extracts (BPRCE) on atherosclerosis biomarkers, making this research crucial to understanding their potential impact in reducing inflammation and oxidative stress. Therefore, this study was performed to evaluate the synergistic effects of the BPRCE on the levels of VCAM-1, ICAM-1, and IL-6, potentially offering a new approach to managing atherosclerosis.

Methods

Study Design and Experimental Animal Inclusion

This was an experimental laboratory design with a post-test control group study conducted from July to September 2024. Thirty-six male white rats (*Rattus norvegicus* L.) weighed 180-200 grams and aged 3-4 months were selected as experimental animals. Rats should be in good health and have a Lee index above 300 to be included in the study. The rats were randomly assigned to 6 groups, with 6 rats in each group. One group was fed with standard feed, while the other 5 groups were fed with a high-fat atherogenic diet (HFAD) to cause atherosclerosis in rats. The HFAD rats were given either no treatment, sodium carboxymethylcellulose (Na-CMC), or BPRCE at 300, 400, or 500 mg/kgBW (Table 1). Each group received specific treatments over 45 days, and the rats were carefully monitored to observe the effects of the interventions. The study has received ethical consideration and approval from the Animal Research Ethics Committees (AREC) of the Faculty of Mathematics and Natural Sciences, Universitas Sumatera Utara (No. 0685/KEPH-FMIPA/2024).

Preparation of HFAD

The standard feed was well-grinded and mixed with 10% goat fat, 1% cholesterol, 5% quail egg yolk, and 1% cooking oil. All ingredients were stirred until well combined and then shaped using a noodle-making machine. The mixture was then cooked until fully cooked.(26,27) HFAD was given to rats to create atherosclerosis rat model as confirmed in previous study.(26)

Preparation of 1% Na-CMC Solution

Ten grams of sodium carboxymethylcellulose (Na-CMC) was dissolved in 200 mL distilled water, then heated and stirred for 15 minutes until suspension was formed.

Table 1. Experimental treatment groups for test subjects.

Group	Treatment Given	Treatment Description
HFAD	HFAD only	Administered the HFAD
Na-CMC	Na-CMC	Administered the HFAD with 1% Na-CMC
CE300	300 mg/kg BPRCE	Administered the HFAD with 1% Na-CMC and BPRE at a dosage of 300 mg/kg body weight
CE400	400 mg/kg BPRCE	Administered the HFAD with 1% Na-CMC and BPRE at a dosage of 400 mg/kg body weight
CE500	500 mg/kg BPRCE	Administered the HFAD with 1% Na-CMC and BPRE at a dosage of 500 mg/kg body weight
SF	Standard feed	Administered standard feed

Subsequently, distilled water was added gradually until the volume reached 1000 mL and stirred continuously until well-mixed.(26) Na-CMC solution was used as a stabilizing agent in preparing BPRCE suspension for rats, ensuring that BPRCE remained well-dispersed and did not settle.

Preparation of BPRCE

The butterfly pea and roselle flower samples were obtained from the same location in the Bangun Sari Flower Tourism area, Bangun Sari Village, Tanjung Morawa District, Deli Serdang Regency, Sumatera Utara, Indonesia. Both samples were identified at the Herbarium Medanense of the Universitas Sumatera Utara, confirming that the butterfly pea sample was classified as *Clitoria ternatea* Lour., and the roselle sample was classified as *Hibiscus sabdariffa* Lour. The two flower samples were washed individually and dried at a temperature of 40-50°C. Once dried, the flowers were blended into simplicia powder and sifted through a 40-mesh sieve. The butterfly pea and roselle flowers were mixed in a 1:1 ratio. Two hundred grams of the combined simplicia powder were placed into a vessel, and 750 mL of 96% ethanol was added. The vessel was tightly sealed and stored in a dark place for 72 hours, stirring every 8 hours. The mixture was then filtered to obtain the first macerate. The remaining solid was soaked again with 250 mL of ethanol for 24 hours and filtered to obtain the second macerate. Both macerates were combined and left overnight to sediment. The supernatant, separated from the residue, was then heated with a rotary evaporator at 40°C until a thick ethanol extract was obtained. This thick ethanol extract was prepared by combining the two flower samples in an equal ratio, and it was referred to as the BPRCE.

BPRCE was formulated at 300, 400, and 500 mg/kg BW doses. The BPRCE combination was mixed with a 1% Na-CMC solution until a total volume of 42 mL was reached, resulting in a homogeneous suspension. This suspension was administered daily to the experimental rats for 7 days. The preparation and administration process for the BPRCE suspension was repeated weekly for 45 days. The 42 mL volume was determined by calculating the total volume needed for administering a daily dose of 1 mL per rat for six rats over a period of seven days (1 mL × 6 rats × 7 days = 42 mL). This suspension was administered daily to the experimental rats for 7 days, with the preparation and administration of the BPRCE suspension repeated weekly over 45 days.

Phytochemical Screening Process of BPRCE

Phytochemical screening was conducted to identify the presence of tannins, saponins, terpenoids, alkaloids, and flavonoids in BPRCE. To detect tannins, a few drops of 5% $FeCl_3$ solution were added to 1 mL BPRCE; the formation of a blue-green color indicates the presence of tannins. To detect saponins, 2 mL of the extract was mixed with distilled water in a test tube and shaken for 10 min; the observed foam indicated saponins. Terpenoids were identified by adding 1 mL of 100% CH_3COOH and 1 mL concentrated sulfuric acid to 1 mL BPRCE, where the result showed a reddish-brown color that confirmed the presence of terpenoids. Meanwhile, to observe alkaloids, 2 mL chloroform and a few drops of concentrated H_2SO_4 were added to 1 mL BPRCE, followed by the addition of Wagner's reagent; the formation of a brown precipitate indicated the presence of alkaloids. Finally, to detect flavonoids, 1 gram of magnesium powder and a few drops of concentrated HCl were added to 1 mL of BPRCE; the development of a yellow color indicates the presence of flavonoids.(25) Phytochemical screening was also conducted for the butterfly pea flower extract (BPFE) and roselle flower extract (RFE). This screening was conducted to identify the presence of tannins, saponins, terpenoids, alkaloids, and flavonoids in both samples, following the same procedures as those used for BPRCE.

Measurement of Atherosclerosis Biomarkers

The measurements were conducted using Sandwich-enzyme-linked immunosorbent assay (ELISA) kits. The Rat sVCAM-1/CD106 (soluble Vascular Cell Adhesion Molecule 1) ELISA Kit (Catalog No: E-EL-R0910; (Elabscience, Houston, TX, USA) was used to detect Rat VCAM-1/CD106 in samples, with a sensitivity of 7.5 pg/

mL and a detection range of 12.5–800 pg/mL. The Rat sICAM-1/CD54 (soluble Intercellular Adhesion Molecule 1) ELISA Kit (Catalog No: E-EL-R0046, Elabscience) was used to detect Rat ICAM-1/CD54, with a sensitivity of 0.19 ng/mL and a detection range of 0.31–20 ng/mL. The Rat IL-6 (Interleukin 6) ELISA Kit (Catalog No: E-EL-R0015, Elabscience) was used to detect Rat IL-6 in samples, with a sensitivity of 7.5 pg/mL and a detection range of 12.5–800 pg/mL. The final absorbance measurements were evaluated using a Bio-Rad ELISA photometer (Model 680).

Results

Phytochemical Screening Results of the BPRCE

The phytochemical screening results for BPRCE using various reagents confirmed that the combination extract of butterfly pea and roselle flowers contains bioactive compounds such as terpenoids, saponins, tannins, and flavonoids. The presence of flavonoids was confirmed through a positive reaction with the Lieberman-Burchard reagent, which produced an intense yellow fluorescence under ultraviolet light, indicating a high content of these compounds. Additionally, a reaction with 2N HCl produced foam, indicating the presence of saponins in the extract. The formation of a brownish ring in the reaction with the Lieberman-Burchard reagent also confirmed the presence of terpenoids. However, the results showed that BPRCE does not contain alkaloids because this compound was undetected. The phytochemical screening results for butterfly pea flower extract (BPFE) and roselle flower extract (RFE) also showed that both extracts contain similar bioactive compounds, namely terpenoids, saponins, tannins, and flavonoids, with no alkaloids detected in either extract.

Administration of BPRCE Reduces VCAM-1 Level in Dose-Dependent Manner

The effect of administering BPRCE on VCAM-1 levels showed that the HFAD group exhibited the highest VCAM-1 levels (98.73±5.92 pg/mL), while the SF group had the lowest (29.42±4.24 pg/mL). Among the groups receiving BPRCE, the highest VCAM-1 level was observed in the CE300 group (57.78±4.15 pg/mL) and the lowest in the CE500 group (32.73±3.57 pg/mL) (Figure 1), showing that BPRCE reduced VCAM-1 level in a dose-dependent manner. A post hoc Tukey HSD result showed significant variation in VCAM-1 levels among the treatment groups (p=0.000). The Tukey test revealed that the CE300, CE400, and CE500 groups had significantly lower VCAM-1 levels than the HFAD group, though there were no significant differences among the CE300, CE400, and CE500 groups. The Na-CMC group had a VCAM-1 level of 89.67±11.63 pg/mL, which was lower than the HFAD group but higher than the other treatment groups. These findings indicate that higher doses of BPRCE, particularly in the CE500 group, may effectively reduce VCAM-1 levels, which is relevant for managing atherosclerosis.

Administration of BPRCE Reduces ICAM-1 Level in Dose-Dependent Manner

The effect of BPRCE on ICAM-1 levels showed that the HFAD group had the highest ICAM-1 levels (19.32±1.43 ng/mL), while the lowest levels were found in the SF group (4.66±1.26 ng/mL). Among the rats treated with BPRCE, the highest ICAM-1 level was in group CE300 (10.25±1.26 ng/mL), and the lowest was in group CE500 (5.68±1.17 ng/mL) (Figure 2), showing that BPRCE reduced ICAM-1 level in a dose-dependent manner. Overall, there was a significant difference in ICAM-1 expression among the treatment groups in white rats (p=0.000). A post hoc Tukey HSD analysis results indicate that ICAM-1 levels in the SF group significantly differed from the HFAD group (p=0.000) and had a mean difference of -73.23250. However, despite a gradual decrease in ICAM-1 levels from group CE300 to CE500, no significant differences were found among the other treatment groups. Administration of BPRCE significantly reduced ICAM-1 levels in atherosclerosis rat models, particularly at the highest dose (CE500). However, differences among the treatment groups were not statistically significant according to the Tukey HSD test.

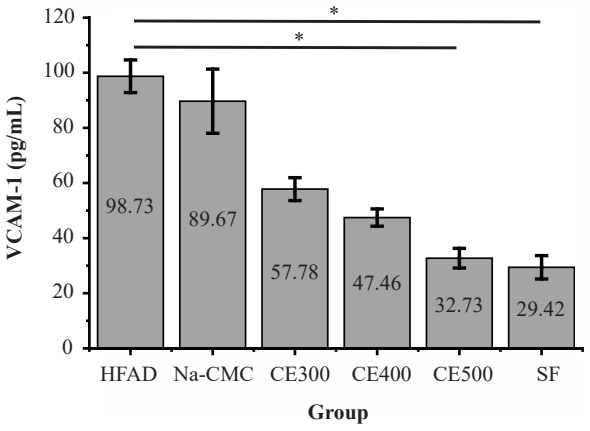

Figure 1. VCAM-1 levels in white rats following BPRCE administration. There are significant differences between HFAD and CE500, as well as HFAD and SF (*p<0.05). Tested using ANOVA, followed by a Tukey post-hoc test.

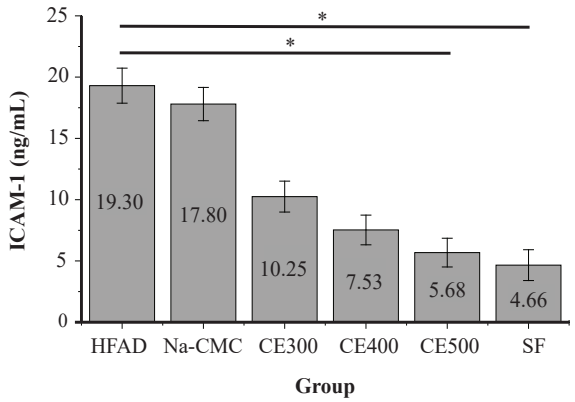

Figure 2. ICAM-1 levels in white Rats following BPRCE administration. There are significant differences between HFAD and CE500, as well as HFAD and SF (*p<0.05). Tested using ANOVA, followed by a Tukey post-hoc test.

Administration of BPRCE Reduces IL-6 Level in Dose-Dependent Manner

The effect of BPRCE on IL-6 levels showed that the HFAD group had the highest IL-6 levels, while the lowest levels were found in the SF group. Among the rats treated with BPRCE, the highest IL-6 levels were observed in group CE300 (53.43±5.12 pg/mL), and the lowest were in group CE500 (21.49±4.62 pg/mL) (Figure 3), suggesting that BPRCE reduced IL-6 level in a dose-dependent manner. A significant difference was observed in IL-6 expression among the treatment groups in white rats (p=0.000). A post hoc Tukey HSD analysis results indicate that IL-6 levels in the SF group significantly differed from the HFAD group (p=0.000) and a mean difference of -72.71750. However, despite a gradual decrease in IL-6 levels from group CE300 to CE500, no significant differences were found among the other treatment groups.

Discussion

The phytochemical screening results confirm that BRECE is rich in compounds with potential pharmacological activities, including antioxidant, anti-inflammatory, and antimicrobial properties. While, both BPFE and RFE also contained similar bioactive compounds, terpenoids, saponins, tannins, and flavonoids, with no alkaloids. These results have been confirmed by previous studies, which demonstrated that both BPFE and RFE contain bioactive compounds such as terpenoids, saponins, tannins, and flavonoids.(28–30) These findings underscore its potential to serve as a foundation for natural plant-based products, offering reassurance in its potential health benefits.(26,28,30)

The administration of BPRCE to white rats over 45 days significantly reduced VCAM-1 levels in the treatment groups (CE300, CE400, and CE500) compared to the HFAD group. This reduction became more pronounced with higher doses of BPRCE (CE500), supporting previous studies that showed higher doses of rosella extract lower VCAM-1 levels in white rats.(30) Flavonoids in BPRCE, with anti-inflammatory and antioxidant properties, are believed to play a vital role in this reduction by inhibiting enzymes involved in arachidonic acid metabolism and reducing NF-kB activity and LDL oxidation.(31,32) These findings strongly suggest that flavonoids contribute to lowering VCAM-1 levels associated with inflammation and cardiovascular disease risk (33), highlighting the potential of BPRCE as a future treatment.

The study on the effects of BPRCE administration on ICAM-1 levels in white rats revealed significant findings. One Way ANOVA showed a marked difference in ICAM-1 levels between the control groups (SF and HFAD) and the treatment groups (CE300, CE400, and CE500), with ICAM-1 levels decreasing as BPRCE dosage increased. This reduction is attributed to the powerful antioxidant properties of rosella and butterfly pea flower flavonoids, which act as anti-inflammatory agents by inhibiting reactive oxygen species (ROS) and reducing ICAM-1 activity. (34) Flavonoids also inhibit Acyl-CoA acyltransferase and Microsomal Triglyceride Transfer Protein, enzymes involved in cholesterol synthesis, supporting their potential for cholesterol-related treatments.(35–38)

The 45-day administration of BPRCE to white rats showed significant reductions in IL-6 levels in the treatment groups (CE300, CE400, and CE500) compared to the HFAD groups. Notably, IL-6 levels decreased progressively

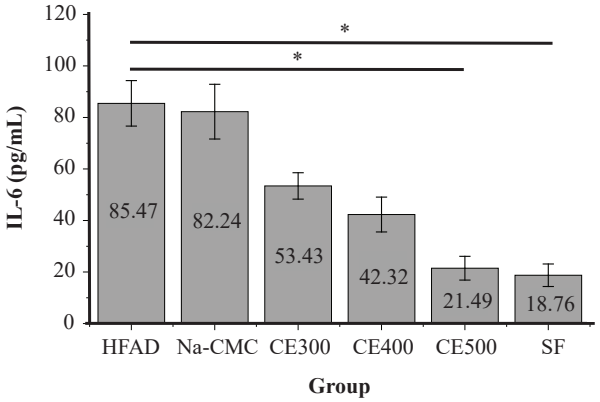

Figure 3. IL-6 Levels in White Rats Following BPRCE Administration. There are significant differences between HFAD and CE500, as well as HFAD and SF (*p<0.05). Tested using ANOVA, followed by a Tukey post-hoc test.

at higher doses, underscoring the effectiveness of the treatment. This reduction aligns with previous studies indicating that increased rosella and butterfly pea extracts lower IL-6 levels, potentially aiding cardiovascular health.(25,39) The decline is attributed to flavonoids in both plants, which act as antioxidants by scavenging free radicals and preventing LDL oxidation, thus reducing IL-6 levels and supporting cellular protection.(22,40)

While this study shows promising results, it highlights the need for further research. The 45-day duration may need to be increased to assess long-term effects and safety, and testing only specific doses (300, 400, and 500 mg/kg) leaves the efficacy of other doses unknown. These limitations suggest more comprehensive studies to better understand BPRCE's benefits and risks. The findings encourage further investigation into BPRCE's potential and instill hope for developing standardized herbal therapies for CHD prevention, raising public awareness of its health benefits, and encouraging its dietary inclusion.

Healthcare practitioners may use these findings to develop holistic, plant-based treatment programs for heart disease prevention, promoting natural alternatives. The promising results encourage future research on BPRCE's long-term effectiveness, interactions with standard treatments, and potential combinations with other herbs. Such studies could offer safer natural options that reduce the dependence on synthetic medications, as well as enhancing quality of life.

Conclusion

Administration of BPRCE for 45 days in white rats reduced atherosclerosis biomarkers (VCAM-1, ICAM-1, and IL-6), with the optimal effect observed at a dosage of 500 mg/kg body weight. This results suggest that the BPRCE has significant potential in preventing and treating CHD due to its flavonoid content, which has antioxidant and anti-inflammatory properties. Therefore, BPRCE might prevent inflammation associated with CHD, highlighting its potential as a natural therapeutic option.

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Authors Contribution

KH and AHR contributed to the conception and design of the research. RS and BA carried out data acquisition and collection. KH and II performed data calculations and analysis. RGS, II and SW assisted in interpreting the results. KH and AHR prepared the manuscript and designed the tables. All authors participated in critically reviewing and revising the manuscript.

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