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Research Article

Effect of Bezafibrate and *Ginkgo biloba* Extract Combination on Doxorubicin-Induced Cardiotoxicity in Rats

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Abstract

Objectives: This study aimed to evaluate the possible synergistic effect of bezafibrate and ginkgo biloba (GKB) extract on cardiotoxicity induced by doxorubicin. ***Methods***: Thirty rats were allocated into 5 groups: The negative control group was treated daily with 1 ml of distilled water orally by gavage tube; the positive control received doxorubicin 3.7 mg/kg on day 11 for 3 days intraperitoneally; the bezafibrate group received 100mg/kg orally by gavage tube; the GKB group received 60mg/kg orally by gavage tube; and the combination of bezafibrate and GKB group. All the groups received the doxorubicin protocol, with an exception for the negative control. The treatment continued for 14 days. On day 14, blood samples were taken for the measurement of serum levels of troponin, natriuretic peptide, creatine phosphokinase (CPK), IL-6, and total lipid profile. The atherogenic index, cardiac risk, and LDL/HDL ratios were calculated. Cardiac tissues were sent for histopathological analysis. ***Results***: Both bezafibrate and GKB exhibited attenuation of troponin, natriuretic peptides, CPK, IL-6, TG, cardiac risk ratio, and atherogenic index, as well as an increase in HDL levels. However, the combination group showed the greatest effect compared to the positive control group. The histopathological findings supported the biochemical outcomes. ***Conclusions***: Combining GKB extract and bezafibrate protects against cardiac injury by restoring injury markers and IL-6, as well as improving the lipid profile, cardiac risk ratio, and atherogenic index.

Keywords: Bezafibrate, Cardiotoxicity, Cardiac injury markers, Doxorubicin, GKB extract.

تأثير تركيبة مستخلص بيزافيبرات والجنكة بيلوبا على السمية القلبية التي يسببها دوكسوروبيسين في الجرذان

الخلاصة

الأهداف: تهدف هذه الدراسة إلى تقييم التأثير التآزري المحتمل لمستخلص بيزافيبرات والجنكة بيلوبا على السمية القلبية التي يسببها دوكسوروبيسين. **الطريقة**: تم توزيع ثلاثين جرذا في 5 مجموعات: تم علاج المجموعة الضابطة السلبية يوميا ب 1 مل من الماء المقطر عن طريق الفم. تلقت المجموعة الضابطة الإيجابية دوكسوروبيسين 3.7 مغ/كغ في اليوم 11 لمدة 3 أيام داخل الصفاق. تلقت مجموعة bezafibrate 100mg / kg عن طريق الفم وتلقت مجموعة GKB 60 مجم / كجم عن طريق الفم ؛ والجمع بين bezafibrate ومجموعة GKB. تلقت جميع المجموعات بروتوكول دوكسوروبيسين ، باستثناء السيطرة السلبية. استمر العلاج لمدة 14 يوما. في اليوم 14 ، تم أخذ عينات الدم لقياس مستويات التروبونين، والببتيد الناتريوتريك، وفسفوكيناز الكرياتين و IL-6، والمستوى الكلي للدهون في المصل. تم حساب مؤشر تصلب الشرايين ومخاطر القلب ونسب LDL / HDL. تم إرسال أنسجة القلب للتحليل النسيجي المرضي. **النتائج**: تسبب كل من bezafibrate و GKB في خفض التروبونين والببتيدات الناتريوتريك و CPK و IL-6 و TG ونسبة مخاطر القلب ومؤشر تصلب الشرايين، بالإضافة إلى زيادة في مستويات HDL. ومع ذلك ، أظهرت المجموعة المركبة أكبر تأثير مقارنة بمجموعة التحكم الإيجابية. دعمت النتائج النسيجية المرضية النتائج الكيميائية الحيوية. **الاستنتاجات**: الجمع بين مستخلص GKB و bezafibrate يحمي من إصابة القلب عن طريق استعادة علامات الإصابة و IL-6 ، وكذلك تحسين ملف الدهون، ونسبة مخاطر القلب ومؤشر تصلب الشرايين.

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INTRODUCTION

Doxorubicin (DOX) is an anthracycline anticancer drug that is frequently used in the treatment of various malignancies. It has been used to treat lymphoma and leukemia, in addition to urogenital, breast, and gastric cancer [1]. The key mechanisms by which doxorubicin produces its anticancer effect include preventing the action of topoisomerase II by intercalation with DNA [2]. The use of doxorubicin as a chemotherapeutic agent has been associated with some serious side effects, especially cardiotoxicity [3,4]. Various mechanisms

contribute to doxorubicin-induced cardiotoxicity; it has been shown to induce oxidative stress through initiating lipid peroxidation through increasing the production of reactive species (RS) [5]. Doxorubicin causes cardiomyocyte death by inducing apoptosis [6]. Other mechanisms of cardiotoxicity include intracellular calcium dysregulation by blocking the sodium-calcium exchanger channel, which has a key role in heart contractility, and mitochondrial damage through binding to different mitochondrial proteins [7,8]. Cardiotoxicity limits the use of doxorubicin; therefore, preventive and therapeutic methods to reduce doxorubicin-induced cardiotoxicity are a priority. Bezafibrate is an agonist of peroxisome proliferator-activated receptor (PPAR), which has been used as an antihyperlipidemic agent [9]. Many studies have shown the antioxidant effect of bezafibrate via reducing reactive oxygen species (ROS) and lipid peroxidation. Additionally, it demonstrated a protective effect in streptozotocin-produced oxidative damage in rats [10,11]. Bezafibrate has been shown to attenuate apoptosis in palmitate-induced apoptosis in osteoblastic cells in rats [12]. Additionally, bezafibrate also demonstrated anticancer [13] and neuroprotective effects [14]. These studies render Bezafibrate to be a good candidate for screening its potential cardioprotective effect in doxorubicin-induced cardiotoxicity. *Ginkgo biloba* is a herbal medicine that has been part of traditional Chinese medicine; it has been known for its therapeutic effects as cardioprotective, hepatoprptective, antidiabetic, antiasthmatic, and CNS stimulant [15-16]. *Ginkgo biloba* is also known for its anti-inflammatory activities [17–18]. In addition, the antioxidant effects of *Ginkgo biloba* are well documented in many studies [19,20]. The current study aimed to evaluate the possible synergistic effect of bezafibrate and ginkgo biloba extract in animal models to ameliorate cardiotoxicity induced by doxorubicin.

METHODS

Chemicals

The chemicals used in the current study were purchased from drug companies as follows: Adriamycin® doxorubicin hydrochloride injection (50mg/25ml) from Pfizer. Ginkgo biloba extract standard powder (EGb 761) from Apollo Healthcare Resources, Singapore, and Bezalip® bezafibrate tablets (200mg) from Actavis.

Experimental animals

In the current study, thirty male Wister albino rats weighing 150–180 g were used and kept in well-ventilated plastic cages under standard conditions of temperature (25 ± 2°C), humidity (55 ± 5%), and 12 hours of dark/light cycle. They were fed a conventional pellet diet and had unlimited access to water. The Ethical Committee of the University of Sulaimani, College of Pharmacy (Certificate no. PH69-22 on 18th October 2022) approved the experimental protocols, adhering to the Institutional Animal Ethics Committee's guidelines. The Canadian Council on Animal Care (CCAC) guidelines guided the performance of the study.

Study protocol

Thirty rats were randomly assigned into five groups (6 rats per group) and received the following treatments: The first group acted as the negative control (NC), receiving 1 mL of distilled water (D.W.) orally for 14 days. The second group functioned as a positive control (PC) and received 1 mL of D.W. orally for 14 days; on day 11, they received DOX as an intraperitoneal injection (3.7 mg/kg/day) for three days [21]. The third group was treated with bezafibrate (100 mg/kg) orally for 14 days, followed by the DOX regimen. The fourth group got Ginkgo biloba (60 mg/kg) orally for 14 days, along with the DOX procedure [23]. The fifth group got a combination of BZF 100 mg/kg and GKB 60 mg/kg orally for 14 days, as well as the DOX regimen. Twenty-four hours following the final dosage of doxorubicin, rats were sedated with intraperitoneal injections of ketamine (50 mg/kg) and xylazine (5 mg/kg) and prepared for biochemical and histological analyses.

Biochemical tests

Blood samples were collected by cardiac puncture and used for the measurement of serum levels of troponin, natriuretic peptide, CPK, IL-6, and total lipid profile using ELISA kits (Bioassay Technology Laboratory, Shanghai, China). The cardiac risk ratio (CRR) is calculated using the following equation [4]:

CRR = Total cholesterol/HDL

The atherogenic index in plasma is also calculated using the following equation [4]:

Atherogenic index in plasma = log (TG/HDL)

Histopathological examination

The histological protocol was performed at the end of the study. The animals were fasted before being sacrificed and then euthanized in a human practice. Consecutively, after animal scarification, necropsy findings were started by collecting tissue samples for histological preparation. Heart samples were fixed into tissue cassettes and then dipped into a 10% buffered formaldehyde solution for about 48 hours. Thereafter, sections were dehydrated by passing through a series of ascending ethanol alcohols, followed by three steps of xylene clearance. Next, the processed sections were infiltrated and embedded in melted paraffin blocks using an automated wax embedder at 60–70 °C. Paraffin-blocked tissues were sectioned to 5 µm using a semi-

automated rotary microtome. After that, tissue sections were placed on glass slides and dried using a hot plate tissue holder. Later on, tissue sections were deparaffinized and cleaned with xylene solution for 30 minutes, then dried for 5 minutes. Finally, tissue sections were stained with Harris's hematoxylin and eosin solution, cleaned with xylene, and cover slipped.

Semi-quantitative lesion scoring

Lesion scoring was determined semi-quantitatively using image analyzer software (AmScope, 3.7) and a microscope eyepiece camera (MD500, 2019), while tissue samples were examined under a light microscope (NOVEL XSZ-N107T, China). Damages in cardiac slices were quantified in µm and statistically assessed as mean percentages. These types of harm included degenerative alterations, fatty infiltration, and vascular congestion. In contrast, inflammatory and cardiac degenerative cells were counted in ten randomly selected fields under high power magnification (1000X), and the mean average was determined statistically as a percentage. The average % of all calculated values was reported as follows: Score 0-10% for no lesions; 10-25% for mild; 25-50% for moderate; 50-75% for severe; and 75-100% for significant lesions.

Statistical analysis

The statistical analysis was performed using GraphPad Prism 8. The values of the measured parameters were expressed as mean ± standard deviation (S.D.). For the comparisons between different groups, one-way analysis of variance (ANOVA) was followed by Tukey's test to compare each group with the positive control group. The results were considered statistically significant when the p-value was less than 0.05.

RESULTS

Serum troponin levels were higher in the positive control (PC) group, but the difference was not statistically significant. When compared to the PC group, all treatment groups had a reduced level (Figure 1A). The natriuretic peptide level increased significantly in the PC group (p= 0.002) compared to the negative control (NC) group. The levels decreased in all treatment groups, although the combination group had a high level (p= 0.035) (Figure 1B). The study discovered that the level of CPK was considerably greater in the PC group (p= 0.0017) than in the NC group. However, CPK levels were considerably lower in the BZF, GKB, and combination groups than in the PC group (p= 0.002, p= 0.0052, and p= 0.007, respectively) (Figure 1C). The study found that the PC group had significantly higher levels of IL-6 than the NC group (p= 0.0073). IL-6 levels dropped significantly in all treatment groups, including those given bezafibrate, GKB, or both (Figure 1D). The combination group showed the greatest benefit (p= 0.0032, p= 0.0037, and p= 0.0003).

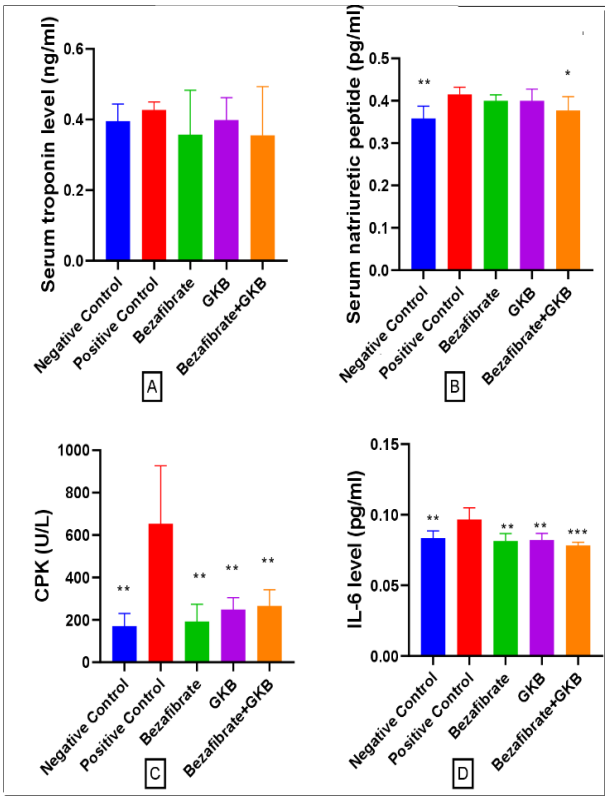

Figure 1: Effect of Bezafibrate and GKB alone or in combination on A) Serum troponin, B) Serum natriuretic peptide, C) CPK, and D) IL-6. Values were presented as mean ± S.D (n= 6 animals in each group); values with (*) are significantly different from the positive control using ANOVA and post hoc test (* p<0.05), (** p<0.01), and (*** p< 0.001).

The total cholesterol levels did not differ substantially across groups (p> 0.05) (Figure 2A). However, TG levels were considerably greater in the PC group than in the NC group (p= 0.011), and all three treatment groups (BZF, GKB, and combination) had significantly reduced TG levels (p= 0.0009, p= 0.0009, and p= 0.0001, respectively) (Figure 2B). All of the examined groups had no significant changes in their LDL levels (p > 0.05) (Figure 2C); however, both the GKB and combination groups had significantly higher HDL levels than the PC (p = 0.04 and p = 0.01, respectively) (Figure 2D). The cardiac risk ratio was higher in the PC group, but it did not reach a significant level when compared to NC, and all treatment groups were able to reduce it; nevertheless, only the combination group achieved a significant level (p= 0.04) (Figure 2E). In terms of the atherogenic index, the PC group had a significant increase when compared to the NC (p= 0.007), whereas all of the treatment groups—BZF, GKB, and the combination groups—had a significant decrease when compared to the PC group (p= 0.0001, p= 0.0001, and p= 0.0001, respectively) (Figure 1F). The LDL/HDL ratio did not alter significantly across treatment groups (Figure 2G).

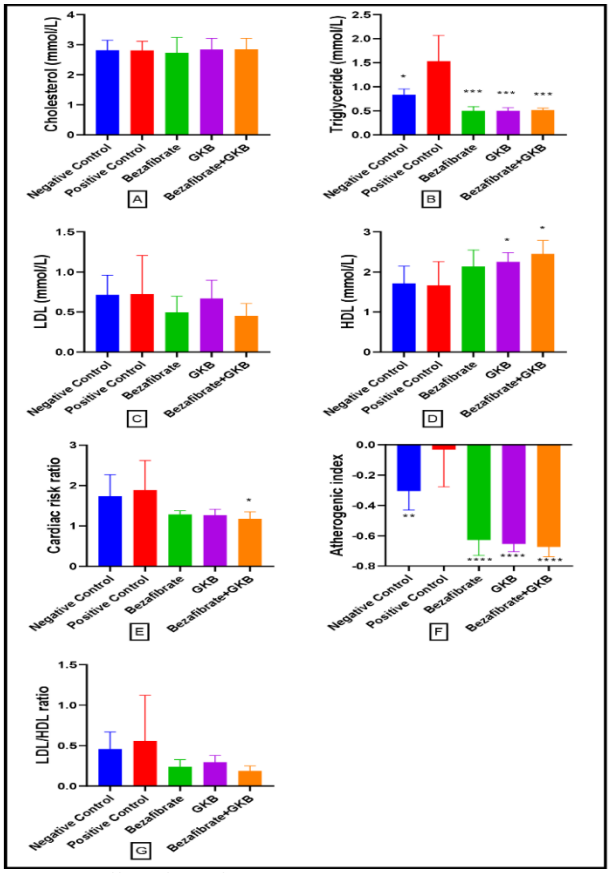

Figure 2: Effect of Bezafibrate and GKB alone or in combination on A) Cholesterol, B) TG, C) LDL, D) HDL, E) Cardiac risk ratio, F) Atherogenic index, and G) LDL/HDL. Values were presented as mean ± S.D (n= 6 animals in each group); values with (*) are significantly different from the positive control using ANOVA and post hoc test (* p < 0.05), (** p< 0.01), (*** p<0.001), and (**** p<0.0001).

Table 1 shows the quantitative morphometric assessment of heart sections. Rats given both Bezafibrate (BZF) 100 mg/kg and Ginkgo biloba (GKB) 60 mg/kg had a substantial decrease (p<0.05) in the mean percentage of cardiac degenerative cells and inflammatory cells. The number of fatty infiltrations and the area of vascular engorgement decreased significantly in the combo group as compared to the PC group that received three doses of doxorubicin (DOX) at 3.6 mg/kg. Furthermore, cardiac slices from the BZF group (treated with 100 mg/kg of BZF) and the GKB group (treated with 60 mg/kg of GKB) reveal that the lesions are not as severe as those in the PC group. In comparison to the other treatment groups, the combination group has significantly lower lesion scoring and grading. Figure 3 depicts the degree of morphological alterations in heart parenchymal tissue in both the positively treated and untreated groups, providing additional information.

DISCUSSION

Drug-induced cardiotoxicity is still one of the contributing factors in restraining the use of cancer therapeutic agents, including doxorubicin [24,25]. In the current study, the deleterious effect of doxorubicin clearly appeared as the high level of troponin, natriuretic peptide, CPK, and IL-6 in addition to the changes noticed in the cardiac tissues. Troponin, natriuretic peptide, and CPK are specific biomarkers raised during cardiac injury. Troponin is a protein released from cardiac muscle in response to cardiac damage or infarction [26]. Meanwhile, atrial natriuretic peptide is a hormone that has crucial roles in regulating neuro-hormonal pathways, vascular tone, cell proliferation, and fluid and electrolyte balance. A high level of this hormone can be used as a diagnostic biomarker for cardiac problems [27]. Another important cardiac biomarker is CPK, which plays an important role in providing the muscles, including the myocardium, with ATP. Its level increases during cardiac damage due to the free level of myoglobin [28]. The use of bezafibrate and GKB together was effective in ameliorating the toxic effects of doxorubicin. Bezafibrate is a medication that is primarily used to treat high cholesterol and triglyceride levels in the blood. It works by reducing the production of triglycerides and increasing the production of HDL, which can help protect the heart and blood vessels from damage [29]. Several studies have investigated the cardioprotective effects of fibrates and have shown to reduce the risk of cardiovascular events, including coronary artery disease, stroke, and peripheral artery disease [30,31]. On the other hand, there is some evidence to suggest that Ginkgo biloba may have a protective effect on the heart by reducing oxidative stress and improving endothelial cell function [32,33]. Interestingly, the hypolipidemic effect of GKB was comparable to that produced by bezafibrate. The findings of the current study showed the hypolipidemic effects and obvious attenuation in the atherogenic index and cardiac risk ratio of both bezafibrate and ginkgo biloba alone or in combination, adding new evidence on the cardioprotective effects of both agents. Bezafibrate has also been shown to attenuate the mortality rate by 10% in patients with coronary heart disease and 25% in those with high TG levels [34]. Furthermore, bezafibrate is known to have antioxidant [10] and anti-inflammatory properties [35], which may help protect against doxorubicin-induced cardiac toxicity. In a study conducted on rats, pretreatment with gemfibrozil (another derivative of fenofibrate) was found to attenuate doxorubicin-induced oxidative stress and reduce cardiac damage [36]. Additionally, GKB is a popular herbal supplement that is known to have antioxidant and anti-inflammatory properties. Ginkgo biloba has been shown to improve cognitive function, including memory, attention, and information processing speed, in both healthy individuals and those with cognitive impairment or dementia [37,38].

Table 1: Micromorphological quantitative assay of heart sections

Experimental Groups n=6	Inflammatory Cells (%)**	Cellular Degeneration (%)	Fatty Infiltration*(%)	Vascular Congestion* (%)	Lesion Scoring (%)	Lesion Grading
NC group †	3.46^{A}#	2.89^{A}	6.39^{A}	6.71^{A}	0-10	No lesion
PC group	81.76^{E}	76.81^{E}	77.19^{E}	82.56^{E}	75-100	Critical
BZF group	68.45^{D}	64.32^{D}	59.11^{D}	65.57^{D}	50-75	Severe
GKB group	59.44^{D}	61.39^{D}	54.91^{D}	66.28^{D}	50-75	Severe
Combination group	44.61^{C}	50.22^{C}	48.72^{C}	51.81^{C}	25-50	Moderate

Notes: *Myocardial degenerative cells and inflammatory cells were calculated in % of a mean of cell numbers. Area of fatty infiltration and vascular congestion were estimated by mean % µm. **Each value represents mean ± SD (n=6). #Statistical comparison among groups: Mean values with different capital letters are significantly different ($p<0.05$). Negative control group (NCG) † Distilled water; Positive control group, Doxorubicin (DOX) 3.7 mg/kg; BZF group: DOX with Bezafibrate (BZF) group 100 mg/kg; GKB group: DOX with Ginkgo biloba (GKB) group 60 mg/kg; Combination group: DOX 3.7 mg/kg with Bezafibrate 100mg/kg with Ginkgo biloba 60 mg/kg.

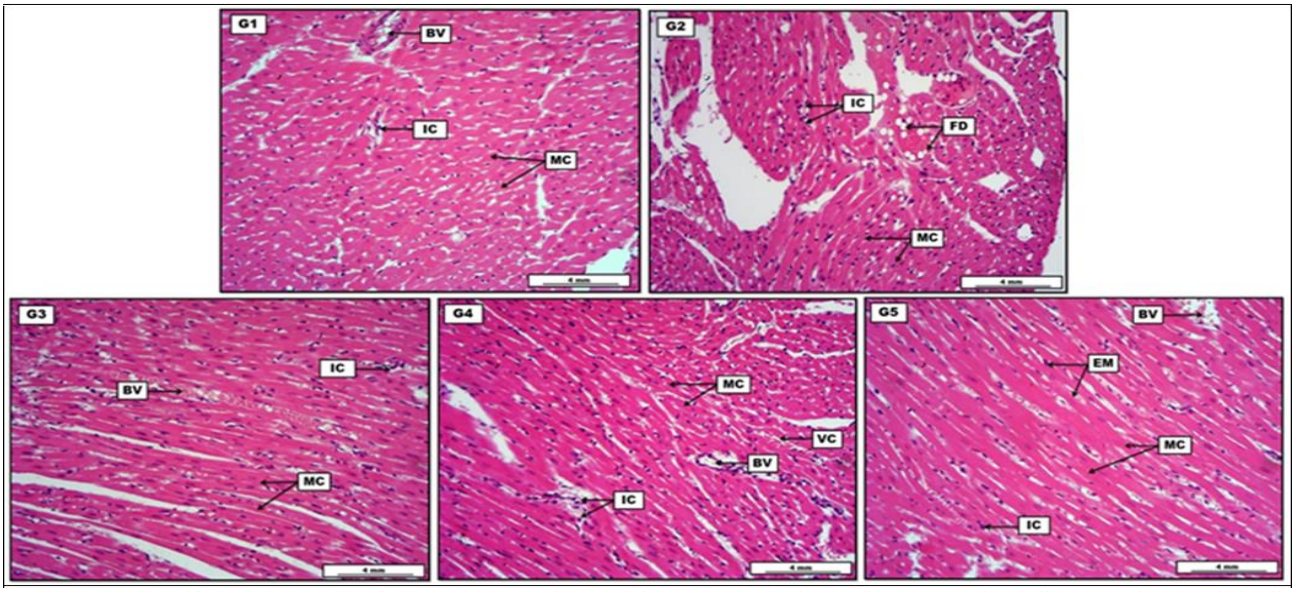

Figure 3: Images of heart tissue sections from groups; (Negative control group): Received D.W, demonstrate classically arranged myocardial cells (MC), with low-grade infiltration of inflammatory cells (IC) within the stromal tissue, the section also reveals the cross-sectional structure of some coronary artery brunches (BV). (Positive control group): Received 3.7 mg/kg Doxorubicin for three days, showing the significant distribution of fatty degenerative lesions (FD) within the myocardial cells (MC) together with diffuse infiltration of inflammatory cells (IC) within the cardiac mesenchymal tissue. (Bezafibrate group): Received Doxorubicin and treated with 100 mg/kg of Bezafibrate, revealing a significant longitudinal section from a congested blood vessel (BV), together with significant infiltration of inflammatory cells (IC), furthermore, the section shows normally appeared myocardial muscle (MC)with their acidophilic cytoplasm. (GKB group): Received Doxorubicin and treated with 60 mg/kg of GKB, demonstrating moderately graded pre-vascular cuffing of inflammatory cells (IC) which border longitudinal sections of some blood vessels (BV). Myocardial cells (MC) display a usual arrangement with deep acidophilic cytoplasm. (combination group): Received Doxorubicin and treated with 100 mg/kg of Bezafibrate and 60 mg/kg of GKB, showing moderate to low-grade infiltration of inflammatory cells (IC). Additionally, the section reveals vascular congestion of many blood vessels (BV). Myocardial muscles (MC) reveal oval nuclei with a cross-striated appearance together with some eosinophilic myocardial muscle cells (EM) with their hyperchromatic nuclei. H&E. Scale bars: 4 mm with 3X magnification power.

The mechanism of action may involve increasing blood flow and oxygen delivery to the brain and/or reducing oxidative stress and inflammation [39]. Ginkgo biloba has been shown to improve vascular function, reduce blood pressure, and decrease platelet aggregation in some studies [40,41]. These effects may be due to its antihypertrophic activity and its ability to increase nitric oxide production and/or reduce oxidative stress [16]. Numerous studies suggested the antioxidant activity of GKB [19,42,43]. In a study conducted on rats, pretreatment with GKB was found to attenuate doxorubicin-induced cardiac damage and improve cardiac function by reducing oxidative stress and inflammation [44]. In the present study, GKB extract effectively restored cardiac markers and the anti-inflammatory marker IL-6; however, more protection was observed in the combination of bezafibrate with GKB. Besides its primary use as a lipid-lowering agent, bezafibrate has been found to have pleiotropic effects, which means it can affect multiple pathways and have beneficial effects such as anti-inflammatory effects, and this was clear in the current study in ameliorating cardiac markers and IL-6. Bezafibrate has been shown to reduce inflammation by inhibiting the production of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6. This effect has been demonstrated in animal studies as well as in human studies of patients with chronic inflammatory conditions such as rheumatoid arthritis and Crohn's disease [45,46]. Moreover, bezafibrate has been found to have antioxidant

properties by reducing oxidative stress and eventually preventing damage to cells and tissues [10,11]. The histopathological finding greatly supports the biochemical results, where the maximum effect shown by the combination group manifested as alleviation in the inflammatory cell, cellular degeneration, infiltration of fatty cells, vascular congestion, and lesion scoring. All the aforementioned mechanisms of both GKB and bezafibrate may explain the cardioprotective effect of this combination.

Conclusion

The current study showed that combining GKB extract with bezafibrate can protect the heart by lowering levels of troponin, natriuretic peptide, and CPK, as well as IL-6, which is a marker of inflammation, and raising levels of lipids, cardiac risk ratio, and atherogenic index. Furthermore, the histopathological findings showed maximum protection from this combination. The results may indicate that this combination has a possible synergistic effect.

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Conflict of interests

No conflict of interests was declared by the authors.

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Data sharing statement

Supplementary data can be shared with the corresponding author upon reasonable request.

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