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Research Article

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Eucalyptus Globulus Ethanol Extract Mitigates Formalin-Induced Acute Cardiotoxicity by Lowering Uric Acid and Lactate Dehydrogenase in Male Swiss Mice

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Abstract Background: *E.globulus* is a medicinal plant which has been shown to possess and exhibit various beneficial properties like anti-inflammatory, anticancer, antibacterial, antiseptic and astringent. Aim: To investigate the cardioprotective properties of *E. globulus* against formalin-induced acute cardiotoxicity in male Swiss mice and to determine the possible mechanism of its cardioprotective effects. Materials and Methods: Thirty animals were randomly selected into six groups of five animals each as follows; Control group received distil water, extract group (500mg/kg *p.o* daily), formalin group (20µl/kg *i.p*) formalin+pro+extract (propranolol, 40mg/kg with *E.globulus* extract 500mg/kg *p.o* daily), formalin+gli+extract group (glibenclamide, 8mg/kg with *E.globulus* extract 500mg/kg *p.o* daily) formalin+nife+extract group (nifedipine, 10mg/kg with *E.globulus* extract 500mg/kg *p.o* daily). The treatment period lasted for two weeks. The mechanism of action was investigated using adrenergic, L type voltage gated calcium channel, and ATP sensitive K^+ channel blockers. Results: Cardiotoxicity was assessed by measuring cardiac uric acid, lactate dehydrogenase, urea, and some haematological indices. Treatment with *E. globulus* (500mg/kg) significantly ($p<0.05$) decreased the levels of cardiac uric acid and lactate dehydrogenase activity. Conclusion: These results suggest that *E. globulus* has the potential in preventing the cardiotoxic effects induced by formalin possibly through a mechanism that may involve the adrenergic system and the L-Type Voltage gated Calcium channel.

Keywords Formalin, *E.globulus*, uric acid and lactate dehydrogenase

Introduction

Medicinal plants play a vital role in world health and have been regarded as one of the primarily used agents since ages for the treatment and prevention of a number of diseases.*Eucalyptus globulus (E. globulus)* is an over green tree which belongs to the family of Myrtaceae. From ancient time, the use of medicinal plants in subcontinent is very common issue. *E. globulus* is a rich source of phytochemical constituents which contain flavonoids, alkaloids, tannins and propanoids, which are present in the leaf, stem and root of the plant. Numerous studies have shown that

E. globulus exhibit various properties like anti-inflammatory, anticancer, antibacterial, antiseptic and astringent. The leaves of *E. globulus* have been used as a natural remedy for the treatment of several diseases such as influenza [19] fungal infections [43] pulmonary tuberculosis [38] and diabetes [23]. The antioxidant properties of the *E. globulus* bark has been reported by scientist; however, the mechanism is not yet established.

Formalin can induce oxidative stress by increasing the formation of reactive oxygen species (ROS) [6][37][42]. Formalin intoxication may stimulate oxidative stress and thus, some secondary toxic effects in cardiac cells and tissue [18]. Uric acid is a strong antioxidant in the body and has been shown toparticipate in the pathophysiology of human disease [27]. Lactate dehydrogenase (LDH), an intracellular enzyme constitutes a major checkpoint of anaerobic glycolysis, by catalyzing the conversion of lactate to pyruvate and indicates cellular damage [17].

Uric acid (UA) is an end product of purine metabolism and acts as an antioxidant which accounts for 50% of the total antioxidant capacity of biological fluids in humans. When present in cytoplasm of the cells or in acidic/hydrophobic milieu in atherosclerotic plaques, UA converts into a pro-oxidant agent and promotes oxidative stress. Through this mechanism, UA participates in the pathophysiology of human disease including cardiovascular disease (CVD). Evidence available also suggests an association between elevated UA and traditional cardiovascular risk factors, metabolic syndrome, insulin resistance (IR), obesity, non-alcoholic fatty liver disease and chronic kidney disease [27][49]. Experimental and clinical studies have evidenced several mechanisms through which elevated UA level exerts deleterious effects on cardiovascular health including increased oxidative stress, reduced availability of nitric oxide and endothelial dysfunction, promotion of local and systemic inflammation, vasoconstriction and proliferation of vascular smooth muscle cells, IR and metabolic dysregulation. Although the causality in the relationship between UA and CVD remains unproven, UA may be pathogenic and participate in the pathophysiology of CVD by serving as a bridging mechanism mediating or potentiating the deleterious effects of cardiovascular risk factors on vascular tissue and myocardium [34][35][15]. The association between UA and CHD or mortality has been extensively investigated [27]. However, epidemiological evidence on the association between UA and CHD or mortality remains controversial with studies supporting [8][21][33][34]] or refuting [12][45][47] such an association. UA-lowering therapy has been used for putative beneficial effects in terms of reduction of CHD events and as an instrument to investigate causality relationship between UA and CHD. UA is correlated closely with almost all known cardiovascular risk factors [35][40][42], IR [27][49], metabolic syndrome [35][15], obesity [31], non-alcoholic fatty liver disease [6] and chronic kidney disease [28][22]. In many instances, there is a mutual relationship between UA and these conditions and teasing out the individual contribution of each factor has proven difficult. In this regard, an elevated UA level may be seen as a correlate of cardiovascular risk or an epiphenomenon of co-existing cardio-metabolic risk factors (a risk marker). Also, UA is a product of XOR, which is known to be one of the most important courses of ROS in organism. Elevated UA level may be a marker or a consequence of up-regulated or increased XOR activity and increased oxidative stress. XOR per se has far reaching implications in CVD mostly but not entirely related to ROS generation and increased oxidative stress [3][5]. XOR is closely related with another major ROS producer, the enzyme NADPH oxidase (ROS produced by XOR activate NADPH oxidase and vice versa) [3]. Recent studies have shown an independent positive association between UA and cardiovascular mortality [14][48]. Earlier, UA was suggested to play a role in protecting man from the damaging action of oxygen free radicals and may be partly responsible for man's relatively long lifespan. This further suggested that UA protects the enzyme lactate dehydrogenase (LDH) from free radical mediated damage [25]. However, recent studies showed that elevated UA is accompanied with overexpression of LDH gene in incidence of cardiotoxicity which is commonly associated with cancer cells muscle damages [7][13]. In addition, report from a study showed that UA can be a prognostic factor of cardiovascular mortality and morbidity in the general population and LDH can be useful prognostic factor for survival time of terminally ill cancer patients as well as a predictor of mortality in general cancer populations [41] but the mechanism remains elusive.

Lactate dehydrogenase (LDH or LD) is an enzyme found in nearly all living cells. LDH catalyzes the conversion of lactate to pyruvate and back, as it converts NAD^+ to NADH and back. LDH is expressed extensively in body tissues, such as blood cells and heart muscle. Because it is released during tissue damage, it is a marker of common injuries and disease such as heart failure. High LDH level was associated with poor therapy response and recurrence

of cancer [26][20]. Thus LDH has been suggested to be a marker of tumour aggressiveness and aggravate muscle damage. LDH is also related to the damage to cardiac muscles, lung, and erythrocytes and thus can be regarded as a marker of sepsis or multiple organ failure [4][9].

Globally, the leading causes of death are heart disease (12.2% of all deaths) related [31] and research has attributed some of deaths to cardiotoxicity. Toxicity problems appear only in the presence of additional cardiovascular disease conditions or develop months/years following the exposure, making the diagnosis difficult. Cardiotoxicity may be mitochondria-related which can further culminate in to cell death [44]. Therefore, this study was set out to investigate the potential cardioprotective effects of *E. globulus* extract and the possible mechanism through which it exhibit the cardioprotective actions.

Materials and Methods

Animals

Adult male Swiss mice (22-30g), obtained from the animal house facility of college of health science, Kogi State University, Anyigba, were used in this study. They were acclimatized for about two weeks, kept under standard laboratory conditions and fed on rodent cubes.

Collection and Extraction of Eucalyptus globulus leaf

Eucalyptus globulus (*E.globulus*) leaves were obtained from the premises of the Faculty of Agriculture Kogi State University, Anyigba Kogi State. Authentication was done at Forest Research Institute of Nigeria (FRIN) Ibadan. The leaves were air-dried at room temperature, finely powdered with blender. The pulverized plant was macerated in ethanol for 72 hours. After the extraction, the extract was sieved and filtered. The filtrate was concentrated in the oven at 40 ºC. The dried extracts were stored at 4ºC until needed. Appropriate dose dilutions were made with distilled water.

Figure 1: Eucalyptus globulus leaves

Acute Oral Toxicity Test

The oral acute toxicity study (LD50) of the ethanol leaf extract of *E. globulus* in mice was estimated according to the biphasic method of Lorke (1983). In the 1st phase, 3 groups of mice (n=3) were administered the extract 10, 100 and 1000 mg/kg. The animals were observed for physical signs of toxicity and death for the first 4 hours and intermittently for 24 hours. In the 2nd phase, 3 groups of mice (n=1) were administered the extract 1600, 2900 and 5000 mg/kg, based on the result from the 1st phase. The mice were also observed for physical signs of toxicity and death for the first 4 hours and intermittently for 24 hours.

Acute Toxicity

The oral median lethal dose of *E. globulus* was estimated to be greater than 5,000 mg/kg in mice. No death was observed throughout the period of the experiment, the plant was considered safe for the experimental purpose.

Preliminary Qualitative Phytochemical Analysis of *Eucalyptus globulus*

The extract was screened to detect the presence of some phytochemicals according to the methods described by [38].

The phytochemical screening revealed the presence of alkaloids,tannins flavonoids,Anthraquinones, Terpenoids, saponins and cardiac glycosides in the ethanol extract of *Eucalyptus globulus* with a high percentage composition of flavonoids.

Table 1: Preliminary qualitative phytochemical analysis of *Eucalyptus globulus*

Phytochemicals	Ethanol Extract
Alkaloids	+
Flavonoids	++
Saponins	+
Tannins	+
Anthraquinones	+
Terpenoids	+
Cardiac glycosides	+

(+ means present; ++ present with high percentage)

Drugs and chemicals

The following drugs and chemicals were used propranolol, nifedipine, formaldehyde, ethanol, glibenclamide. They were of high analytical value. This drugs are used in this present study to determine the possible mechanism through which the *E. globulus* works

Experimental protocol

Thirty animals were randomly selected into six groups of five animals each as follows; **Control group** received distill water, **extract group** (500mg/kg *p.o* daily), **formalin group** (20µl/kg *i.p*) **formalin+pro+extract** (propranolol, 40mg/kg with extract 500mg/kg *p.o* daily), **formalin+gli+extract group** (glibenclamide, 8mg/kg with extract 500mg/kg *p.o* daily) **formalin+nife+extract group** (nifedipine, 10mg/kg with extract 500mg/kg *p.o* daily). The treatment period lasted for two weeks.

Table 2: Experimental protocols

Groups	Treatment
Control	Distill water daily
E. globulus extract	*E. globulus* extract 500mg/kg *p.o* daily
formalin	Formalin 20µl/kg
F+pro+extract	Formalin 20µl/kg, propranolol, 40mg/kg with extract 500mg/kg *p.o* daily
F+gli+extract	Formalin 20µl/kg, glibenclamide, 8mg/kg with extract 500mg/kg *p.o* daily
F+nife+extract	Formalin 20µl/kg, nifedipine, 10mg/kg with extract 500mg/kg*p.o* daily

At the end of the experimental period, animals were anaesthesized with sodium pentobarbital and blood samples were collected through retro-orbital route. The heart was excised and washed in an isotonic solution after which it was homogenised and centrifuged at 3000 rpm for 5 minutes. Blood samples were immediately taken for haematological analysis using an automated method while the supernactant were frozen prior to biochemical analysis.

Statistical Analysis

Data were presented as mean±SEM. Comparisons between groups were made using the one way analysis of variance (ANOVA) followed by Bonferonni's post hoc test, 95% confidence level and at P value less than 0.05 was considered statistically significant.

Results

Effects of *Eucalyptus globulus* on some haematological indices in formalin-induced cardiotoxicity in male Swiss mice.

Formalin treatment increased white blood cell count compared to the control however, treatment with *E. globulus* decreased the WBC count when compared to the formalin treated group. Also treatment with some selected standard drugs such as proponalol, glibenglamide and nifedipine with extract decreased WBC count when compared to the

formalin treated group (Figure 2). Formalin treatment also increased the lymphocyte count when compared to the control however *E. globulus* treatment only or with the standard drugs propanalol, glibenclamide and nifedipine decreased lymphocyte count compared to the formalin treated group (Figure 2). Formalin treatment as well as the *E. globulus* treatment does not have difference on the granulocyte count (Figure 2).

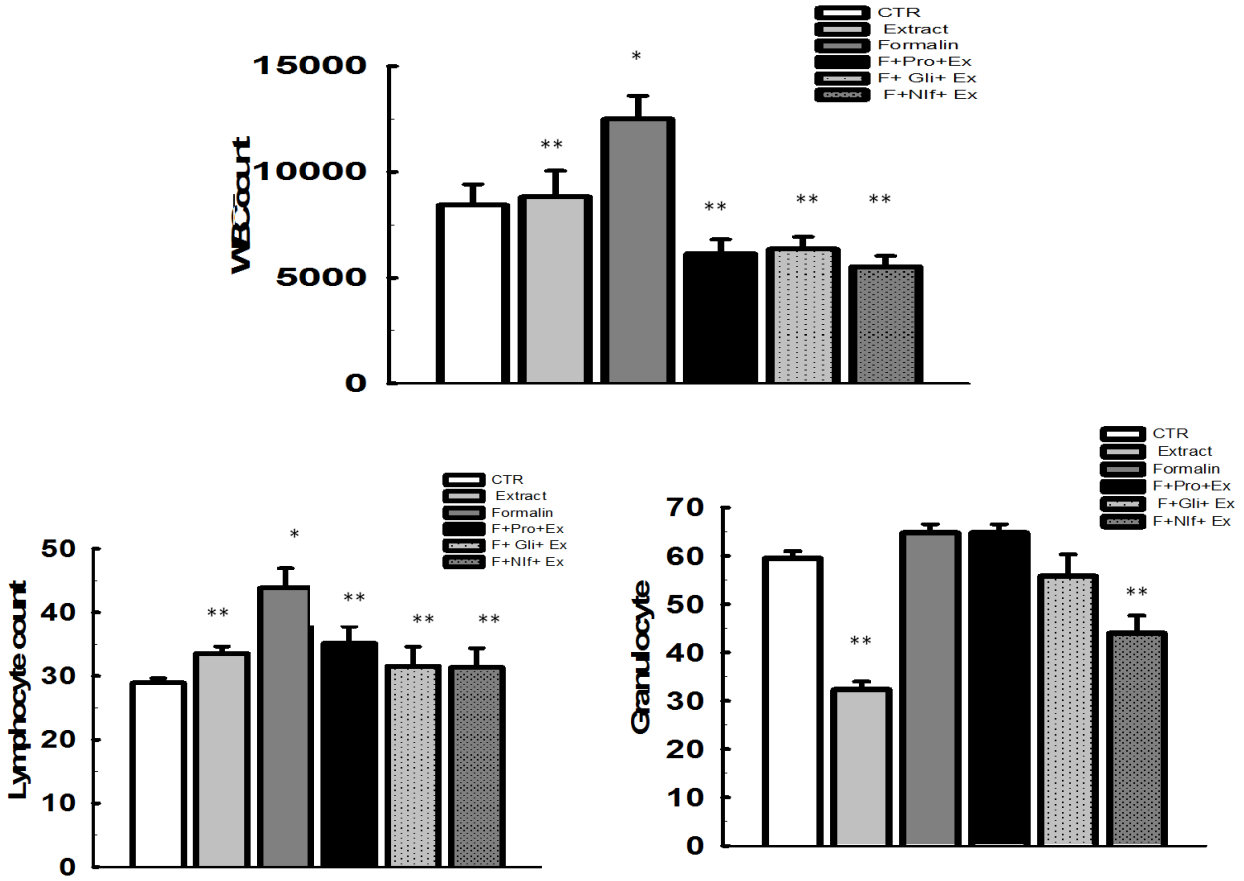


*Figure 2: effects of E.globulus extract on heart (a) uric acid (b) LDH (c) urea in formalin-induced male Swiss mice. P<0.05 *CTR vs Formalin; **Formalin vs Extracts/drugs. Values are mean ± SEM, ANOVA followed by Bonferonni post-hoc test*

Effects of *Eucalyptus globulus* on cardiac uric acid, lactate dehydrogenase (LDH) and urea in formalin-induced cardiotoxicity in male Swiss mice.

Formalin treatment increased uric acid level compared to the control however, treatment with *E. globulus* decreased the uric acid when compared to the formalin treated group. Also treatment with some selected standard drug nifedipine with extract decreased uric acid when compared to the formalin treated group (Figure 3) but not with propranolol or glibenclamide. Formalin treatment also increased the LDH activity when compared to the control however *E. globulus* treatment only or with the standard drugs nifedipine decreased LDH acitivity compared to the formalin treated group (Figure 3). There were no significance difference when the extract was used in combination with propanalol or glibenclamide. Formalin treatment also increased the urea level when compared to the control however *E. globulus* treatment only or with the standard drugs propanalol, glibenclamide and nifedipine decreased urea level compared to the formalin treated group (Figure 3).

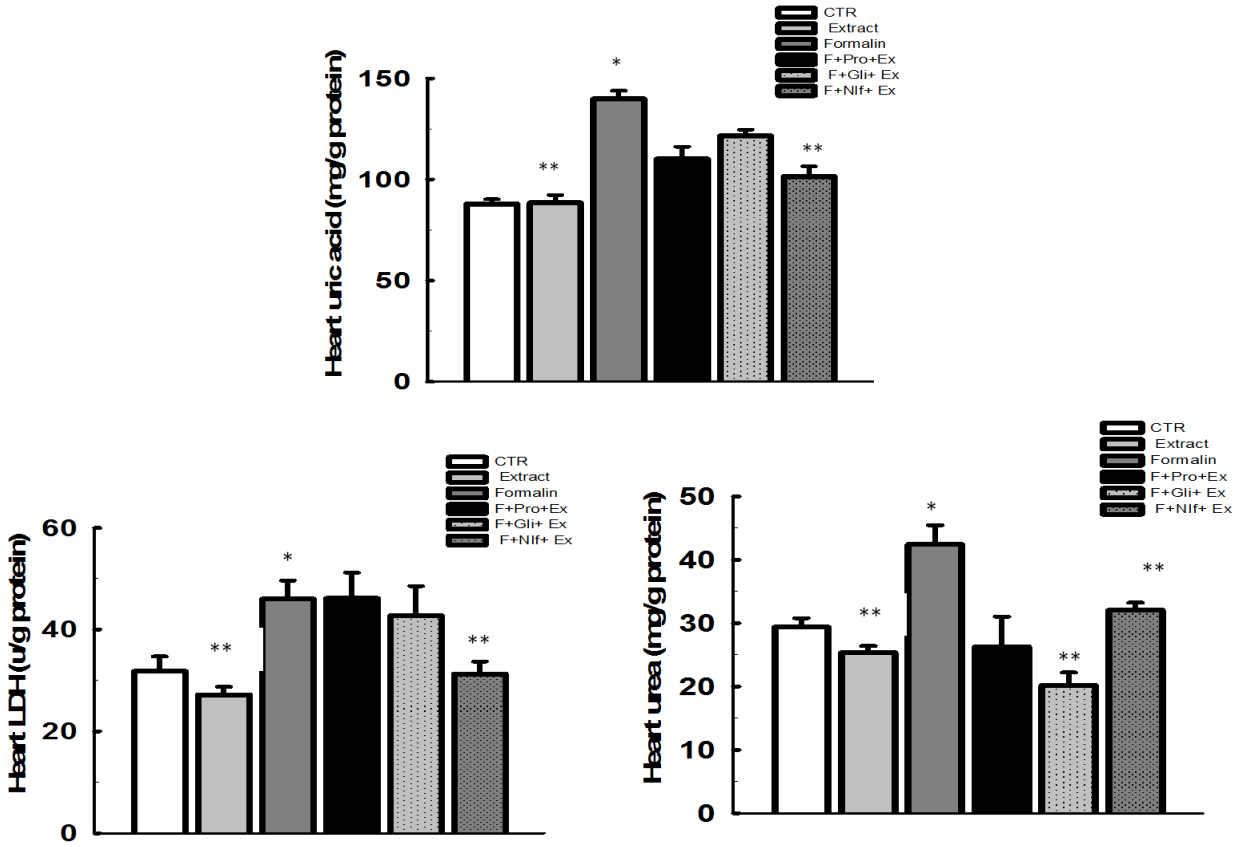
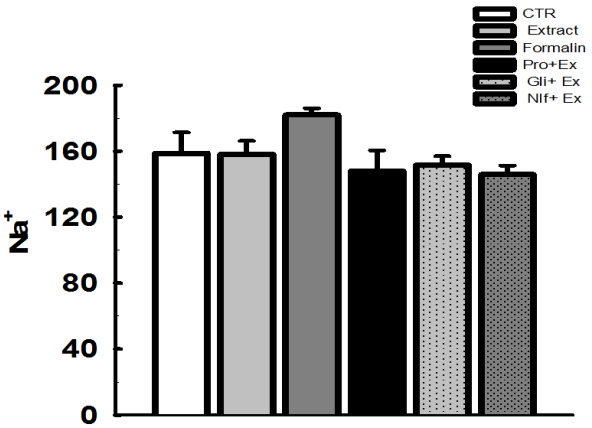


*Figure 3: effects of E. globulus extract and nifedipine on heart (a) uric acid (b) LDH (c) urea in formalin-induced male Swiss mice. P<0.05 * CTR vs Formalin; ** Formalin vs Extracts/drugs. Values are mean ± SEM, ANOVA followed by Bonferonni post-hoc test.*

Effects of *Eucalyptus globulus* on some electrolytes (Ca^{2+}, $Na^{+}$and K^{+}) in formalin-induced cardiotoxicity in male Swiss mice.

Formalin treatment increased Ca^{2+} compared to the control however, treatment with *E. globulus* decreased the Ca^{2+} when compared to the formalin treated group. Also treatment with some selected standard drugs such as proponalol, glibenglamide and nifedipine with extract decreased Ca^{2+} when compared to the formalin treated group (Figure 3). Formalin treatment, *E. globulus* as well as the standard drugs does not alter Na^{+} and K^{+} (Figure 3).



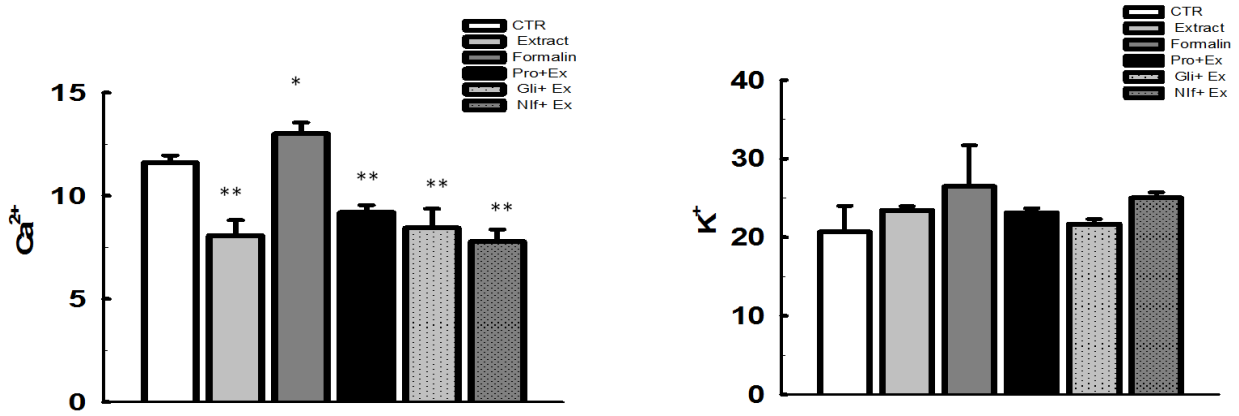


*Figure 4: Effects of E. globulus extract and nifedipine on heart (a) Na^+ (b) Ca^{2+}(c) K^+ in formalin-induced male Swiss mice. P<0.05 *CTR vs Formalin; **Formalin vs Extracts/drugs. Values are mean ± SEM, ANOVA followed by Bonferonni post-hoc test.*

Discussion

The present study aimed at investigating the cardioprotective benefits of ethanol leaf extract of *E. globulus* and to evaluate the pharmacological mechanism for its cardioprotective effects.

Formalin has been shown to stimulate oxidative stress and thus, some secondary toxic effects in cardiac cells and tissue [18]. From the result of the present study, formalin caused the elevation uric acid and LDH which are indicators of cellular damages. This resulted to the elevation observed in the WBC and lymphocyte counts.

Propranolol is a non-cardioselective β-blocker which has been reported to have membrane-stabilizing properties. It has been used to control hypertension, pheochromocytoma, myocardial infarction, cardiac arrhythmias, angina pectoris, and hypertrophic cardiomyopathy [1]. Glibenclamide is widely used and effective antihyperglycemic drug which has also been reported to be used in treating myocardiac infarction [16]. Nifedipine is a dihydropyridine calcium-channel blocker (CCB) introduced approximately 30 years ago for the prophylaxis of angina symptoms, and then later utilized as an anti-hypertensive agent [11] Nifedipine acts by inhibiting the trans-membrane influx of calcium into cardiac and vascular smooth muscle cells, thus reducing muscle contraction and has predominantly vasodilatory effects on arteries with minimal effects on the myocardium and cardiac conduction [11].

From the present study, formalin induced increase in WBC count and the lymphocyte count was ameliorated by the treatment with *E. globulus* leave extract and the standard drugs; propoanalo, glibenclamide and nifedipine.Numerous studies have shown that E. globulus exhibit various properties like anti-inflammatory, anticancer, antibacterial, antiseptic and astringent [19][23][38][43]. Also the present study elevated level of uric acid in the heart and this was followed with overexpression of LDH activity within the heart. However, *E. globulus* leave extract only and in combination with the standard drugs; nifedipine lowered the uric acid level and the LDH expression. This is suggestive to mitigate the deleterious effects on cardiac muscles. Elevated UA is accompanied with overexpression of LDH gene in incidence of cardiotoxicity which is commonly associated with cancer cells and muscle damages [7][13]. Several mechanism have been reported through which elevated cardiac UA exerts deleterious effects on cardiovascular health including oxidative stress, systemic inflammation and metabolic dysregulation [7]. High cardiac LDH activity is related to cardiac muscle damages and aggravates organ failure [9][26].

The possible mechanism via which *E. globulus* mitigates cardiotoxicity is suggestive to be by lowering UA level and LDH expression in the heart as shown in this study. Also the possible inhibitory effect on the expression of LDH is through adrenergic pathway because compared to other standard drugs used in this study, the beneficial effects of the *E. globulus* extract was more when it was used in combination with nifedipine. Therefore in conclusion, *E. globulus* possess cardioprotective effects in experimental animal models.

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