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BETEL LEAF EXTRACTS (PIPER BETLE L.) PREVENT ENDOTHELIAL DYSFUNCTION BY REDUCING THE LEVELS OF URIC ACID AND ICAM-1 EXPRESSION OF THE HYPERURICEMIA WISTAR RATS (RATTUS NORVEGICUS) AORTIC ENDOTHELIAL

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ABSTRACT
Hyperuricemia as a risk factor for cardiovascular disease causes endothelial dysfunction by increasing oxidative stress and inflammation. Extracts of betel leaf (*Piper betle L.*) contain bioactivity as an antioxidant, anti-inflammatory and XO inhibitor. The aim of the research is to determine the betel leaf extracts can prevent endothelial dysfunction caused by hyperuricemia by lowering uric acid levels and the expression of ICAM-1 endothelial aorta. The experimental research was conducted by the design of The Post Test Only Randomized Control Group Dsign, performed on untreated animals and rats fed with oxonic potassium (hyperuricemia), and compared to hyperuricemic rats treated with either betel leaf extract or allopurinol. After the experiment, the blood uric acid levels and the expression of aortic endothelial ICAM-1 were determined. The research results showed that the betel leaf extract lowered significantly ($p < 0.05$) the blood uric acid levels and the expression of ICAM-1, rat aortic endothelial hyperuricemia. There is a positive correlation between the levels of uric acid with the expression of aortic endothelial ICAM-1 ($p < 0.05$). It can be concluded that the betel leaf extracts can prevent endothelial dysfunction caused by hyperuricemia by lowering the uric acid levels and the expression of ICAM-1 aortic endothelial.

Keywords: Piper betle L., Endothelial Dysfunction, Oxidative Stress, Hyperuricemia and ICAM-1.

INTRODUCTION

Hyperuricemia is a precipitating factor of gout and kidney stones as well as the risk factors for metabolic syndrome and cardiovascular disease.[1] As a risk factor for cardiovascular disease, hyperuricemia affects endothelial function through conventional risk factors of oxidative stress, inflammation, and decreased nitric oxide (NO) that contribute to initiate the development of atherosclerosis.[2]

Hyperuricemia cause oxidative stress with increased formation of reactive oxygen species (ROS) through enzymatic oxidation system with enzyme NADPH-oxidase, xanthine oxidase (XO) and endothelial nitric oxide synthase (eNOS). In the reaction system with NADPH oxidase uric acid activates NADPH oxidase resulting in increased production ROS[3]. This system is reckoned to be the main controller and the O_2^- formation as the inducer is angiotensin II[4,5,6]. The reaction system with XO, catalyzes the reaction hypoxanthine and xanthine to uric acid involves the transfer of electrons to oxygen molecules to form H_2O_2 and O_2^- (ROS).[6] While the reaction system with eNOS, uric acid inhibits eNOS in synthesizing nitric oxide (NO) endothelium, by inhibiting the absorption of arginine aorta and activate arginase, arginine deficiency causes eNOS uncoupling with BH_4 prosthetic group, resulting in the transfer of electrons to oxygen to form ROS.[7]

In a state of oxidative stress, endothelial function is dysfunctional, its cells increase the expression of adhesion molecules such as intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1) to recruit inflammatory cells on the blood vessel walls to facilitate interaction endothelial-leukocyte. The expression of adhesion molecules is controlled by the transcription regulatory proteins of NF-$_\kappa$B.[8,9]

In hyperuricemia, uric acid can also be absorbed into the vascular smooth muscle cell (VSMC) causes proliferation of the cells and secondary atherosclerosis[10] that stimulate the formation of monocyte chemoattractant protein-1 (MCP-1), inflammation and increase cyclo-oxygenase 2 (COX-2) to thromboxane form, through the activation of

nuclear transcription factor (NF-κB), and mitogen-activated protein kinases (MAPKs).[11,12]

Betel leaf (*Piper betle* L.) has been used as traditional medicine for centuries. The use of betel leaves as traditional medicine is promising, causing a lot of chemical and biological research done on its extracts. The study results revealed that, betel leaf extract has activity as antimutagenic, anticarcinogenic, antidiabetic, anti-inflammatory and anti-bacterial activities.[13,14,15] It has been shown that the fractionation and pure compounds from betel leaf extract, has antidiabetic activity, cardiovascular, anti-inflammatory, antioxidant, and anti platelet aggregation.[16] Besides, betel leaf extract can also inhibit the enzyme xanthine oxidase (XO).[15]

Based on the above, and the results of research, it is estimated that the betel leaf extract can prevent / inhibit endothelial dysfunction caused by hyperuricemia by lowering uric acid levels and the expression of ICAM-1 aortic endothelial.

METHODS

Fresh betel leaf (*Piper betle L*.) were collected from farmers in the village of Senganan Tabanan, Bali, sanitized and air dried for 3-5 days in the shade, and then finely ground. Weighed 1 kg of macerated with 96% ethanol for 72 hours at room temperature (28-30^o C). The extract was filtered and the filtrate is collected and then the residue was macerated again with 96% ethanol for 72 hours at room temperature until the filtrate is colorless. The filtrate is collected together and evaporated to dryness with a rotary evaporator at 40 ° C (yield 86.96 g) and stored at -20^o C until it was used.[13,17]

Wistar rats (Rattus norvegicus) males aged 10-12 weeks weighing 200-250 g was obtained from the Laboratory of Animal Unit, Section of Pharmacology of the Faculty of Medicine of Udayana University. Housed in standard environmental conditions and fed and watered *at libitum*.

The chemicals used were of analytical grade unless otherwise stated in another that is 96% ethanol, oxonic potassium and allopurinol (from Sigma-Aidrich USA), 0.5% CMC solution, Uric Acid Assay Kit, and materials for the immunohistochemical expression of ICAM-1 aortic endothelial such as: 10% formalin, citrate buffer (PBS) 0.1 M (pH 7.4), LSAB kit (Dako, Denmark) and primary antibody Rabbit Anti-ICAM-1 polyclonal antibody (Bioss, Cat. Bs-0608R) as well as other materials from Sigma-Aldrich (USA) such as methanol p.a, 0.25% trypsin, 3% H_2O_2, 10% phosphate buffered formalin, 70%, 90%, 96%, 100% alcohol, xylene, liquid pure paraffin, PBS, 5% FBS, Biotinilatid Coat Anti-polyvalent, Streptavidin peroxidase, aquabidest, Gill hematoxylin and trisodium citrate buffer.

The research was conducted with true experimental method by the Randomized Post-test Only Control Group Design. The protocol followed the instructions and approval of the Animal Care Committee of the Faculty of Veterinary Medicine of Udayana University. The experiments used 24 Wistar rats that were divided into 4 groups respectively of 6 rats (n = 6). (1) the normal group were fed a standard feed / pellet (50 g / kg bw / day) and CMC 0.5% as Vehicle. (2) the negative control group (hyperuricemia / oxonic potassium) were fed a standard feed / pellet (50 g / kg bw / day) and oxonic potassium 750 mg / kg bw / day. (3) the positive control group (oxonic potassium + allopurinol) were given a standard feed pellets (50 g / kg bw / day), oxonic potassium 750 mg / kg bw / day and allopurinol 5.0 mg / kg bw / day. (4) The treatment group with betel leaf extract (oxonic potassium + betel leaf extract) were given a standard feed pellets (50 g / kg bw / day), oxonic potassium 750 mg / kg bw / day and betel leaf extract 300 mg / kg bw / day.

At the end of the experiment (7 weeks) the rat blood was collected through the retro-orbital plexus of rats by using micro hematocrit tubes, for specified levels of uric acid by the method of spectrophotometry using Uric Acid Assay kit (Sigma-Aldrich, USA) until obtaining the data of uric acid levels in the blood of rats in mg / dL. Then the rats were killed by injecting i.m. using a 30 mg / kg bwxylazine, and it was followed by laparotomy to take their aorta to determine the expression of ICAM-1 endothelial by immunohistochemical methods using kit techniquesof LSAB (Dako, Denmark) and primary antibody Rabbit Anti-ICAM-1 polyclonal Antibody (Bioss, Cat. Bs-0608R) to obtain the data of ICAM-1 expression of rats' aortic endothelial in the form of the scoring average number of endothelial cells in the visual field that express ICAM-1 positive (cells / visual field) of 5 visual field of 30 μm that was observed in the rats' aortic tissues.

The data of blood uric acid levels and the score of the number of positive aortic endothelial cells which express ICAM-1 is shown in mean ± SE and statistically analyzed by One-way ANOVA and followed by Post-hoc analyzes using LSD to compare between groups. Correlation analysis of Pearson Product Moment was conducted between the blood uric acid levels with ICAM-1 expression of rats' aortic endothelial.

RESULTS

After 7 weeks of administration of oxonic potassium experiment, it significantly increases the rats' blood uric acid levels compared to the normal rats (3.467 ± 0.3018 vs. 1.983 ± 0.1682 mg / dL, $p < 0.05$, n = 6). When the administration of oxonic potassium jointly given with allopurinol or betel leaf

extract, the increase was actually prevented or reduced (3.467 ± 0.3018 vs. 1.833 ± 0.1542 or 1.950 ± 0.1384 mg / dL, p <0.05, n = 6) (Table 1). The administration of oxonic potassium also increased the expression of ICAM-1 aortic endothelial although it was not significant compared with the normal rats (13.667 ± 1.5377 vs. 11.267 ± 1.2293, p> 0.05, n = 6) (Table 1). When the administration of oxonic potassium jointly given with allopurinol or betel leaf extract, it significantly decreased the expression of ICAM-1 rat aortic endothelial (13.667 ± 1.5377 vs. 7.967 ± 1.0462 or 8.567 ± 1.144, p <0.05, n = 6) (Table 1).

There is a positive correlation between blood uric acid levels in rats with ICAM-1 expression of rat aortic endothelial (r_{count} = 0.460 (*)> r_{table}= 0.404 at α = 0.05 and n = 24 or p <0.05).

Table 1. The Mean (± SE) of Rat Blood Uric Acid Levels and ICAM-1 Expression of Rats'Aortic

Group of Treatment Rats	Mean of Uric Acid level (mg/dL)	Mean of Cell Score ICAM-1 Expression
N(Untreated)	1.983 ± 0.1682[a]	11.267 ± 1.2293[ab]
C- (Hyperuricemia /OP)	3.467 ± 0.3018[b]	13.667 ± 1.5377[a]
C+ (OP + All)	1.833 ± 0.1542[a]	7.967 ± 1.0462[b]
TE (OP + BLE)	1.950 ± 0.1384[a]	8.567 ± 1.1447[b]

Notes: 1. The average value with the same letter in the same column shows insignificant differences (p> 0.05).
2. The average value with different letters in the same column indicates significant difference (p <0.05)
3. N = Normal, C- = Control Negative, C+ = Control Positive, TE = Treatment with Extract, OP = Oxonic Potassium, All = Allopurinol, BLE = Betel leaf extract

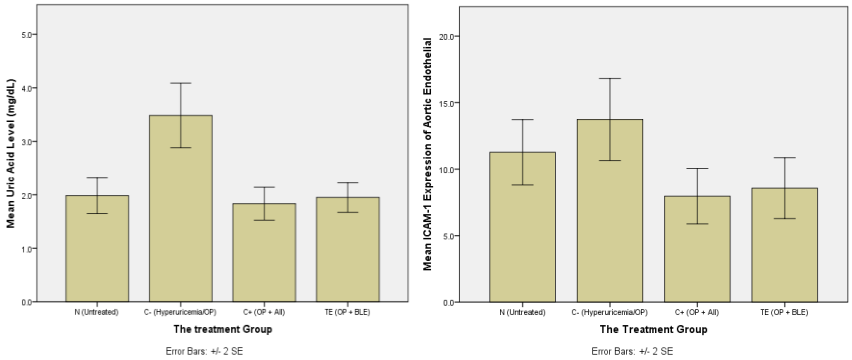
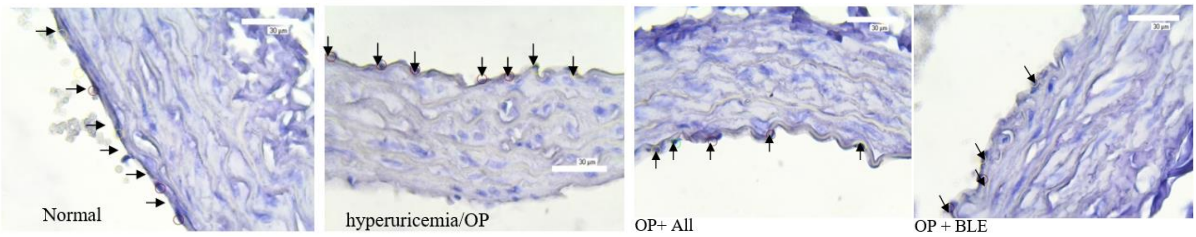


Figure 1. Uric Acid Levels (A) and ICAM-1 Expression of Aortic Endothelial (B) in The Various Treatment Group Notes : N = Normal, C- = Control Negative, C+ = Control Positive, TE = Treatment with Extract, OP = Oxonic Potassium, All = Allopurinol, BLE = Betel leaf extract.



Notes : OP = Oxonic Potassium, All = Allopurinol, BLE = Betel leaf extract

Figure 2. Positive Aorta Endothelial Cells Expressing ICAM-1 Marked with Arrows (→) on Normal Group, hyperuricemia / OP, OP + All, and OP + BLE.

DISCUSSIONS

The results showed that oxonic potassium significantly (p <0.05) can increase blood uric acid levels in rats. These results are consistent with results of previous studies[7,18]. These results indicate that oxonic potassium (OP) 750 mg / kg bw / day can induce hyperuricemia in the rats because oxonic potassium is an enzyme inhibitor of uricase / urate oxidase that has been used widely to increase the uric acid levels in rats. When oxonic potassium was administered jointly with allopurinol or the betel leaf extract, it significantly (p <0.05) lowered the blood uric acid levels in rats (Table 1). These results are also consistent with the results of previous studies.[19,20,21]

Betel leaf extract can lower the blood uric acid levels of rats (as an enzyme inhibitor of XO),

because the betel leaf extract contains phenol compounds particularly eugenol that can inhibit the enzyme activity of XO in the oxidation reaction of hypoxanthine into xanthine and oxidation of xanthine to uric acid, in the degradation pathway of purines into uric acid, so the buildup of uric acid can be reduced.

The administration of oxonic potassium also increased the expression of ICAM-1 aortic endothelial compared to normal rats, although not significantly ($p > 0.05$). But the administration of oxonic potassium jointly with allopurinol or betel leaf extract can actually decrease significantly ($P < 0.05$) the expression of ICAM-1 rat aortic endothelium. This result is also supported by the positive correlation between the levels of uric acid with the expression of ICAM-1 rat aortic endothelial ($r_{count} = 0.460$ (*)$> r_{table} = 0.404$ at $\alpha = 0.05$ and $n = 24$ or $p < 0.05$). This is due to allopurinol or betel leaf extract can reduce levels of uric acid that is also expected to decrease ROS, the expression of IL-1β and TNFα that can stimulate the expression of ICAM-1.[22,23] Besides, uric acid also activates the transcription factor of NF-$_K$B[11,12], which acts to control the expression of ICAM-1.[8,9]

Under stress, oxidative endothelial function will be dysfunctional, its cells increase the expression of adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule (VCAM-1)[8,9] so that ICAM-1 as one biomarker of endothelial dysfunction.[24,25]

CONCLUSIONS

Based on the results of research and discussion, it can be concluded that the betel leaf extract can prevent / inhibit the endothelial dysfunction by lowering the blood uric acid levels and the expression of ICAM-1 of the rat aortic endothelial hyperuricemia.

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