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Original Article

In vivo study of cardioprotective effect of bay leaf (*Syzygium polyanthum*) extract

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A B S T R A C T

Background: The excessive use of chemotherapy drugs such as doxorubicin might induce cardiovascular diseases. Heart-specific biomarker enzymes such as LDH and BNP indicated the early signs of cardiotoxicity. However, there is no report on the effect of bay leaf on LDH and BNP nor its bioactive compounds and histopathology analysis.

Objective: This study aims to determine the cardioprotective effect of Ethanol Extract of Bay Leaf (EEBL) based on phytochemical analysis, LDH and BNP levels, and cardiac histology of rats administered with doxorubicin.

Methods: About 24 Wistar rats were divided into six groups treated with 100 mg/kg BW, 300 mg/kg BW, and 500 mg/kg BW of EEBL with CMC-Na 0.5 %, vitamin E, and doxorubicin. Preconditioning was 14 days, followed by 21 days of intraperitoneal administration. LDH and BNP parameters were measured on days 1st, 7th, 14th, and 20th. Histology analysis was conducted on day 21st.

Results: Dark green EEBL showed sufficient physical quality and properties for practical application in pharmacy. EEBL has significantly affected LDH, BNP, and cardio recovery dose-dependent. The most effective doses were observed at 500 mg/kg BW, and its performance is not statistically different from those of the commercial vitamin E. The histopathological images revealed significant improvements in interstitial edema, wavy fibers, hemorrhage, intracellular vacuole, and inflammatory cell infiltration. Here we report six bioactive compounds obtained from EEBL have cardioprotective effects; Neophytadiene, squalane, phytol, methyl palmitate, stigmasterol, and 9,12-Octadecanoic acid methyl ester.

Conclusion: This study has shown the promising potential of bay leaf extract as a cardioprotector with sufficient quality pharmacological standards.

INTRODUCTION

Cardiac diseases such as ischemic cardiomyopathy, reperfusion injury, and cardiac infarction may be related to administering anthracyclines drugs such as doxorubicin during chemotherapy.[1] Doxorubicin could trigger the formation of free radicals and decrease endogenous antioxidants, which result in cardiomyocyte apoptosis.[2] The mechanism of doxorubicin inducing cardiomyocyte apoptosis is proposed by intercalation of the DNA and topoisomerase II inhibition.[3] Cardiac mitochondrial damage is supposed to occur after a few hours following the revelation of doxorubicin.[4] Doxorubicin-induced cardiotoxicity is related to several signaling pathways. Oxidative stress has been implicated in several cases of doxorubicin-induced cardiotoxicity. Therefore, the use of antioxidants is an effort to prevent damage to the myocardium.[5]

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Medicinal plants have been known for their protective effects, encouraging people to consume them as health supplements.[6–8] A study reported that *Oroxylum Indicum* extract protects against cardiotoxicity caused by doxorubicin and cyclophosphamide by inhibiting ROS-mediated apoptosis and blocking the impact of tyrosine hydroxylase,[9] and by restoring redox balance.[10] Another study has reported ten sesquiterpenes extracted from *Laurus nobilis* leaves. Most of these compounds were moderate to significantly hazardous to K562 leukemia cells, with one molecule being even more toxic than doxorubicin.[11] Another study discovered that chia seed oil protects female Wistar rats from doxorubicin-induced cardiotoxicity.[12]

Since 1955, Lactate Dehydrogenase (LDH) has been considered a useful biomarker in diagnosing acute coronary syndrome.[13] LDH has five isoenzymes, with LDH-1 being the most abundant in the heart. Within 6-12 hours following the start of chest discomfort, LDH-1 levels in the blood rise. It reaches its height in 1-3 days and gradually returns to normal in 8-14 days. LDH is exclusively used to distinguish acute from subacute myocardial infarction in patients who arrive in the hospital with positive troponins but normal CK and CK-MB readings.[14] BNP and NT-proBNP are considered the most valuable and reliable biomarkers for diagnosing heart failure and heart disease by determining severity, guiding relevant treatment strategies, and assessing the prognosis of heart disease, according to the American College of Cardiology Foundation/American Heart Association (ACCF/AHA) and the European Society of Cardiology (ESC) guidelines. BNP and NT-proBNP are employed as postmortem biomarkers in forensic medicine to represent the cardiac function of the deceased before death, as diagnostic biomarkers for heart failure and cardiac dysfunction in clinical medicine.[15]

Based on the literature search, there is no report on the cardioprotective effect of the ethanolic extract of bay Leaf (EEBL) by measuring Lactate Dehydrogenase (LDH) and B-type Natriuretic Peptide (BNP) as biomarkers of myocardial function damage, as well as analyzing the histological changes of the heart in doxorubicin-induced rats. Moreover, the details of bioactive compounds that role in the cardioprotective effect are not reported yet, so this research is essential for advancing knowledge in this field.

METHOD

Study Design

The present study used a true-experimental pretest-posttest control group research design with Wistar rats as the research subjects.

Study Site

This experiment was conducted at the Laboratory of Biomolecular and Biochemistry, Universitas Prima Indonesia, and the Pharmacology and Toxicology Laboratory of the Faculty of Pharmacy, University of North Sumatra. The bay leaf samples were obtained from the Harjosari II Medan Amplas, North Sumatra. The species name of *Syzygium polyanthum* (Wight.) has been confirmed by the Herbarium of the University of North Sumatra (USU).

Materials

Materials used were fresh bay leaves, ethanol 96%, aqua dest, chloroform, filter paper, parchment paper, aluminum foil, doxorubicin, Nature-E, CMC-Na, 2N HCl, Mayer's reagent, Bouchardat's reagent, Dragendorff's reagent, magnesium powder, powder zinc, concentrated HCl, amyl alcohol, isopropanol, methanol, Molisch's reagent, concentrated H_2SO_4, $FeCl_3$, lead(II) acetate 0.4M, anhydrous sodium sulfate, ether, Lieberman-Bourchard reagent, toluene, 10% formalin. Medical gloves, surgical scissors, anatomical tweezers, petri dish, glass, stainless-bottom wax surgical board, closed container, caliper, and millimeter block paper were among the tools used in the surgery. A rat cage, trays, scissors, labels, markers, syringes, measuring flask tubes, oral probes, and digital scales are additional instruments.

Plant Extraction Process

The collection of the bay leaf was carried out purposively. After separation from the stem, washed, cleaned, dried, blended, and the powdered Simplicia was stored and weighted for the next step. About 1.000 g Simplicia were macerated by 7.5 L ethanol 96% in a maceration container for five days and stored in a dry place to protect from sunlight, stirring occasionally. After filtration, the dregs were washed with 2.5 L ethanol 96% to get 10 L filtrate and transferred to a closed vessel, left for two days in a cool place protected from sunlight, and then poured or filtered to get the crude extract; the obtained macerate was concentrated with a rotary evaporator at 45 ºC.[16]

Before cardioprotective tests, suspension solution was prepared by sprinkling 0.5 g CMC-Na in a mortar containing sufficient hot water, allowed to stand for 15 minutes until it expands, and ground until a transparent and homogeneous mass is presently obtained. Added with a bit of water, homogenized, put into a 100 mL volumetric flask, then the volume was made up to the marked line. The EEBL suspension was made of 100 mg, 300 mg, and 500 mg of crude EEBL added slowly by 0.5% CMC-Na suspension while grinding until a homogeneous mass was obtained. Then it was put into a 10 mL volumetric flask, and the volume was made up with CMC-Na suspension up to the marking line. Then EEBL suspension is ready for the cardioprotective activity test.

Physical Evaluation of Extract

The organoleptic examination included the color, smell, texture, and taste. The viscosity value was measured with

the NDJ-8S viscometer. Determination of the pH of the extract was carried out using a digital pH meter. After calibration and electrode washed, then weighed 1 g of EEBL dissolved in 100 mL of distilled water to make a 1% sample solution concentration, pH was measured. The characteristics of EEBL include the level of water-soluble extract content, total ash content, water content, ethanol-soluble extract content, and acid insoluble ash content.[16,17]

Phytochemical Screening of Extract

The phytochemical screening of the extracts was carried out with the standard protocols.[17] The phytochemical screening of EEBL includes flavonoids, alkaloids, glycosides, steroids/triterpenoids, saponins, and tannins compounds.

Bioactive Compounds Analysis

The GCMS was used to analyze the secondary metabolites compounds in the EEBL. About 1 µL EEBL was injected into a capillary model with Agilent number 19091S-433HP-5MS 5% of Phenyl Methyl Siloxane with specifications as follows; length 30 m, diameter 250 1 µL, thickness 0.25 1 µL, oven 100-220ºC, temp rate 15ºC, flowrate 1.0 ml. min, Helium 10.5 psi, total speed 140 ml. min, and split ratio 1:50.

In Vivo Procedure

Animal Preparation

In this study, the animal used was 24 male Wistar rats with body weights ranging from 150 g - to 200 g divided into six groups, with four individuals per group. Before the study, the experimental animals were acclimatized for two weeks to adjust to the environment at a temperature of 22ºC - 25ºC, given pellets. They drank everyday ad libitum under a 12-hour light/dark cycle.[18]

Experimental Procedure

After two weeks of animal preparation, all six groups were then treated as follows;
(1) Normal group was treated with regular food;
(2) Negative control group was treated with 1% (w/w) of Na-CMC 0.5% and 5 mg/kg BW of doxorubicin via intraperitoneal on days 1st, 7th, 14th, and 20th;
(3) Positive control group was treated with 1% vitamin E, which is commercially available, namely as Nature E, and also 5 mg/kg BW of doxorubicin via intraperitoneal on day 1st, 7th, 14^{th,} and 20th;
(4) Treatment group I was treated with 100 mg/kg BW of EEBL suspension and 5 mg/kg BW of doxorubicin via intraperitoneal on days 1st, 7th, 14th, and 20th;
(5) Treatment group II was treated with 300 mg/kg BW of EEBL suspension and 5 mg/kg BW of doxorubicin via intraperitoneal on days 1st, 7th, 14th, and 20th;
(6) Treatment group III was treated with 500 mg/kg BW of EEBL suspension and 5 mg/kg BW of doxorubicin via intraperitoneal on days 1st, 7th, 14th, and 20th;

Analysis of LDH and BNP

On the last day of animal model treatment, all groups get fasting for 18 h and then anesthetized with ketamine at a 70 mg/kg BW dose by i.v. The chest cavity was dissected, and 3 mL of blood in the heart was taken using a 5 mL syringe. The blood was then transferred to a blood tube and centrifuged for 10 min at 3,000-4,000 rpm to get serum and sediment. The serum layer was taken by 1 mL syringe, collected in a microtube, and stored in a refrigerator at -4°C before use.[19] Then serum obtained was send for LDH and BNP analysis at the Laboratorium Kesehatan Daerah Provinsi Sumatera Utara with an established protocol.[16]

Histology Analysis

Histology analysis was conducted through the fixation, trimming, dehydrating, embedding, sectioning, and staining following the Guidance of the Ministry of Health.[20]

Statistic Analysis

All obtained LDH and BNP data were analyzed for outliers, normality, and homogeneity. The boxplots were used to assess the outliers, while the Shapiro-Wilk tets were used to evaluate the normality of the data. As for assessing the homogeneity of variance, we used the Levene test. The mean difference between groups was evaluated using the One Way ANOVA test. The Tukey Post Hoc test was used to determine the differences between groups in data that met the homogeneity assumption. In contrast, the Games-Howell test compared groups that did not meet the homogeneity assumption.

Ethical Consideration

The ethical clearance was issued by Komisi Etik Penelitian Kesehatan-Universitas Prima Indonesia No. 008/KEPK/UNPRI/XI/2020.

RESULTS

Physical Evaluation of Extract

The crude extract yielded by maceration was 123.5 g or 12.35% (w/v). The obtained bay leaf extract is dark green with a distinctive odor, thick texture and tastes bitter and chelating. The viscosity of EEBL almost showed 99.9% at 12, 30, and 60 rpm with 2497, 999, and 499.5 mPa.s, respectively. The mean pH value (triplicate) was 5.83, acceptable for biological experimental conditions.[16]

Table 1. Physical Quality of Obtained Bay Leaf Extract

Parameter	Percentage
Water-soluble extract content	21.64 %
Total ash content	1.79 %
Water content	9.98 %
Ethanol-soluble extract content	45.38 %
Acid insoluble ash content	0.39 %

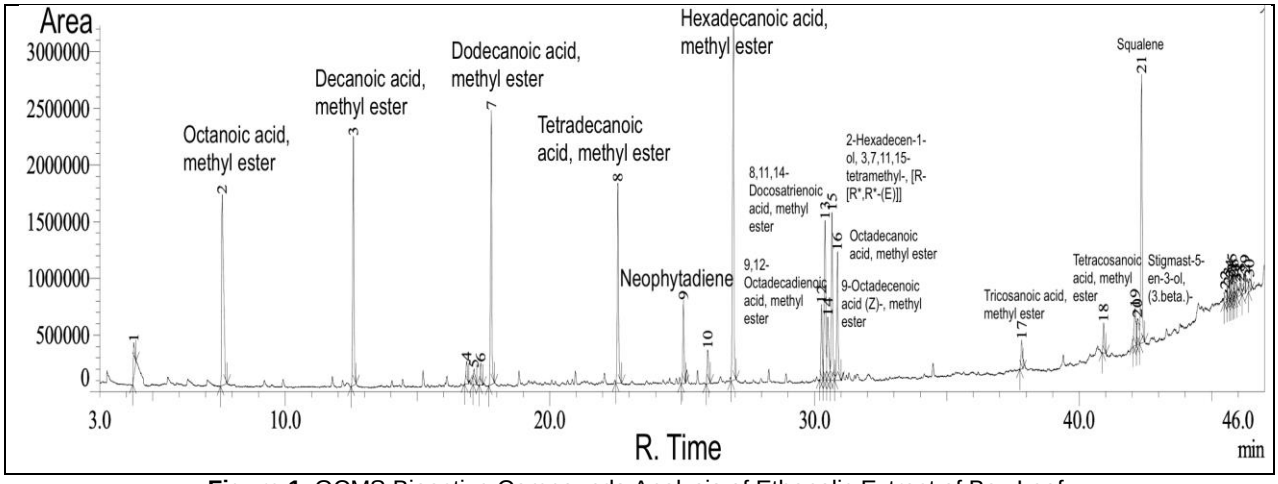

Figure 1. GCMS Bioactive Compounds Analysis of Ethanolic Extract of Bay Leaf.

The physical parameters in Table 1 have met the standard quality of extract preparation. It was even better than the minimum requirements of up-to-date regulation.[17] Our results on the physical quality and characteristics of EEBL might be used as an excellent practical standard for the pharmacological industries' application.

Phytochemical Analysis

By preliminary screening of EEBL, it has positively confirmed that flavonoid, alkaloid, glycoside, steroid/triterpenoid, saponin, and tannin contents were detected. We used GCMS analysis to explore more details about the bioactive compounds in the EEBL. GCMS data of ethanolic extract of bay leaf are visualized in Figure 1. The data showed that the majority group of decanoic and hexadecanoic acid methyl ester, families of saturated fatty acids. Interestingly, among 28 compounds detected in our extract, we found six bioactive compounds correlated to cardioprotective effects; Neophytadiene, squalane, phytol, methyl palmitate, stigmasterol, and 9,12-Octadecanoic acid methyl ester.

LDH and BNP Data Analysis

Visual examination of boxplots did not show any outliers. The Shapiro-Wilk was used to evaluate the normality of the data, and the test showed that data were normally distributed ($p > 0.05$). Levene test for LDH showed that the data met the assumption of homogeneity of variance ($p > 0.05$). However, the BNP data did not meet the assumption of homogeneity of variance ($p < 0.05$). For this reason, we use different post hoc methods, the Tukey test for LDH and the Games-Howell test for BNP.

A one-way ANOVA was run to determine the LDH mean differences between six groups. The groups are: normal (525.33 ± 100.85), negative control (1050.67 ± 53.27), positive control (598.67 ± 102.00), treatment group I (822.00 ± 219.80), treatment group II (669.33 ± 131.72), treatment group III (629.67 ± 132.72). The values represented mean ± SD. One-way ANOVA showed significant differences between the groups, $p = 0.005$, F (5,12) = 6.172. Follow up posthoc analysis using the Tukey test showed statistically significantly different LDH levels between treatment group II (EEBL 300 mg/kg BW) and negative control group (doxorubicin), $p = 0.039$, *95% CI* [15.62, 747.04], as well as between treatment group III (EEBL 500 mg/kg BW) and negative control group (doxorubicin), $p = 0.021$, *95% CI* [55.29, 786.71]. The LDH level for treatment group I (EEBL 100 mg/kg BW) was slightly lower than the negative control group but not significantly different, $p = 0.348$, *95% CI* [-137.04, 594.38]. The posthoc test also showed that the LDH levels of the treatment groups were not different from the positive control group (Vitamin E), as shown by a non-statistically significant difference between the two groups, $p > 0.05$. Details of multiple comparison tests are shown in Table 2.

Table 2. LDH and BNP Levels of All Groups After 21 Days of Treatment.

Groups	LDH (U/L)*	BNP (pg/mL)*
Normal	525.33 ± 100.85	86.88 ± 5.20
Negative control	1050.67 ± 53.27[ac]	217.43 ± 20.94[ac]
Positive control	598.67 ± 102.00[b]	88.27 ± 1.49[b]
Treatment I	822.00 ± 219.80[a]	181.52 ± 5.03[ac]
Treatment II	669.33 ± 131.72[b]	143.58 ± 6.50[ac]
Treatment III	629.67 ± 132.72[b]	86.99 ± 15.05[b]

*Values represented by mean ± SD.
[a]$p < 0.05$ vs.normal group
[b]$p < 0.05$ vs.negative control group
[c]$p < 0.05$ vs.positive control group

A one-way ANOVA was also run to examine the BNP mean differences between the six groups. The mean and standard deviation for each group are: normal (86.88 ± 5.20), negative control (217.43 ± 20.94), positive control (88.27 ± 1.49), treatment group I (181.52 ± 5.03), treatment group II (143.58 ± 6.50), treatment group III (86,99 ± 15,05). The values represented mean ± SD. One-way ANOVA showed statistically significant differences between the groups, $p = 0.000$, F (5,12) = 74.825. Follow-up posthoc analysis using the Games-Howell test showed-

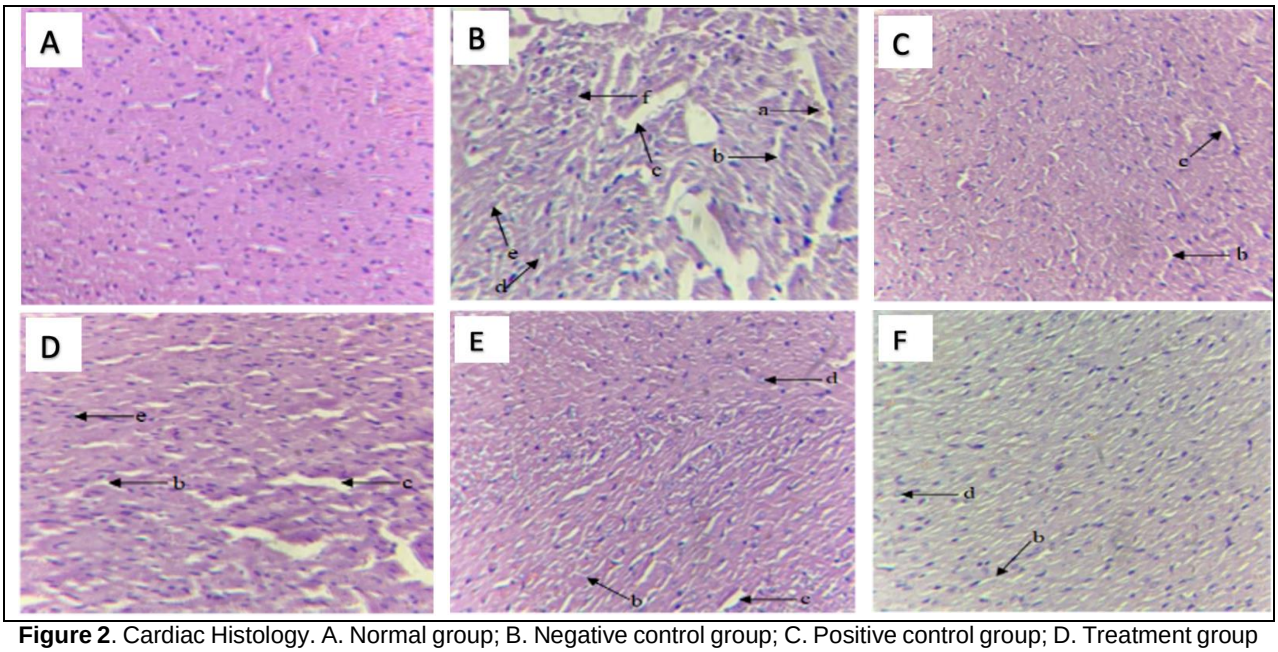

Figure 2. Cardiac Histology. A. Normal group; B. Negative control group; C. Positive control group; D. Treatment group I; E. Treatment group II; F. Treatment group III. The arrows indicated a. Hemorrhage; b. Myocardial fibers; c. Interstitial edema; d. Intracellular vacuole; e. Necrosis; f. Infiltration of inflammation cells.

a statistically significantly different BNP level between the negative control groups (doxorubicin) and all treatment groups, $p > 0.05$. The BNP levels decrease as the dose increases from 100 to 500 mg/kg BW. The results showed a dose-response relationship indicating a cardioprotective effect of EEBL. Interestingly, the best performance was shown by treatment group III (500 mg/kg BW), which was not statistically different from positive control (Vitamin E), $p = 1.000$, *95% CI* [-69.59, 72.15]. Details of multiple comparison tests are shown in Table 2.

Cardiac Histology

Based on Figure 2, the treatment with EEBL improved the histological picture of cardiac tissue in a dose-response relationship. The higher the dose of the EEBL given, the more significant improvement will be obtained. Treatment III (Figure 2F) showed the best recovery of cardiac tissues under EEBL cardioprotective effect.

DISCUSSION

Further analysis of the GCMS data of EEBL showed an agreement with another study on the ethanolic extract of bay leaf for p53 expression of HeLa cell lines,[21] which also shows the same groups of metabolite compounds. Based on literature studies, we strongly suggest Neophytadiene, squalane, phytol, methyl palmitate, stigmasterol, and 9,12-Octadecanoic acid methyl ester in EEBL play a role in the cardioprotective effect in many ways. Moreover, decanoic acids are antibacterial agents, anti-inflammatory agents, human metabolites, volatile oil components, and plant metabolites.[22]

These results might explain the potential of EEBL as a cardioprotector through several possible mechanisms. Neophytadiene has bioactivity as an anti-inflammation, antioxidant, and cardioprotective effect by modulating the expression of TNF-α, IL1β, NF-κB, iNOS, PI3k/Akt, and MAPK in the heart tissue.[23] Squalane has potential antitumor, antioxidant, anti-bacteria, anti-fungi, anti-cancer, and cardioprotective effects by inhibiting lipid accumulation by its hypolipidemic or antioxidant properties.[24,25] Phytol could be used as anti-inflammation, antioxidant, anticancer, anti-microbes, and cardioprotective by attenuating doxo-mediated cardiotoxicity via antioxidant, free radical scavenging, anti-lipoperoxidation, and anti-thrombotic mechanisms.[26]

Methyl palmitate showed bioactivity as a phagocytosis inhibitor and cardioprotective by preventing the cardiomyocyte death mediated through the GPR40-activated PI3K/AKT signaling pathways.[27] Stigmasterol also showed potential as an antioxidant, anti-inflammation, anti-diabetic, anti-apoptosis, and cardioprotective by decreasing the cardiac marker enzyme level and restoring antioxidant status.[26,28] Moreover, fatty acid methyl esters such as 9,12-Octadecanoic acid-methyl ester showed anti-inflammation, anti-arthritis, hepatoprotective, anti-hypercholesterolemia, and anti-oxidative cardiotoxicity by suppressing lipid dysmetabolism and modulating cardiometabolic activities linked to cardiac dysfunctions.[29]

The significant effect of EEBL on reducing LDH and BNP levels is shown in Table. 2. These effects might be due to

the bioactive compounds and their mechanism, as described previously. A recent study on the protective effect upon cardiotoxicity also showed that ethanol extract of *Vernonia amygdalina* reduces the level of AST, ALT, Ureum, Creatinine, CK- MB, LDH, Troponin T, and BNP significantly.[30] Another study also reported the effect of rutin as a cardioprotector in rats induced by pirarubicin. In contrast, the treatment with a middle dose of rutin affects the BNP and LDH values. Treatment with rutin and dexrazoxane resulted in an increase in Bcl-2/ Bax ratio, reduction in JNK, and Caspase-3 protein levels.[31]

Regarding histopathology analysis on the cardiac tissues, based on Figure 2A, there were no damages such as bleeding, interstitial edema, inflammatory cell infiltration, wavy myocardial fibers, necrosis, etc. Doxorubicin administration on histological results in Figure 2B showed the occurrence of hemorrhages, wavy myocardial fibers, interstitial edema, intracellular vacuoles, necrosis, and inflammatory cell infiltration. Cellular changes in Figure 2B were similar to those that occur during apoptosis, such as pyknosis, karyolysis, karyorrhexis, condensed chromatin, and fringe nuclei which are the hallmark of apoptosis associated with caspase-targeted proteins in the nuclear lamina, i.e., between peripheral chromatin, disorganized muscle fibers, decreased cytoplasmic acidophilia, and blocked blood vessels.[29,32,33]

Administration of vitamin E in Figure 2C showed an improvement by a reduced level of damage approaching the normal condition. Only slightly wavy myocardial fibers and interstitial edema were observed. Treatment with EEBL at various doses showed an improvement in the histological picture of the heart tissues. The higher the dose of EEBL being given, the more significant improvement in heart tissue will obtain. A 100 mg/kg BW dose showed that interstitial edema was more critical than other doses, and many wavy fibers were still visible. However, there was no evidence of hemorrhage or inflammatory cell infiltration. At a dose of 300 mg/kg BW, the interstitial edema decreased, indicating a better chance of the level of edema with fewer intracellular vacuoles. Likewise, the administration of EEBL at a dose of 500 mg/kg BW showed a better improvement close to the Vitamin E and the regular groups.

Another study on the effect of curry extract showed a smaller area of damage, smaller necrosis, and reversible histological changes in myocardial tissue supported by its antioxidant and anti-inflammatory properties.[29] A cardioprotective effect of garlic extract has been reported to the up-regulated expression of the cardiac apoptotic caspase-3 gene at the initial stage. However, the expressions gradually get downregulated and improve the cardiac damage caused by apoptosis.[35] Based on the histology analysis, we can conclude that there was a significant improvement in the cardiac tissues by administration of EEBL in experimental rats induced by doxorubicin. The cardioprotective effect shown by EEBL might be due to the bioactive components in the obtained EEBL, i.e., Neophytadiene, squalane, phytol, methyl palmitate, stigmasterol, and 9,12-Octadecanoic acid methyl ester. These compounds regulate gene expressions such as TNF-α, IL1β, NF-κB, iNOS, PI3k/Akt, MAPK, GPR40-activated PI3K/AKT, inhibiting lipid accumulation, free-radical scavenging, and other mechanisms as reported in the literature.

CONCLUSIONS AND RECOMMENDATION

Ethanolic extract of bay Leaf (EEBL) showed its potential as a cardioprotector in Wistar rats induced by doxorubicin. The protective effect showed at 300 and 500 mg/kg BW of EEBL suspension. The recovery level of LDH and BNP was almost close to commercially available vitamin E. Additionally, we have also reported several data on the physical characteristics of sample preparation. Furthermore, we proposed that the cardioprotective effect shown by EEBL was due to the bioactive components, i.e., Neophytadiene, squalane, phytol, methyl palmitate, stigmasterol, and 9,12-Octadecanoic acid methyl ester. The bioactive compounds reported from this research might share new insight and inspire the further analysis of *in silico* studies in the future. Investigating the more specific biomolecular parameters such as cTnI and cTnT and High sensitive C reactive protein is also recommended to reveal the molecular mechanism of the cardioprotective effect of EEBL.

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