ABSTRACT

Aim To investigate effect of bay leaf extract in endothelial integrity, observed by vascular endothelial growth factor (VEGF) level, VEGF and CD31 expression.

Methods Thirty-two acute coronary syndrome surgery-induced Wistar Rats (Rattus novergicus) were divided into 16 bay leaf extract (treatment) groups and 16 control groups, sacrificed on day 1, 4, 7, and 14 after the induction. Serum VEGF level was determined by ELISA and expression of VEGFR-2 and CD31 were detected on immunohistochemistry.

Results This study showed increased expression of serum VEGF level, and VEGFR-2 expression was found significantly on day 7 and 14 in the treatment group compared to the control group. CD31 expression was significantly different compared to the control groups on day 4, 7, and 14 of administration.

Conclusion The potential effect of bay leaf extract on angiogenesis in acute coronary syndrome (ACS) as adjuvant for the treatment. Bay leaf extract has been shown to support angiogenesis and maintain endothelial integrity that leads to better prognosis for reperfusion on ischemic tissue.

Key words: ischemia, flavonoids, herbal medicine
INTRODUCTION

Cardiovascular disease is projected to be the single leading cause of death. The World Health Organization (WHO) stated that by 2030, almost 23.6 million people would suffer from CHDs, mainly from heart disease and stroke (1). As an ever-growing developing country, Indonesia faces an increasing trend of non-communicable disease (NCD) cases (2). This explains the fact published by the Ministry of Health of the Republic of Indonesia in 2018 that stated coronary heart disease (CHD) as one of the diseases with the highest prevalence in Indonesia, leading to the second highest number of deaths in Indonesia (3). As the highest cause of mortality, more studies are needed to investigate further on the pathogenesis of CHD, specifically on interventions needed for the prevention of CHD cases and/or the progression of the disease.

One of the important parts of CHD pathogenesis is arteries’ supply blockage caused by atherosclerosis (4). The progression of atherosclerosis begins in childhood or adolescence to progress over time. Atherosclerosis would accumulate to the point of shear stress as its essential feature (5). This would correlate to the level of endothelial shear stress (ESS), as low ESS induces inflammation (tribute to its potent proinflammatory profile) and signals atherogenesis process (6). These processes are associated with the development and progression of coronary atherosclerosis (7). In response, this condition further upregulates the expression of potent vascular smooth muscle cells (VSMC) mitogens, including growth factors such as vascular endothelial growth factor (VEGF) (8). The expressions of these factors are amplified with the ongoing formation of reactive oxygen species (ROS) and pro-inflammatory cytokines signalled from low ESS (9). In the condition of shear stress, mechanosensors play an important role. A study by Tzima et al. has identified a mechanosensory complex in atherosclerotic plaque pathophysiology consists of CD31, vascular endothelial–cadherin (VE-cadherin) and vascular endothelial growth factor receptor-2 (VEGFR2) that responded towards external shear stress (10), with regards to VEGF receptor 2 (VEGFR2) as the principal receptor of VEGF in blood vessels (11).

Platelet endothelial cell adhesion molecule (PECAM-1), known as a cluster of differentiation 31 (CD31), is considered as one of the most important mechanosensors, which is highly expressed at endothelial cell junctions. In thrombotic process that challenges the shear system, CD31 is responsive for direct transduction of mechanical forces, not only in the macrophages but also in the endothelial cells (10). CD31 signals to regulate all critical aspects related to the pathogenesis of atherosclerosis: from leukocyte detachment, T-cell activation, platelet activation, to angiogenesis (12). Corresponding to VEGF level, high CD31 expression is related to its function to protect vascular endothelial barrier integrity (13). A study by Xia has proven that low ESS promotes expression of CD31 in vivo, in atherosclerotic rat models (14). The study was done by constricting abdominal aorta; and opportunities are open to assess whether the result is analogous to occur on coronary arteries or acute coronary syndrome-induced models in vivo. Serum VEGF levels, VEGFR-2 and CD31 expressions in heart tissue were chosen as inflammatory markers in this study.

Indonesia has great resources of plants biodiversity, due to its geographic location along Equator line. Indigenous people residing in Indonesia have consumed traditional herbs for health. Empirically, bay leaf (Syzygium polyanthum) or salam leaf (in Bahasa Indonesia) stew was used as home remedy for hypercholesterolemia, diabetes mellitus, and hypertension. Bay leaf contains flavonoid (15), which is a natural antioxidant polyphenol compound and proven to protect arterial endothelium by preventing damage of the cells and reducing cholesterol deposition on the surface of the arterial endothelium (16,17). In response to the shear stress, VEGF and CD31 are upregulated to secure endothelial integrity. A study published by Bassino proved that flavonoid promoting VEGFR2 phosphorylation was significantly increased (18); specifically, the study proved it occurs only by stimulation with HSP (Hesperidin), one example of flavonoid compound.

The aim of this experimental study was to demonstrate protective effect of bay leaf extract on endothelial integrity on rat model with myocardial infarction. Hypothesis of the study accentuates that the bay leaf administration would target the atherogenesis by targeting mechanosensory pathway, which involves CD31 and VEGFR2 expressions.
MATERIALS AND METHODS

Sample and study design
This study involved thirty-two Wistar rats (*Rattus norvegicus*) weighing around 200 grams, 10-12 weeks of age. Rats were divided into two groups consisting of sixteen rats each, namely treatment and control groups. Rats were housed in steel cage given free access to self-feed and conditioned with 12 hours dark/light cycle at temperature of 20-25 °C. Samples resided in the Animal House, Research Laboratory, School of Medicine, Universitas Brawijaya, Malang, Indonesia. All procedures were conducted in December 2019. The institutional Ethics Committee of Universitas Sumatera Utara has approved procedures and interventions conducted in this study.

Methods

Surgical induction of myocardial infarction. To induce samples with acute coronary syndrome, coronary arteries of rats were ligated, according to a study by Wu et al. (19). Ketamine was given as an anaesthetic agent. Samples from both groups underwent thoracotomy followed by ligation of anterior descending (LAD) artery, where sign of infarct is proven by blanching in myocardium presented in distal of the ligation site. The surgical wound was later sutured.

Administration of bay leaf (*Syzygium polyanthum*) extract. An extract of *Syzygium polyanthum* was prepared by maceration in the Research Unit laboratory, School of Medicine, Universitas Brawijaya, Malang, Indonesia. The suspension was given in a dose of 3.6 mg (0.72 mL in volume), administered via orogastric tube.

Sample collection. Samples were sacrificed on day one, four, seven, and fourteen after administration. Blood samples were collected from the coronary artery of all rats and followed with surgical removal of the heart. Blood samples were subjected to measure serum VEGF level, heart specimens were fixated using formaldehyde buffer solution of 10% for 24 hours, paraffin embedded then cut into four to six microns thick.

VEGF ELISA. Blood samples were centrifuged at 1500 g at room temperature. Collected supernatant was assessed using Rat VEGF-A (Vascular Endothelial Cell Growth Factor A) ELISA Kit (Elab Science, US). With sandwich-ELISA principle, the micro plate wells had been pre-coated with an antibody specific to Rat VEGF-A. After samples were added to the wells and combined with the specific antibody, a biotinylated detection antibody specific for Rat VEGF-A and avidin-horseradish peroxidase (HRP) conjugate were added to each well and incubated. Free components were washed away, then substrate solution was added. Wells containing Rat VEGF-A, biotinylated detection antibody and Avidin-HRP conjugate appeared blue in colour. The enzyme-substrate reaction was halted by adding the stop solution when the colour of the solution in wells turned yellow. The optical density (OD) value was measured at the wavelength of 450 nm. The OD value was proportional to the concentration of VEGF level. Therefore, the concentration of VEGF level was calculated as a result of OD value of the samples compared to the standard curve.

Immunohistochemistry staining of VEGFR-2 and CD31. The tissue specimens were embedded by paraffin and cut into four to six micron of thickness using microtome. Slides were deparaffinised, rehydrated, and then heated on microwave with EDTA antigen repair solution for 20 minutes. Slides were left to cool by putting it in cold water, and rinsed three times using phosphate buffered saline (PBS), pH 7.4, each 3 minutes long. Hydrogen peroxide 3% solution was incubated for 15 minutes to halt endogen peroxidase activity, and then the slides were rinsed. Primary antibody was detected by biotinylated detection antibody and avidin-conjugated horseradish peroxidase (HRP) from then rinsed. Anti-VEGFR-2 (sc-6251, Santa Cruz Biotechnology, USA) and Anti-CD31 (sc-376764, Santa Cruz Biotechnology, USA) were added at room temperature for 20 minutes, then rinsed. A signal was detected using diamino-benzidine (DAB) solution. The slides were then counterstained with hematoxylin followed by histological slides examination by two blinded pathologists. Final slides score was calculated as a result of the positive cells score multiplied by intensity of staining. Positive cells were scored as (0) for less than 10% cells stained, (1) for 10 – 25% stained, (2) for 25 – 50% stained, (3) for 50 – 75% stained, and (4) for more than 75% cells stained. Intensity of the staining was categorized into (1) weak, (2) moderate, and (3) strong intensity.
Statistical analysis

Samples fit to normal distribution were assessed using independent samples t-test, to compare serum VEGF level and expressions of VEGFR-2 and CD-31 between the treatment and control groups. If data were not normally distributed, data would be compared by using Mann-Whitney U-test. P value under 0.05 indicated statistical significance.

RESULTS

Thirty-two drug-naive rats, weighing in average 200 gr, were divided into two experimental groups. Serum VEGF level, immunohistochemistry scoring of VEGFR-2, and CD31 were compared between the treatment group to the control group on day one, four, seven and fourteen of bay leaf extract administration. Higher serum VEGF levels in the treatment group on day one, seven and fourteen days compared to the control group were found (Table 1).

<table>
<thead>
<tr>
<th>Day</th>
<th>Serum VEGF level in the group (mean, SD)</th>
<th>Control</th>
<th>Treatment</th>
<th>Mean difference (95% CI)</th>
<th>df</th>
<th>p</th>
</tr>
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<td></td>
<td>38.98</td>
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<td>49.39</td>
<td>59.91</td>
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<td>-2.57</td>
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<td>65.32</td>
<td>-9.97</td>
<td>-4.86</td>
<td>(-15.31, -0.47)</td>
</tr>
</tbody>
</table>

Table 1. Serum vascular endothelial growth factor (VEGF) level between the control and the treatment group

Both slides of VEGFR-2 and CD31 expression showed clear contrasting results since the first day of administration, presented in brown-stained cells. There was a significantly higher immunohistochemistry score of VEGFR-2 in the treatment group compared to the control group on day seven (p=0.010), and fourteen (p=0.011) (Figure 3).

Slides were examined for the expression of VEGFR-2 (Figure 1) and CD31 (Figure 2).

DISCUSSION

This study found higher serum VEGF levels in groups with one, seven and fourteen days of bay leaf extract administration. Serum VEGF level is elevated in acute phase of ACS and therefore
has the potential to be the marker of myocardial injury (13). Increased VEGF expression in myocardium after acute onset correlates to both of reperfusion in ischemic area and evolving progression of the ACS (19). This pattern could be explained as VEGF expresses differently to infarcted myocardium, noninfarcted myocardium, and the border zone. As the ischemia process undertakes, VEGF expression would reach its peak differently: 2 hours after onset at infarcted myocardium, 12 hours after onset at border zone, while at noninfarcted myocardium it remains unchanged (20). This explained the significant difference even in the first day of administration.

To date, there is no sufficient research to reach a consensus of certain VEGF level as a benchmark (21). A study found high VEGF level and expression associated to well-developed coronary collateral arteries in which improved survival in patients with coronary artery disease (22) as increased VEGF expression has a potential role in cardiac repair following the acute onset of myocardial infarction (20). Prolonged expression afterwards is resulted after HIF-alpha identifies hypoxia in border zone, as VEGF expression is needed for angiogenesis meant to supply collateral vessels. If the area of ischemic is large enough, it needs more time to build adequate density of vascular collateral to supply affected area. This co-expression may be consistently different up to 14 days as shown in this study, supported by previous studies (23,24), noting serum VEGF levels elevated gradually and reaching a peak on day 14 after onset of acute myocardial infarction. VEGFR2 expressions showed consistent results with serum VEGF levels of increased expression after bay leaf administration, considering the role of VEGF-A/VEGFR2 signal-dependent angiogenesis pathway (25). Our results suggest that VEGF and its receptor pathways may be responsible for outcome range shown in this study.

To further understand ACS progression, it is important to measure both VEGF functions of as angiogenesis regulators and their anti-angiogenic isoform that inhibits angiogenesis, feasibly through its receptors (26). Overexpression of VEGF must be avoided as it may worsen the clinical manifestation rather than support the improvement of ischemic tissues (27). CD31 expression confirmed improved neovascularization within the infarct border zone, thereby contributing to protection of nonischemic areas (28). This study is concordant with a previous study, which found increased CD31 concentration in patients with coronary artery stenosis (29). The study by Serebruany et al. stated that CD31 level in plasma and platelet were observed to increase to peak of 3 hours after acute onset and followed by a significant decrease later at 24 hours (30). Platelet CD31 was known to decline early and rise as the second phase of ‘re-expression’ takes place and this may explain the significant difference in our study, as proven in our study by the result of day seven and fourteen. Co-expression of CD31 reflects that myocardial reperfusion takes place and the expression was concentrated from the affected area. However, high expression may also result as the effect of angiogenesis itself, which is not distinguished in this study. Angiogenesis related to increased expression of CD31 has also been shown in a previous study in rat model, proving that CD31 expression correlates with hypoxia inducible factor-1 alpha (HIF-1α) that responded to ischemic tissue (31).

Flavonoid compound, especially quercetin, has proven to improve prognosis in rat models with ischemic brain injury. The study was done in *C. argagana sinica* (32), which shares the same property of quercetin with *Syzygium polyanthum*. In case of acute onset of infarction, nitric oxide (NO) level is upregulated in response to ischemia, as well as being a starter for reperfusion. At certain NO concentration, it activates cNOS enzyme to start reperfusion. By modulating HIF-1 alpha in the pathway, quercetin upregulates VEGF expression in ischemic condition (33). Overconcentration of NO responds to high ROS, which contributes to ischemia-reperfusion injury. Flavonoid, given as adjuvant, let reperfusion occur in myocardium but avoid the injury, as it directly scavenge excessive harmful reactive species (34). Quercetin also induces upregulation of CD31 (32). This is concordant to the result of the study, which shows an increased CD31 expression after bay leaf extract administration. An earlier study published by Flego at al. found that an increase in CD31 expression had protective effect against helper T cell dysregulation and lymphocyte overreaction observed during acute onset (25).
Effective dosage of bay leaf extract or flavonoid active compound may alter changes in CD31 expression and serum VEGF level; this is within the limitation of this study. More samples may present clearer results within each group. Further studies are needed to evaluate dose-dependent and active flavonoid compound from bay leaf extract to seek beneficial effects of bay leaf on endothelial integrity and angiogenesis post myocardial infarction. Expressions of VEGF-related angiogenesis may be done at different place and time, considering that different areas of infarction express VEGFR-2 and CD31 differently.

In conclusion, this study shows the potential effect of bay leaf extract on angiogenesis in ACS as adjuvant for the treatment. Bay leaf extract has been shown to support angiogenesis and maintain endothelial integrity that leads to a better prognosis for reperfusion on ischemic tissue.

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**TRANSPARENCY DECLARATION**

Conflict of interest: None to declare

**REFERENCES**


