

Protection by Ethanolic Extract from *Ulva lactuca* L. against Acute Myocardial Infarction: Antioxidant and Antiapoptotic Activities

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Original Article

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Abstract

Background: Reactive oxygen species (ROS) play a major role in myocardial damage during acute myocardial infarction (AMI). This study aimed to determine the antioxidant and antiapoptotic activities of an ethanolic extract from *Ulva lactuca* L. (EEUL) against AMI.

Methods: Thirty-six male Wistar rats were divided into six groups: one control group and five treatment groups. Treatment group II was given 85 mg/kg body weight (BW) of isoproterenol (ISO). Group III, IV and V were given ISO and EEUL at 250, 500 and 750 mg/kg BW, respectively. Group VI were given 10 mg/kg BW of ISO and melatonin. EEUL and melatonin were orally administered for 28 days. ISO was injected subcutaneously on day 29 and 30 to chemically induce AMI. On day 31, blood was collected for antioxidant assay and heart tissues were collected for histological examination.

Results: The activity of catalase (CAT), an endogenous antioxidant, in the EEUL-treatment groups was significantly increased compared to the ISO-treatment group ($P < 0.001$). The EEUL-treatment groups showed significantly decreased expression of caspase-3 ($P < 0.001$) and better myocardial tissue morphology.

Conclusion: EEUL possibly protects against AMI because of its antioxidant and antiapoptotic properties.

Keywords: *Ulva*, isoproterenol, antioxidant, apoptosis, melatonin

Introduction

Acute myocardial infarction (AMI) is responsible for considerable human morbidity and mortality worldwide (1-2). Previous studies have reported that reactive oxygen species (ROS) are responsible for myocardial damage during AMI (3). ROS elevated lipid peroxidative products malondialdehyde (MDA), which led to a decrease of endogenous antioxidant levels, such as superoxide dismutase (SOD), catalase (CAT) and glutathione (GSH) (4-5). ROS are reported to induce myocardial apoptosis, which plays an important role in the pathogenesis of cardiac diseases, including myocardial infarction (6-7).

Cardiac diseases have been linked to oxidative stress initiated by the reaction of free radicals with biological macromolecules such as proteins, lipids and DNA. Antioxidants, preferably from natural sources, are considered effective treatment for AMI. *Ulva lactuca* L. is a rich source of bioactive secondary metabolites important in the development of new pharmaceutical agents. Previous studies have shown that *U. lactuca* L. compounds contain several active chemicals, such as chlorophyll, carotenoids, vitamin C, polyphenols, polysaccharide sulphate (8-9) and melatonin (10). Melatonin has been demonstrated to have a cardioprotective effect in male rat SD induced by ISO and antiadrenergics (11). Endogenous antioxidants, such as SOD, glutathione peroxidase, glutathione reductase, glucose 6-phosphate dehydrogenase and nitric oxide synthases, are significantly increased by administration of melatonin in rat models (12).

Recent research has shown that medicinal plants with antioxidant properties can be used for cardioprotection (13). The current study investigated the preventive effect EEUL against AMI by measuring its antioxidant activity and antiapoptotic properties in an animal model. ISO was used to chemically induce AMI.

Materials and Methods

Materials

Fresh *U. lactuca* L. algae were collected from Drini Beach, Yogyakarta, Indonesia. Plant identification and authentication were carried out by Sujadmiko from the Systematical Plant Laboratory, Faculty of Biology, Gadjah Mada University, Yogyakarta (Identification No. 0626/S.Tb/I/2015). A specimen voucher was deposited at the herbarium unit, Faculty

of Pharmacy, Ahmad Dahlan University, Yogyakarta. Isoproterenol hydrochloride and melatonin were purchased from Sigma Aldrich. MDA levels and the activities of SOD and catalase were analysed using commercial reagent kits (BioVision Inc., Milpitas, CA). Anti-caspase-3 antibody was purchased from Abcam, USA, and secondary antibodies were purchased from Biocare Medical LLC, CA.

Plant Extraction

The algae were cleaned, dried and powdered. An ethanol extract was obtained from the powder using 96% ethanol and maceration. The extract was then evaporated in a vacuum rotary evaporator (Heidolph Instruments GmbH & Co, Schwabach, Germany) at 40 °C. The now thickened extract was stored at 4 °C until use.

Dosage Determination of EEUL

A previous *in vivo* study by Widyarningsih et al. (2015), employing 200 mg/kg BW and 400 mg/kg BW of EEUL showed antioxidant activity, reduced MDA levels and increased SOD activity in CCl₄-induced rat liver (14). Therefore, the dosages of EEUL in the current study were set at 250, 500 and 750 mg/kg BW. The extract was mixed with 1% CMC Na and given orally.

Phytochemical Screening and Identification of Melatonin

Phytochemical screening was carried out in accordance with standard protocol described by Trease and Evans (1983) (15) to determine the presence of reducing sugars (Fehling's test), anthraquinones, terpenoids (Salkowski test), flavonoids, saponins, tannins, alkaloids and cardiac glycosides (Keller-Kiliani test). Identification of melatonin was performed using the thin-layer chromatography (TLC) method (16). TLC elution was carried out on silica gel F254 eluted with a mobile phase of n-butanol-acetic acid-water (12: 3: 5) with a melatonin standard and then identified under UV light (254 nm).

Animals and Experimental Groups

Animals (180-200 g) were obtained from the Animal Experimental Unit, Animal Research Centre, Gadjah Mada University. The animals were kept under standard conditions of temperature (25 ± 1.0 °C) and humidity (55 ± 10%) with a 12 hours light/12 hours dark cycle and fed with a standard pellet diet

(Comfeed Industries Ltd) and allowed *ad libitum* water. They were kept in individual, standard polypropylene cages and allowed to acclimatise to the laboratory environment for one week before experimentation. All equipment and handling and sacrificing methods were in accordance with European Council Legislation for the protection of experimental animals. The animal experimental procedures were approved by the Ethical Committee of Integrated Laboratory of Research and Testing (LPPT) Gadjah Mada University (No. 205/KEC-LPPT/XII/2014).

Thirty-six male Wistar rats aged 7–8 weeks were divided into six groups. Group I was the control group. Group II was given 85 mg/kg BW of ISO. Groups III, IV and V were given ISO plus EEUL at 250, 500 and 750 mg/kg BW, respectively. Group VI was given ISO plus 10 mg/kg BW of melatonin. Both EEUL and melatonin were administered by daily oral gavage for 28 days. ISO was injected subcutaneously on day 29 and day 30. At the end of study (day 31), all animals were anaesthetised using an intraperitoneal injection of 50 mg/kg BW of pentobarbital sodium, and blood samples were collected from the infraorbital sinus for antioxidant enzyme activity analysis. Subsequently, all animals were sacrificed and the hearts removed by a transverse cut across the left ventricle and fixed in 10% formalin buffer for histological examination.

Analysis of Antioxidant Activity

The activities of SOD and catalase were determined by commercial diagnostic kits (BioVision, Milpitas, CA). The inhibition activity of SOD was determined by a colorimetric method with an OD of 450 nm and the CAT activity was determined by a colorimetric method (570 nm) with an H_2O_2 standard curve.

Lipid peroxidation marker level, MDA, was determined using thiobarbituric acid reactive substances (TBARS) (BioVision, Milpitas, CA) determined by a colorimetric method with an OD of 532 nm.

Histological Examination of Myocardial Tissue

Haematoxylin and Eosin Staining

Haematoxylin and eosin (H&E) staining was used to visualise cardiomyocyte architecture. Sections of 2–3 mm thick heart tissue were

immediately washed in NaCl solution and fixed in 10% formalin buffer for microscopic examination. Histological changes in the myocardial tissue sections were examined under a light microscope (DPX-20; Olympus Co. Ltd., Japan) in the Pathology Department, Faculty of Veterinary Medicine, Gadjah Mada University, Indonesia, and recorded using a digital camera (Optilab Advance; PT Miconos, Indonesia). Scoring of myocardial infarction was determined by the modification method of Mehdizadeh et al. (17): score 0 = no myocardial infarction; score 1 = myocardial infarction > 1% to < 3%; score 2 = myocardial infarction 3%–6%; 3 = myocardial infarction 7%–9%; and score 4 = myocardial infarction > 10% (17).

Immunohistochemistry Staining for Caspase-3

Immunohistochemistry staining for caspase-3 was performed using a routine immunohistochemistry streptavidin-peroxidase method at the Pathology Laboratory, Faculty of Medicine, Gadjah Mada University. The tissue sections were deparaffinised with xylol and graded ethanol solutions to 70%. Endogenous peroxidase was quenched by incubation in 0.3% (vol/vol) hydrogen peroxide in methanol (JT Baker®, USA). Sections were then incubated for 1 hour with a primary antibody against caspase-3 (Abcam, USA). Sections were then rinsed (PBS) and incubated for 1 hour with peroxidase-conjugated secondary antibodies (Biocare Medical LLC, CA). Finally, the immunoreactions were visualised using avidin-biotin peroxidase complexes (Biocare Medical LLC, CA), and the peroxidase reaction was developed in 3,3'-diaminobenzidine chromogen (Biocare Medical LLC, CA). Tissue sections were counterstained with haematoxylin followed by dehydration with graded ethanol solutions to 100% and then mounted in DPX. Five randomly selected fields from each section were examined with 400× magnification, analysed using Image-Pro Plus (Version 6.0) and then formulated as:

$$\% \text{ of caspase 3 expression} = \frac{\text{number of myocardium expressed caspase 3}}{\text{total number of cells}} \times 100$$

Data analysis

Antioxidant activity levels, myocardial infarction area scores and the expression of apoptotic protein were expressed as means

(SD). The data was tested using analysis of covariance (ANCOVA) with the area of infarction as the covariate (Minitab, Version 16, Statistical Software), and the results were considered significant at $P < 0.05$.

Results

Phytochemical Screening and Identification of Melatonin in EEUL

The results of the phytochemical screening are shown in Table 1. Flavonoids, saponins, alkaloids and cardiac glycosides were present in the EEUL samples. Compounds, such as reducing sugars, anthraquinones, terpenoids, tannin and polyphenols were absent. Identification using TLC under UV light (254 nm) indicated the presence of melatonin in the EEUL with a retardation factor (Rf) of 0.78 (co-chromatographed with a melatonin standard).

Table 1. Phytochemical screening of ethanolic extract from *Ulva lactuca* L.

No	Constituents	Ethanolic Extract
1	Reducing Sugars	Absent
2	Anthraquinones	Absent
3	Terpenoids	Absent
4	Flavonoids	Present
5	Saponins	Present
6	Tannins	Absent
7	Alkaloids	Present
8	Cardiac Glycosides	Present
9	Polyphenols	Absent

Effect of EEUL on antioxidant activity

As shown in Table 2, the ISO-treatment group demonstrated a 59.0% reduction of catalase activity ($P < 0.001$). However, administration of EEUL at 250, 500 and 750 mg/kg BW and 10 mg/kg BW of melatonin significantly increased catalase activity to 58.31%, 57.13%, 45.99% and 56.33%, respectively. The ISO-treatment group demonstrated a 21.73% increase of plasma MDA compared with the control group. Treatment with EEUL at 250, 500 and 750 mg/kg BW and 10 mg/kg BW of melatonin reduced the level of MDA to 11.70%, 11.98%, 23.40% and 6.69%, respectively, compared with the ISO group. Administration of EEUL at 250, 500 and 750

mg/kg BW and melatonin at 10 mg/kg BW also significantly increased the level of SOD activity. However, statistical analysis showed there was no significant difference between the ISO group and the various doses of EEUL and the levels of MDA ($P = 0.533$) and SOD activity ($P = 0.180$).

Effect of EEUL on myocardial tissue histological features

Microscopic examination of myocardial tissue by H&E staining determined that ISO had induced acute myocardial infarction (Figure 1). The control group showed normal myofibril structures with striations, branched appearances and continuity with adjacent myofibrils. In the group given ISO, myocardial infarction was marked by necrotic areas of heart muscle cells and the muscle fibres were shrunk from the infiltration of neutrophils. Administration of the various doses of EEUL showed better myocardial tissue morphology. Scoring of the myocardial infarction areas supported the histological features of the myocardial tissue in the EEUL-treatment group compared to the control and ISO groups (Table 3). As shown in Table 2, all treatments accounted for 31.8%, 61.0% and 36.7% of the variations in MDA, CAT and SOD, respectively, whereas, infarct areas accounted for 13.1%, 24.3% and 4.3%, respectively. Thus, the level of CAT was affected by infarct area and treatment.

Effect of EEUL on the expression of caspase-3 protein

Microscopic examination of myocardial tissue by IHC staining is shown in Figure 2. Caspase-3 expression in normal rat hearts and ISO-administered rats are shown in Figure 3. Immunohistochemical analysis showed that ISO injection significantly increased the expression of caspase-3 ($P < 0.001$) by 71.96% in the myocardium compared to the control group. Administration of EEUL at 250, 500 and 750 mg/kg BW and 10 mg/kg BW of melatonin showed a significantly decreased expression of caspase-3 in the myocardium of 33.31%, 46.99%, 52.56% and 61.48%, respectively, compared to the control group.

Discussion

The results of this study showed that the administration 85 mg/kg BW of IOS for two consecutive days in male Wistar rats resulted in increased levels of lipid peroxidation followed

Table 2. Effect of ethanolic extract from *Ulva lactuca* L. on antioxidant activity in rats without and with

Group(s)	Without Infarct Area as Covariate		
	MDA (IU/L)	CAT (IU/L)	SOD (IU/L)
Control	2025.23 (301.07)	29.07 (3.26)	95.04 (2.98)
ISO	2587.39 (144.14)*	11.91 (2.31)*	89.54 (0.78)*
EEUL 250 mg/kg BW	2284.68 (337.66)	28.57 (1.65)*#	90.7 (3.35)*
EEUL 500 mg/kg BW	2277.48 (401.77)	27.78 (3.79)*#	92.25 (1.89)#
EEUL 750 mg/kg BW	1981.98 (120.59)#	22.05 (2.58)#	91.48 (2.97)*
Melatonin	2414.41 (186.21)*	27.27 (4.66)*#	93.64 (3.63)#

It are expressed as means (SD)

* $P < 0.05$ vs control; # $P < 0.05$ vs ISO

Group(s)	With Infarct Area as Covariate		
	MDA	CAT	SOD
R ² covariate	0.13	0.24	0.04
R ² covariate + treatment	0.45	0.85	0.41

Result are expressed as coefficients of determination (R²)

Multiple Comparison of Treatment Groups after Adjusted Covariate

Group(s)	MDA	CAT	SOD
Control vs ISO	593.74 (279.82)*	-19.94 (2.84)*	-8.37 (3.48)*
Control vs EEUL 250 mg/kg BW	279.55 (223.81)	-2.10 (2.22)	-5.77 (2.38)*
Control vs EEUL 500 mg/kg BW	266.61 (201.68)	-2.59 (2.03)	-4.22 (2.30)
Control vs EEUL 750 mg/kg BW	-20.28 (236.59)	-8.80 (2.32)*	-5.71 (2.89)
Control vs Melatonin	463.52 (307.50)	-5.23 (2.99)	-4.28 (3.98)

Result are expressed as coefficients of regression (SE)

* $P < 0.05$ significant difference

MDA = malondialdehyde

CAT = catalase

SOD = superoxided dismutase

ISO = isoproterenol

EEUL = ethanolic extract from *Ulva lactuca* L.

Table 3. Effect of ethanolic extract from *Ulva lactuca* L. on the area of myocardial infarction.

Group(s)	Score of myocardial infarction
Control	0
ISO	2.50 (1.05)
EEUL 250 mg/kg BW	1.40 (0.89)
EEUL 500 mg/kg BW	1.17 (0.41)
EEUL 750 mg/kg BW	1.83 (0.75)
Melatonin	2.33 (0.58)

Result are expressed as means (SD)

ISO = isoproterenol

EEUL = ethanolic extract of *Ulva lactuca* L.

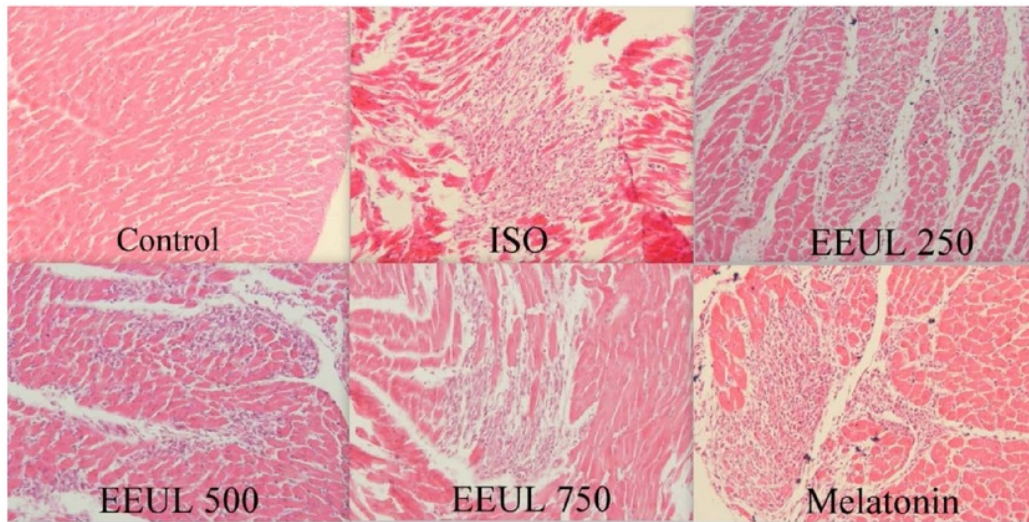


Figure 1. Effect of EEUL on heart tissue histological features in the control and EEUL-treatment groups. The control group showed normal myofibril structures with striations, branched appearances and continuity with adjacent myofibrils. The ISO-treatment group showed myocardial infarction marked by necrotic areas of heart muscle cells and shrinkage of muscle fibres with infiltration of neutrophils. The EEUL-treatment group demonstrated better myocardial tissue morphology compared to the ISO-treatment group (Haematoxylin and eosin staining, 200× magnification). ISO = isoproterenol; EEUL = ethanolic extract from *Ulva lactuca* L.

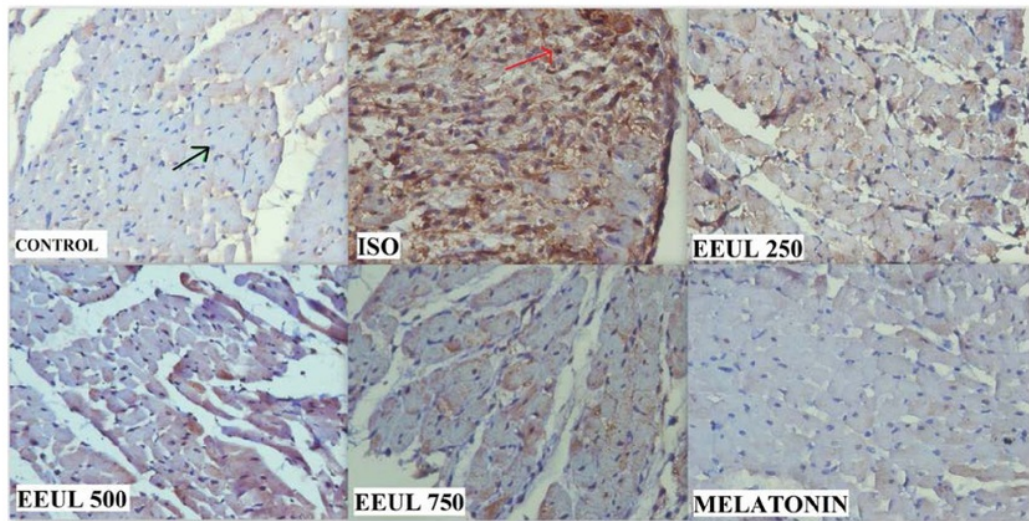


Figure 2. Microscopic examination of caspase-3 expression in myocardium. The expression of caspase-3 is indicated by the brown colour of the nucleus and cytoplasm of the myocardial cells. ISO = isoproterenol; EEUL = ethanolic extract from *Ulva lactuca* L.

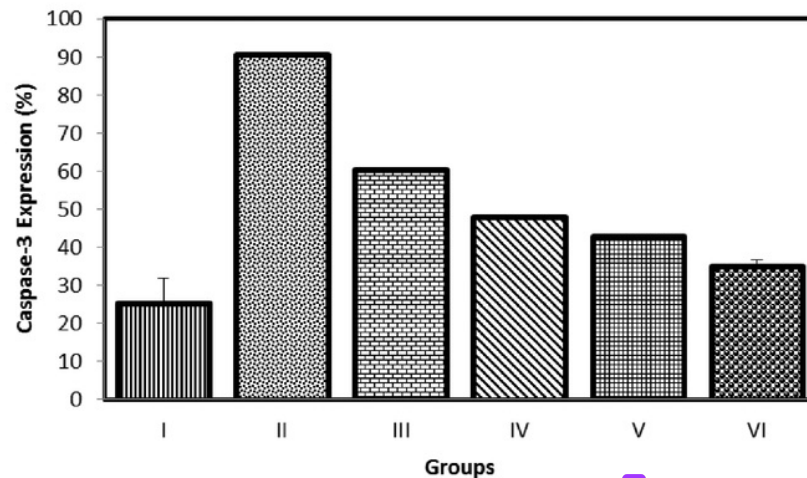


Figure 3. Effect of EEUL on caspase-3 expression in the myocardium. Group I: control group; Group II: isoproterenol group; Group III: 250 mg/kg BW of EEUL-treatment group; Group IV: 500 mg/kg BW of EEUL-treatment group; Group V: 750 mg/kg BW of EEUL-treatment group; and Group VI: melatonin group. ISO = isoproterenol; EEUL = ethanolic extract from *Ulva lactuca* L.

by the elevation of plasma MDA levels. It has also been reported in previous studies that administration of ISO increased MDA levels (11, 17, 18). Administration of ISO has been reported to induce oxidative stress and necrotic injuries in the myocardium of rats. Lipid peroxidation is an important pathogenic event linked to altered membrane structures and enzyme inactivation in myocardial infarction (19). Increased production of free radicals may have been responsible for the observed membrane damage in the current study as evidenced by the elevated lipid peroxidation in terms of MDA levels. Additionally, pre-treatment with EEUL did not significantly decrease the level of lipid peroxidation and SOD activity compared to the ISO group. *U. lactuca* has been reported in previous studies to exhibit antioxidant activity (13, 20), hence, the decrease of lipid peroxidation in the EEUL-treatment group was possibly a result of its antioxidant activity.

The current study also demonstrated that administration of ISO decreases endogenous enzyme (CAT and SOD) activity; this may be due to the involvement of superoxide and hydrogen peroxide free radicals in myocardial cell damage mediated by ISO (21). A previous study found decreased CAT and SOD activities in ISO-treated rats (22).

Administration of EEUL at the various doses and 10 mg/kg BW of melatonin significantly increased the activity of CAT. This indicates the protective effect of EEUL and melatonin to prevent membrane permeability and myocardial cell damage from ISO. Previous research has demonstrated that EEUL has in vitro antioxidant activity (23). Generation of ROS occurs by leakage of electrons into oxygen from various systems. Antioxidants play a vital role in scavenging ROS and protect cells from oxidative damage. Endogenous antioxidant enzymatic defence is very important for neutralising oxygen free radical-mediated tissue injury (24). SOD and CAT, the primary free radical scavenging enzymes, are the first line of cellular defence against oxidative injury; they decompose into O_2 and H_2O_2 before interaction to form the more reactive hydroxyl radical (25–26).

The protective effect of EEUL and melatonin against myocardial infarction was further supported by histological features. Administration of EEUL at the various doses showed better myocardial tissue morphology and significantly decreased the area of myocardial infarction. This protection effect is probably related to EEUL's ability to strengthen myocardial membrane by stabilising membrane action, or alternatively, scavenge free radicals as a result of its antioxidant properties (27).

Study of myocardial infarction has demonstrated that infarction may lead to changes in the forms of apoptosis and necrosis (28). In the current study, it was demonstrated that administration of ISO significantly increased the expression of caspase-3 protein: two-fold compared to the control group. This result is consistent with prior research that showed ISO can increase the expression of caspase-3 (29). Administration of isoproterenol has been reported to increase the activity of caspase-3 and increase DNA damage, which indicates a process of apoptosis in heart muscle cells (29-30). It has been reported that oxidative stress causes damage to DNA and antioxidants inhibit DNA fragmentation and apoptosis (31). Increased iNOS in myocardium is reported to increase the rate of apoptosis in the myocardium (30). In this context, apoptosis in myocardial cells may be commonly regulated by endogenous and exogenous apoptosis mechanisms that initiate the apoptotic factor caspase-3, which results in apoptosis of myocardial cells (32).

Administration of EEUL at the various doses and 10 mg/kg BW of melatonin significantly decreased the expression of caspase-3 protein. This result demonstrated that EEUL and melatonin have anti-apoptotic activity. This indicates that the protective effect of EEUL against ISO-induced myocardial infarction in rats may be related to its antioxidant and antiapoptotic activity.

The phytochemical screening and identification of melatonin in the EEUL demonstrated that it contains flavonoids, saponins, cardiac glycosides and melatonin. Antioxidant activities and cardioprotective effects of melatonin have been reported in previous studies (33-34). Melatonin and its metabolites, N1-acetyl N2-formyl-5-methoxykynuramin (AFMK) and N-acetyl-5-methoxykynuramine (AMK), protect against oxidative stress by scavenging free radicals as ROS, RNS and hydrogen peroxide (33). Melatonin also reduces oxidative stress by increasing endogenous antioxidant enzymes, such as SOD, CAT and glutathione peroxidase (35). Flavonoids exhibit antioxidant activity in vitro because of their ability to reduce free radical formation and scavenge free radicals. In vivo antioxidant capacity of flavonoids have an effect on endogenous antioxidants (36). Saponins contain unique, residue-like 2,3-dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one (DDMP), which is able to scavenge superoxides by forming

hydroperoxide that prevents biomolecular damage (37). Cardiac glycoside from the Castor plant also exhibits antioxidant activity as evidenced by DPPH assay (38). Cardiac glycoside has been reported to treat congestive heart failure and cardiac arrhythmia (39).

Thus, the protective effect of EEUL against myocardial infarction was not only the result of melatonin, but also, possibly, by a combination of the effects of melatonin and the various compounds previously mentioned. In the current study, the antioxidant activity of EEUL compounds, such as reducing sugars, anthraquinones, terpenoids, tannin and polyphenols, possibly prevented apoptosis via intrinsic and extrinsic pathways. Antioxidants have been reported to attenuate myocyte apoptosis in non-infarcted myocardium following large myocardial infarction (40).

Conclusion

The results of the current study indicate that the protective effect of EEUL against ISO induced myocardial infarction in rats could be related to its antioxidant and antiapoptotic properties.

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Authors' Contributions

Conception and design: WW, SW, SP, S
Analysis and interpretation of the data: WW, SW, SP, S, Z
Drafting of the article: WW
Critical revision of the article for important intellectual content: SW, SP, S
Final approval of the article: SW, S
Statistical expertise: Z
Collection and assembly of data: WW

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