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Protective effect of Hibiscus (*sabdariffa Linn*.) against H_2O_2 induced oxidative stress in Cardiomyocytes

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Oxidative stress has been highly reported to be participating in the development of cardiac diseases. Studies that have been conducted previously showed that there are antioxidant properties in Hibiscus *sabdariffa Linn* (HSL). This study focused on examining the likely defensive effects of HSL on H9c2 cardiomyocytes facing hydrogen peroxide (H_2O_2)-induced damage. These cells were pre-treated 24 h with HSL 1 h before exposure to 200 µM H_2O_2. The cells viability was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT) test. Fluorometric methods were used to determine the level of reactive oxygen species (ROS) and lipid peroxidation. H_2O_2 substantially reduced the viability of the cell, followed by increased production of ROS and lipid peroxidation and decreased activity of superoxide dismutase. Pre-treatment of HSL has improved cardiomyocyte viability and decreased the production of raised ROS and lipid peroxidation. HSL has also been able to improve the activity of superoxide dismutase. In conclusion, the damage caused by oxidative stress on cardiomyocytes was reduced by the protective factor found in HSL, which can prevent cardiovascular diseases as it acts as a natural cardioprotective agent.

Keywords: Oxidative stress, cardiomyocytes, Hibiscus, super oxide dismutase, H9c2 cells.

INTRODUCTION

In many critical cardiovascular diseases, in particular heart failure, myocardial ischemic reperfusion injury, atherosclerosis and hypertension (Higashi et al., 2009), oxidative stress has a crucial role to engage in the pathophysiology of these diseases. The reactive oxygen species accumulation that is produced by oxidative stress may lead to several cardiovascular disorders (Dhalla et al., 2000). Reactive oxygen species (ROS) generation in the heart from a cellular source include cardiac myocytes, neutrophils and endothelial cells. Obtaining ROS in cardiac myocytes may be done from the mitochondria, NADPH oxidase, xanthine oxidase, and uncoupled nitric oxide synthases (Tsutsui et al., 2011). High levels of ROS leads to significant damage to myocardial cells. In the neutralization of elevated reactive oxygen species, catalase, natural antioxidant enzymes: superoxide dismutase (SOD), and glutathione play an important role. By stimulating lipid peroxidation, ROS causes damage to the cellular membrane. Malondialdehyde is a main product of lipid peroxidation and needs to be taken into account when measuring the degree of cellular injury (Dhalla et al., 2000). Hydrogen peroxide (H_2O_2) is the main source of ROS it causes damage to the cell membrane and DNA damage as well as inducing lipid peroxidation. Moreover, antioxidants can prevent cell damage from H_2O_2 via decreasing ROS production (Winstead et al., 2005; Sun et al., 2012). It has been proclaimed in previous studies that H9c2 cells can be protected

against H_2O_2 by certain herbal medicine.

Hibiscus *sabdariffa Linn*. (HSL) or Roselle from the Malvaceae family is used as herbal medicine worldwide. Its high nutritional value can be found in the leaves, seeds and calyx with the calyx being utilized in establishing many drinks, jam and jellies most often (Lin et al., 2011). It has a substantial amount of organic acids such as ascorbic acid, citric acid pectin and polyphenols including flavonoids, phenolic acids, anthocyanin and others (Lin et al., 2011). As a result of the numerous active ingredients present in HSL flower, it is difficult to assess a specific psychopharmacological mechanism underlying its effects. HSL was evaluated to treatment of the risk factors of cardiovascular disease by explicating the randomized clinical trial. HSL extract contain low level of toxicity with LD50 between (2,000 to over 5,000) mg/kg/day (Walton et al., 2016). No toxicity is possible if it is not consumed in high doses of HSL extract on liver or kidneys (Hopkins et al., 2013). Daily consumption of HSL extract from HSL calyxes or tea decrease blood pressure "systolic and diastolic blood pressure" in adult, and reduce level of total cholesterol, low-density lipoprotein cholesterol (LDL-C) while high-density lipoprotein cholesterol (HDL-C) are not affected by using HSL extract (Chen et al., 2003). The effect of antioxidant on anthocyanins is inhibition of LDL-C oxidation which is risk factor of cardiovascular disease, and obstruction atherosclerosis. However, anthocyanins, polyphenols considered phytochemicals and HSL acid as they response to antihypertensive and hypocholesterolemia effects (Hopkins et al., 2013). Studies have recently demonstrated that HSL has a number of pharmacological activities, including anti-microbial (Hopkins et al., 2013; Sorachai et al., 2011), antioxidant, anti-inflammatory, and anticarcinogenic effects (Forman and Ursini, 2011). There is a beneficial impact from HSL products on the treating and preventing cardiovascular diseases, cancers, neurological disorders and diabetes (Hopkins et al., 2013; Lin et al., 2011; Cid-Ortega and Guerrero-Beltrán, 2015; Agoreyo et al., 2008). Previous investigations have reported that HSL causes regeneration of epidermal tissue (Lin et al., 2011), in vivo boosting of the immune system, reduction of triglycerides in the liver and has a colon and prostate cancer chemo preventive property (Pérez-Torres et al., 2013; Wu et al., 2018; Hirunpanich et al., 2005). The therapeutic effects of HSL seeds can be associated with having an array of active compounds, notably polyphenols with its antioxidant properties (Pérez-Torres et al., 2013; Wu et al., 2018). Moreover, HSL seed has a large supply of polyunsaturated fatty acids (PUFAs) along with conjugated linolenic acid that can improve human health as it is an important therapeutic agent (Al-Okbi et al., 2017).

It was derived from these findings that HSL could be considered to have protective effects against H_2O_2-induced cardiomyopathy. As a result, this study was conducted to analyze the effect of HSL on the activity of ROS content in H9c2 cardiomyocyte, antioxidant enzyme SOD and lipid peroxidation level.

MATERIALS AND METHODS

Preparation of HSL

HSL's calyces were collected from the market in Jeddah, Saudi Arabia. In a 48 h Soxhlet apparatus, the calyces were dried, powdered and extracted with 70% ethanol. The HSL was then dried in a water bath and dissolved the yield in Dimethyl sulfoxide (DMSO) (24% w/w).

Cell Culture line and Treatment

Thermo Fisher Scientific Inc. (Waltham, MA, USA) provided the cell culture materials. The American Type Culture Collection produced the H9c2 cells originating from the rat embryonic cardiomyocytes. H9c2 cells line were cultured in Dulbecco's Modification of Eagles Medium (DMEM) and with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 µg/ml streptomycin. H9c2 cells were maintained at 37 °C in the presence of 5% CO_2 and 95% humidified air. Cells were developed for a 96-well and 24-well cultivation plates for the experiments, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT)/ ROS assay and malondialdehydebis-(dimethyl acetal) (MDA) assay, respectively. The cells were incubated twice once with HSL (1-100 µM) for 24 h in well plates and then, incubated with 200 µM H_2O_2 for 3 h. For the assessment of HSL toxicity, cells were also exposed to HSL (25-1200 µg/ml) alone for 24 h.

Cell Viability Assay

The viability of the cell was measured using a modified MTT assay as mentioned in previous studies (Mortazavian et al., 2012; Ghorani et al., 2014). At a final concentration of 0.05 %, the cells were cultivated with MTT solution in phosphate buffered saline (5 mg/ml). Finally, the purple formazan precipitate was dissolved in DMSO. The

absorbance of each culture well was tested using a StatFAX303 plate reader at a wavelength of 570 and 620 nm.

Measurement of ROS

A fluorescent probe, 2,7-dichlorofluorescin diacetate (DCF-DA) sample was used to evaluate intracellular ROS levels (Wu and Yotnda, 2011). Later, the incubation of cells was done with DCFH-DA (10 µM) at 4˚C in the dark for 30 min. Then, the fluorescence intensity was noted at a wavelength of 485nm and 530nm for the excitation and emission, respectively. The experiment was conducted 3 times.

Lipid Peroxidation Assay

Malondialdehydebis-dimethyl acetal (MDA) was identified as a product of the lipid peroxidation (Sadeghnia et al., 2013). After incubation, the scraped cells were centrifuged for 30 min (Buega and Aust, 1978), with 400 µl of Trichloro acetic acid (TCA) (15%) and 800 µl of Thiobarbituric acid (TBA) (0.7%) added to 500 µl of cell samples. These were put in a vortex mixer, after which it was heated in a water bath for 40 min. Finally, 200 µl of the sample was relocated to a 96-well plate and the fluorescence intensity was noted at a wavelength of 485nm and 530nm for the excitation and emission, respectively.

Determination of SOD

The SOD activity was detected with an assay kit. The assay used is determined by the inhabitation of the color formed from the reaction of the superoxide radicals with 2-(4-iodophenyl)-3-(4-nitrophenol-5-phenyltetrazolium chloride) forming a red formazan dye and its absorbance at was determined at 505 nm.

Statistical Analysis

The experiments were conducted in triplicate with 3 replicates of each sample. Statistical analysis was performed using SPSS version 22 (Chicago, IL, USA). The results were presented as the means ± standard deviation (SD). Statistical significance was determined using one-way analysis of variance (ANOVA) between the difference in variables within and between groups, respectively. A P-values < 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Frequently oxidative stress related injury is targeted at the heart. Oxidative stress interacts greatly in the pathophysiology of numerous cardiovascular diseases including myocardial ischemic reperfusion injury, heart failure, atherosclerosis and hypertension (Higashi et al., 2009). Cardiomyocytes are exposed to oxidative damage when it undergoes a poor anti-oxidative capacity (Dhalla et al., 2000). Hence, the application of pharmacological methods to avoid oxidative damage in the heart appears to be a successful approach to cardio protection. H9c2 is a cardio myoblast cell line derived from rats that were used to examine heart functions (L'Ecuyer et al., 2004). H9c2 cells present similar morphological traits to immature embryonic cardio myocytes but also maintain many fundamental pathways of the hormonal and electrical signals found in adult cardiac cells. Evidence has shown that the same H_2O_2-induced oxidative stress in H9c2 cells is found in primary cultured rat cardiomyocytes Panche et al., 2016), making the cell line functional to be a model of cardiomyocyte damage induced by oxidative stress. Antioxidants avert the process of oxidation by preventing the start or continuation of the oxidation of the chain reaction. For this current study, the protective effect of HSL on H_2O_2-induced oxidative stress was investigated.

Fig. 1 showed that % cell viability of H9c2 cells in the presence of HSL alone at various concentrations over 24 h. The results revealed that there was a significant decrease ($p < 0.05$) in the cell viability with a concentration of 1200 µg/ml (79.3 ± 2.11 % of control). Other concentrations have not reduced the viability of cells. Fig. 2 represent the effect of HSL on cell viability against H_2O_2. The results showed that H_2O_2 significantly decreased cell viability ($p < 0.05$) to 51.3 ± 2.1 % of control. Pretreatment with 50, 100, 200, 400 and 800 µg/ml of HSL could increase the viability of H9c2 cells to 69.3 ± 1.1 %, 76.2 ± 1.7 %, 82.5 ± 1.3 %, 85.1 ± 1.1 % and 88.2 ± 1.23 %, respectively ($p < 0.05$). However, when H_2O_2 was used with the dose of 25 µg/ml there was no significant change in the increase of cell viability. As seen in fig. 3, H_2O_2 lead to a considerable increase in the level of ROS in H9c2 cells in relation to the control (188.3 ± 2.1 %). HSL at concentrations of 100, 200, 400 and 800 µg/ml were (143.5 ± 2.1%, 126.5 ± 1.6%, 117.9 ± 2.3 % and 111.7 ± 2.23%), respectively. There was a significant decreased in ROS level ($p < 0.05$). No significant reduction in ROS at concentrations of 25 and 50 µg/ml was found. On the other hand, the amount of lipid peroxidation was assessed through the level of its end product, MDA**.**

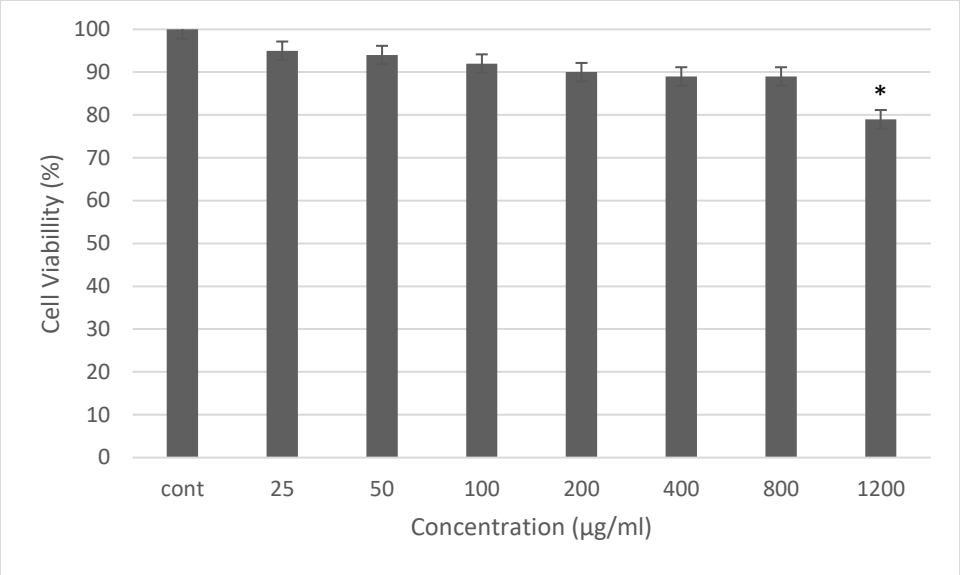

Figure. 1: Effect of HSL alone on cell viability in H9c2 cells.
The cells were treated for 24 h with different concentrations of HSL. Data are expressed as mean ± SD of three separate experiments. * p <0.05 versus control.

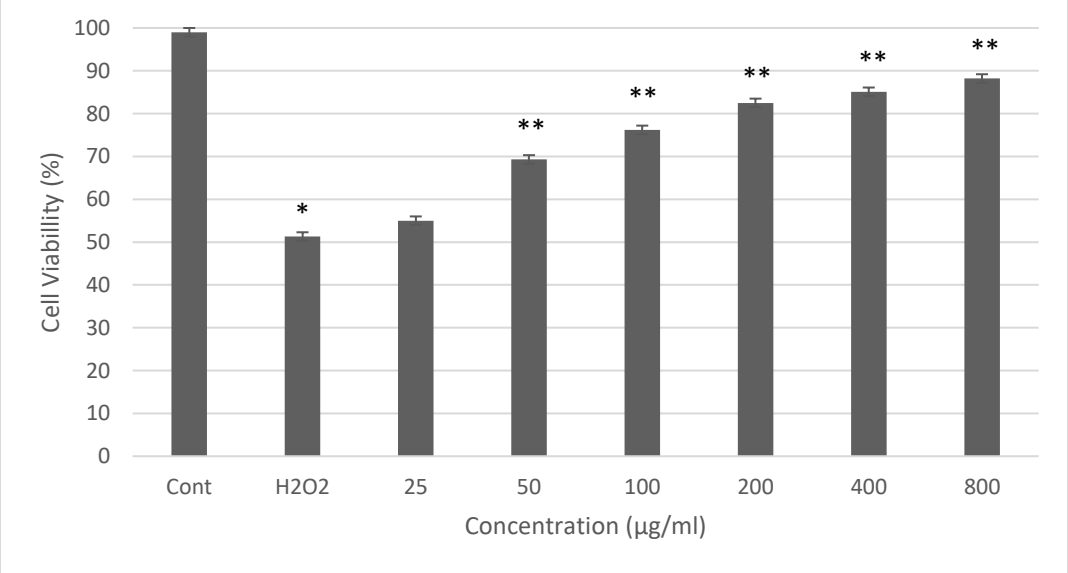

Figure. 2: Effect of HSL on H_2O_2-induced cytotoxicity in H9c2 cells.

The cells were pretreated for 24 h with different concentrations of HSL before to exposure for 1 h to 200 µM of H_2O_2. Data are expressed as mean ± SD of three separate experiments. * p <0.05 versus control, ** p <0.05 versus H_2O_2.

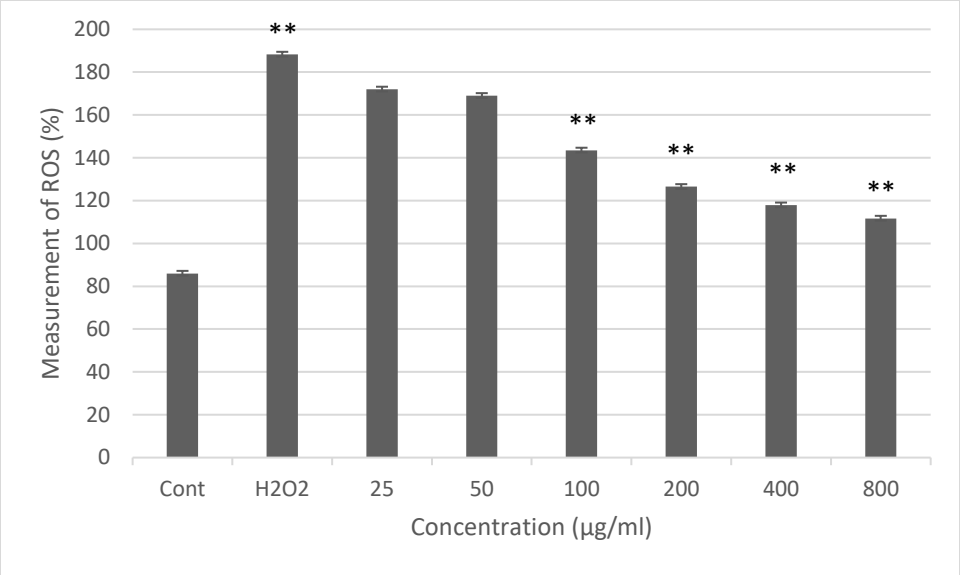

Figure. 3: Effect of HSL on H_2O_2-induced reactive oxygen species (ROS) measurement in H9c2 cells.

The cells were pretreated for 24 h with different concentrations of HSL before to exposure for 1 h to 200 µM of H_2O_2. Data are expressed as mean ± SD of three separate experiments. * p <0.05 versus control, ** p <0.05 versus H_2O_2.

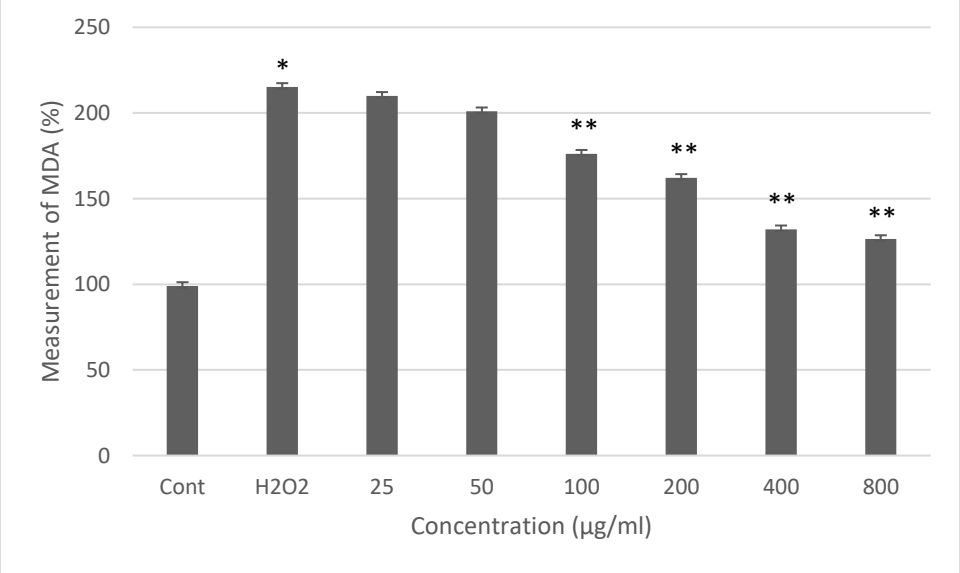

Figure. 4: Effect of HSL on H_2O_2-induced malondialdehydebis-dimethyl acetal (MDA) measurement in H9c2 cells.

The cells were pretreated for 24 h with different concentrations of HSL before to exposure for 1 h to 200 µM of H_2O_2. Data are expressed as mean ± SD of three separate experiments. * p <0.05 versus control, ** p <0.05 versus H_2O_2.

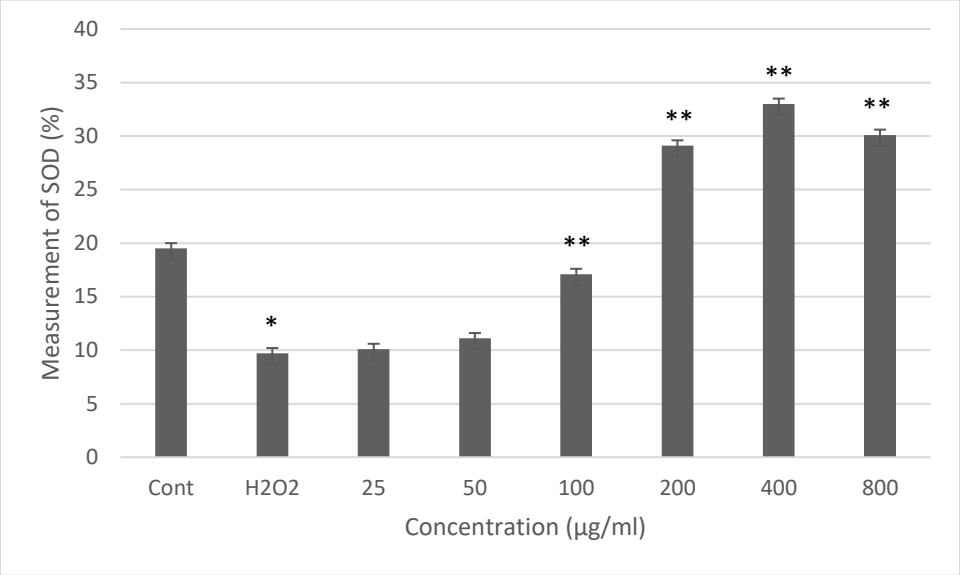

Figure. 5: Effect of HSL on H_2O_2-induced superoxide dismutase (SOD) measurement in H9c2 cells. The cells were pretreated for 24 h with different concentrations of HSL before to exposure for 1 h to 200 µM of H_2O_2. Data are expressed as mean ± SD of three separate experiments. * p <0.05 versus control, ** p <0.05 versus H_2O_2.

As shown in fig. 4, exposing cells to H_2O_2 led to a substantial increase in MDA level (215.2 ± 1.2%) as compared to the control (99 ± 1.1%). The content of MDA in the pretreated cells has significantly decreased giving 100 µg/ml (176.2 ± 3.4%), 200 µg/ml (162.1 ± 1.1%), 400 µg/ml (132.1 ± 1.1%) and 800 µg/ml (126.4 ± 1.3%).

Measuring the level of SOD was intended to deduce the impact of HSL on cellular antioxidant defenses, the level of SOD was calculated (fig. 5). H_2O_2 induced oxidative stress lowered the level of SOD from 19.5 ± 2.1 U/ml (control) to 9.7 ± 1.1 U/ml (p <0.05). In comparison to the untreated cells, the level of SOD has inflated in pretreated cells at HSL concentrations of 100 µg/ml (17.1 ± 1.1 U/ml), 200 µg/ml (29.1 ± 1.1 U/ml) and 400 µg/ml (33 ± 1.6 U/ml) 800 µg/ml (30.1 ± 0.57 U/ml). Latest investigations have proclaimed that polyphenols and flavonoids are beneficial in preventing certain diseases such as inflammatory disorders and cardiovascular by oxidative stress inhibition (Li et al., 2013). Antioxidant compounds of HSL are found in the juice and seeds based on previous studies Singh et al., 2017; Khaghani et al., 2011). The antioxidant mechanism of HSL aids in the cardioprotective activity by free radical scavengers, reduction of lipid peroxidation, inhibition of xanthine oxidase activity and promotion of antioxidant enzymes activity (Costa-Rocha et al., 2014). In-vitro and in-vivo studies have revealed that antioxidant activity of HSL decreased the oxidative stress primary hepatocytes of rats and free radicals scavenged (Tseng et al., 1997). A study has documented that HSL has a preventive effect on lipid peroxidation induced by Pro-oxidant in an isolated brain of rat (Oboh and Rocha, 2008). The mentioned useful effects of HSL were noticed in both water extracts from seeds, flowers or leaves and from ethanolic (Costa-Rocha et al., 2014). Accordingly, the protective effect of HSL attribute by the occurrence of a range of biologically active compounds, for example the antioxidant compounds protect the tissue of HSL. Furthermore, HSL calyces carry effective antioxidant components such as vitamin C and tocopherol, describing the protective effect as it serves in altering α-tocopheroxy radical to α-tocopherol or reducing Ca^{+2} dependent permeabilization of renal cortex mitochondria (Okoko and Oruambo, 2008). Alternatively, antioxidant and radical scavenger of these calyces are linked to the attendance of flavonoids known as anthocyanins (Sini et al., 2011). These findings on the protective properties of HSL on cardiomyocyte signify that it is possible to use these effects to compete with drug induced cardiotoxicity. Doxorubicin, for example, is an antibiotic anthracycline commonly used in cancer

therapy, despite its limited clinical use due to its toxic effects on cardiomyocytes (Xin et al., 2009). Doxorubicin harms metabolic pathways some enzymes and transporter proteins found in the cardiac muscle and can eventually lead to irreversible heart failure (Turakia et al., 2007). Cardiotoxicity induced by doxorubicin works by increasing the production of ROS (Xin et al., 2009). As stated by the results of the present study, HSL and its antioxidant effect on cardiac cells might decrease the doxorubicin-induced cardiotoxic effect, although further studies should be conducted to support this evidence.

CONCLUSION

To summarize, the data has indicated that HSL prevents cardiomyocyte damage induced by hydrogen peroxide, which was managed by reducing lipid peroxidation and ROS production. Thus, HSL could be regarded as a natural source for preventing heart diseases, with further investigations towards the process of these essential effects.

CONFLICT OF INTEREST

The author declared that present study was performed in absence of any conflict of interest.

AUTHOR CONTRIBUTIONS

NMA designed, analyzed the data, and written all aspects of the manuscript. As well as reviewing and approving the final version of the manuscript.

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