Section A-Research paper
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Cardioprotective impact of α-phellandrene against cyclophosphamide-induced cardiotoxicity in Wistar albino rats

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ABSTRACT

The frequency of cardiotoxicity is a worrying trend on a global scale. Currently, it is proposed that the causes of cardiotoxicity are oxidative stress, cardiac inflammation, and apoptosis. Numerous cardioprotective drugs have been developed to treat cardiotoxicity, but sadly, their potential side effects have made physicians seriously concerned about their use. We, therefore, intended to investigate the effect of α-phellandrene against cyclophosphamide (CP) in Wistar rats. Animals were divided into 6 groups: Normal control; Toxic (CP 200mg); α-phellandrene + CP [I]; α-phellandrene + CP [II]; α-phellandrene per se; Propranolol (PLN) +CP. Dosing was completed for 14 days laterally with a dose of CP once on the seventh day. After the final day of treatment, the weight of each animal was measured and animals were sacrificed on the 15th day, and after the serum had been separated using a homogenizer (3000 RPM) for 15 minutes. Various biochemical markers including Total Antioxidant Capacity (TAC), Creatinine Kinase- Myoglobin Binding (CK-MB), Lactate dehydrogenase (LDH) in blood, and other biochemical parameters such as Thiobarbituric acid reactive substances (TBARS), Reduced Glutathione (GSH), Catalase and superoxide dismutase (SOD) were also measured. When cyclophosphamide is given to rats, histology studies show that there are pathological changes, tissue damage, and an increase in relative heart weight, as well as an increase in serum marker enzymes, a substantial rise in the heart lipid concentrations, and a substantial drop in body weight at the end. The level of heart lipids, cardiac mitochondrial

activity, and serum marker enzymes was all markedly normalized by the addition of α-phellandrene. In Conclusion, our research discovered that α-phellandrene had a strong cardioprotective impact against cardiotoxicity caused by cyclophosphamide.

Keywords: Cardiotoxicity, α-phellandrene, cyclophosphamide, Propranolol, Histology.

1. INTRODUCTION

The leading global contributor to fatalities and disabilities is cardiovascular diseases (CVDs). Even though the mortality rate from CVDs has declined over the past few decades, the number of preventable CVD deaths in poor countries is still concerningly high [1]. Low- and middle-income countries account for over 80% of all CVD cases worldwide. Myocardial infarction is the most frequent form of CVD and the main reason for deaths [2]. Numerous studies have demonstrated that dietary and nutritional adjustments can significantly lower the risk of CVD [3]. It has been demonstrated that plant-based foods including whole grains, fruit, and vegetables reduce the risk of mortality for a number of illnesses, including CVD [4] [5].

A frequently occurring cyclic monoterpene, - α-phellandrene is found in numerous essential oils, including those from turmeric leaf (54%), Boswellia sacra (42%), Eucalyptus elata (35%), dill weed (30%), and Eucalyptus dives (17%) [6]. Additionally, it is present in a number of other plant species, including Cannabis sativa, Gossypium hisutum, Heracleum antasiaticum, E. camaldulensis, Cryptomeria japonica, and Cistus ladanifer. It demonstrates a wide range of biological activity, including larvicidal, insecticidal, antifungal, anti-inflammatory, antitumoral, and antinociceptive. It has strong antioxidant capabilities that stop lipid peroxidation. By preventing lipid oxidation and the development of rancid odours, it prolonged the shelf life of fried salted peanuts [7]. Alpha-phellandrene has been designated by the Federal Emergency Management Agency (FEMA) as a drug that is Generally Recognized as Safe (GRAS) since 1965. Since the 1940s, the general public has utilized it. [6] It was recently re-evaluated and determined to be safe for the public's health. It has been approved for use in food by the FDA (21 CFR 172.515). It is used to make creams, lotions, soaps, detergents, and fragrances. The farming, food, and feed industries find its biological activity appealing, and healthcare and cosmetic businesses find their pharmacological qualities appealing. [8].

7.6 million (13%) of all deaths globally are caused by cancer, making it a global threat. By the end of 2030, when there may be 13.1 million cancer-related fatalities [9]. Anticancer

medication organ toxicity causes structural cell damage, weakens antioxidant defence, and raises levels of inflammatory cytokines and apoptotic proteins [10]. Cyclophosphamide is one of the most popular anticancer drugs that work against a variety of cancers [11]. It is a potent immunosuppressant that is commonly used in managed care after organ transplantation to prevent graft rejection. It is also typically given as a bolus. It is a prodrug that rapidly degrades in the liver to form 4-hydro-cyclophosphamide, aldophosphamide, phosphoramide mustard (PM), and acrolein. Acrolein is thought to be cardiotoxic, while PM is an anticancer moiety that works on DNA's N-7 guanine residues and kills cells [12]. According to reports, fatal cardiotoxicity occurred in 11–43% of patients who received CP for the treatment of haematological malignancies, bone marrow transplantation, and other forms of cancers. Symptoms began to manifest one to three weeks after injection. Cardiotoxic consequences of CP include membrane damage, oxidative stress, inflammation, apoptosis, and changes to the ultrastructure of cells [13]. During the Second World War, when the US used nitrogen mustard gas to retaliate against German air strikes on the Italian city of Bari, alkylating medicines were first used. This incident had a very small influence on people but had a significant impact on white blood count (WBC) [14]. Smaller dosages of CP or its metabolite (1–5 mg/kg, p.o.) inhibit the immune system, while greater doses (120–200 mg/kg, i.v.) are toxic to many organ systems [15].

Propranolol is a non-cardio selective -blocker medication. Although it is claimed to have membrane-stabilizing properties, it does not function in a sympathomimetic manner. Propranolol hydrochloride is used to treat a variety of ailments, including angina pectoris, pheochromocytoma, myocardial infarction, cardiac arrhythmias, hypertension, and hypertrophic cardiomyopathy [16]. Additionally, it is used in the therapy of tremors, anxiety-related disorders, and hyperthyroidism to lessen the indications of sympathetic overactivity. Preventing upper gastrointestinal hemorrhage and migraines in people with portal hypertension are two other indications [17].

2. MATERIALS AND METHOD

2.1. Chemicals and Reagents

α-phellandrene (CAS No.-4221-98-1), Cyclophosphamide (CAS No.- 6055-19-2), Propranolol (CAS No.- 318-98-9) and all other materials, including chemicals and reagents,

were of analytical grade and came from reputable suppliers like SDFCL and Sigma-Aldrich. LDH and CK-MB biochemical kits were purchased from Reckon Diagnostics.

2.2. Animal Model

Wistar Albino rats (180-200 g) was obtained from the R. V. Northland Institute Greater Noida G.B. Nagar Central Animal House facility. The animals were kept on a pellet diet with access to food and water allowed. They were kept in polypropylene crates and kept at a temperature of 21°C with a 12-hour day/night cycle and 50% humidity. The experiment received approval in accordance with CCSEA criteria from India's New Delhi.

2.3. Experimental Design

The rats were divided into six groups, each group consisting of 6 animals (n=6).

Group I: Normal control rats provided with normal saline (0.5ml/day).

Group II: Rats received a standard diet and water throughout the experiment and a single dose of CP (200mg/kg) via intraperitoneally once on a day 7 was given.

Group III: Rats were administered with α-phellandrene (5mg/kg p.o.) to the CP-induced group of rats continuously for 14 days.

Group IV: Rats were administered with α-phellandrene (10mg/kg p.o.) to the CP-induced group of rats continuously for 14 days.

Group V: Rats were treated with α-phellandrene per se (10mg/kg) intraperitoneally daily for 14 days.

Group VI: Rats were treated with the standard drug of propranolol in the CP-induced group of rats daily for 14 days.

Blood was drawn by the retro-orbital method 24 hours after the last dose, and the serum was separated from the blood via centrifugation (3000RPM) for a period of fifteen minutes before being kept at 20°C for further calculations. In order to conduct the histological and biochemical analyses, heart tissue was taken, cleaned in normal saline, and used.

2.4. Biochemical Parameters

The non-haemolysed serum was used to determine Total Antioxidant Capacity (TAC) [18], Creatinine Kinase- Myoglobin Binding (CK-MB) [19] [20] [21], and Lactate dehydrogenase (LDH) [22] [23] [24].

Estimates of Thiobarbituric acid reactive substances (TBARS) [25], Reduced Glutathione (GSH) [26], Catalase and Superoxide dismutase (SOD) [27] were made using the heart tissue homogenate.

2.5. Histological Studies

After the animal was sacrificed, the heart was taken out and dried after being immersed in ice-cold normal saline. Then, 10% formalin was used to preserve it. To establish the extent of tissue damage caused by free radicals and the influence of the test drug, lower and higher doses in undoing this damage, tissue was stained with H&E, and paraffin slices of tissues were analyzed [28].

2.6. Statistical Analysis

Mean SEM was used to express the data. Following Tukey's t-test, an ANOVA was utilized to link the data groups together. When p 0.05, results were deemed statistically significant. The program from GraphPad, Inc. (version 5.06) was used for the statistical analysis.

3. RESULTS

3.1. Biochemical estimations in Serum

a. Total anti-oxidant capacity (TAC)

TAC level was found in CP 200-treated rats to be noticeably low ($^{\#\#}P<0.01$). When treated with PLN 5 and PLN 10, the level of TAC increased non-significantly and significantly ($^{ns}P>0.05$ and $^{*}P<0.05$). The TAC levels in the propranolol-treated group were significantly rose (**P0.01) (Figure 1).

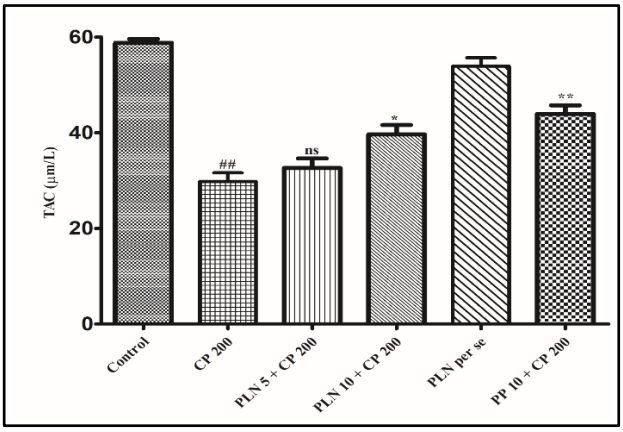

Figure 1: Results are mean±SEM & n=6 viewing $^{\#\#}P<0.01$ (Control vs CP 200); $^{ns}P>0.05$ (PLN 5 + CP 200 vs CP 200); $^{*}P<0.05$ (PLN 10 + CP 200 versus CP 200), and $^{**}P<0.01$ (PP 10 + CP 200 versus CP 200), applying ANOVA (one way) through Tukey's test.

b. Creatine kinase-MB (CK-MB)

In rats administered with CP 200, the CK-MB level was initially found to be noticeably elevated (###P<0.001). When CK-MB was treated with PLN 5 and PLN 10, its level was

dramatically lowered (*P<0.05 and **P <0.01). The propranolol-treated group also experienced a significant reduction in CK-MB levels. (***P<0.001) (Figure 2).

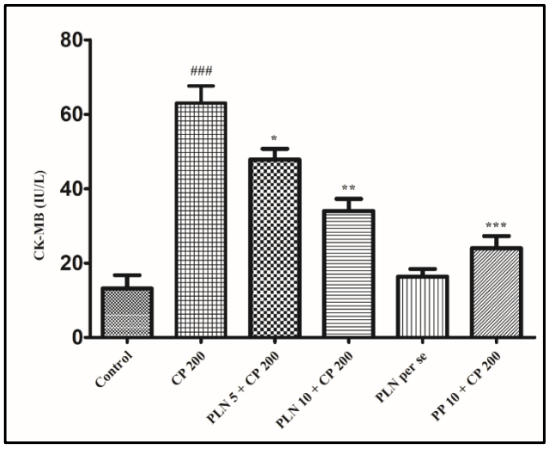

Figure 2: Results are mean±SEM & n=6 viewing ###P<0.001 (Control vs CP 200); *P<0.05 (PLN 5 + CP 200 vs CP 200); **P<0.01 (PLN 10 + CP 200 versus CP 200), and ***P<0.001 (PP 10 + CP 200 versus CP 200), applying ANOVA (one way) through Tukey's test.

c. Lactate dehydrogenase (LDH)

In rats administered with CP 200, LDH levels were found to be abnormally high (###P<0.001). When treated with PLN 5 and PLN 10, the level of LDH was considerably and non-significantly reduced (nsP>0.05 and **P<0.01). LDH levels were significantly reduced in the propranolol treatment group as well (**P<0.01) (Figure 3).

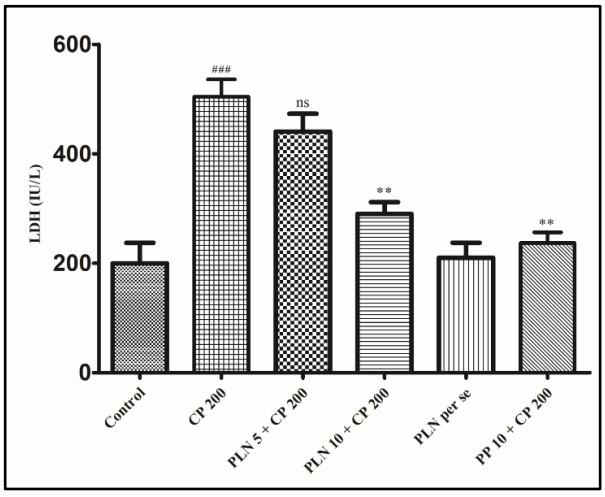

Figure 3: Results are mean±SEM & n=6 viewing ###P<0.001 (Control vs CP 200); nsP>0.05 (PLN 5 + CP 200 vs CP 200); **P<0.01 (PLN 10 + CP 200 versus CP 200), and

**P<0.01 (PP 10 + CP 200 versus CP 200), applying ANOVA (one way) through Tukey's test.

Table 1: Biochemical estimation of serum parameters in different groups

Groups	TAC (µM/L)	CK-MB (IU/L)	LDH (IU/L)
Control	59.02±0.47	14.23±1.94	200.65±2.27
CP 200	28.67±0.18$^{\#\#}$	63.11±3.42$^{\#\#\#}$	503.39±4.27$^{\#\#\#}$
PLN 5 + CP 200	32.00±0.13^{ns}	49.544±4.85*	292.464±3.41^{ns}
PLN 10 + CP 200	38.13±0.15*	34.44±2.45**	220.154±5.36***
PLN *perse*	55.87±0.19	17.77±3.01	208.07±2.26
PP 10 + CP 200	43.23±0.60**	26.408±1.89***	225.629±1.83**

Biochemical estimation of serum parameters in different groups. Data represents mean ± SEM of six rats per group. Data were noted by applying ANOVA (one way) through Tukey's test showing $^{\#\#\#}$p < 0.05 with control vs CP.

3.2. Biochemical estimations in tissue

a. Thiobarbituric Acid Reactive Substances (TBARS)

In rats treated with CP 200, the amount of TBARS was found to be significantly elevated ($^{\#\#\#}$P<0.001). When treated with PLN 5 and PLN 10, the amount of TBARS was considerably and non-significantly reduced (^{ns}P>0.05 and *P<0.05). The levels of TBARS were, however, significantly lower in the propranolol-treated group (***P<0.001) (Figure 4).

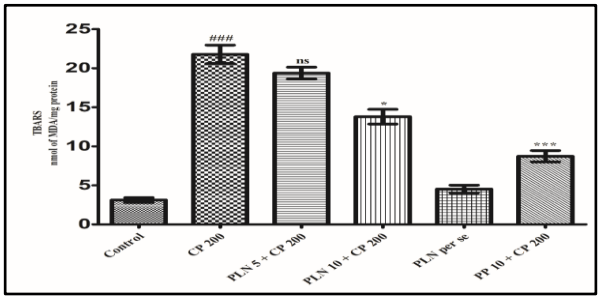

Figure 4: Results are mean±SEM & n=6 viewing $^{\#\#\#}$P<0.001 (Control vs CP 200); ^{ns}P>0.05 (PLN 5 + CP 200 vs CP 200); *P<0.05 (PLN 10 + CP 200 versus CP 200), and ***P<0.001 (PP 10 + CP 200 versus CP 200), applying ANOVA (one way) through Tukey's test.

b. Reduced glutathione (GSH)

In rats treated with CP 200, the GSH level was found to be significantly low ($^{###}P<0.001$). When treated with PLN 5 and PLN 10, the level of GSH increased non-significantly and significantly (nsP>0.05 and *P<0.05). However, the GSH levels in the propranolol-treated group were significantly higher (***P<0.001) (Figure 5).

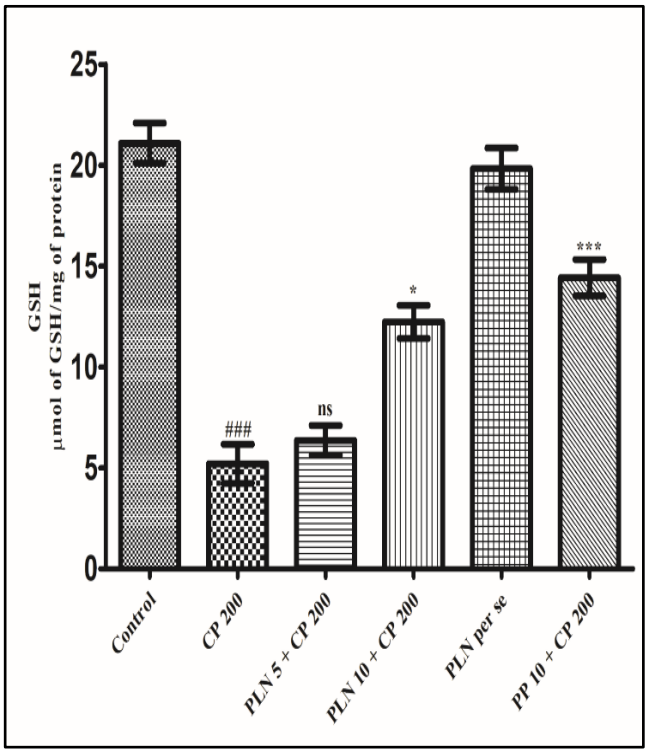

Figure 5: Results are mean ± SEM & n=6 viewing $^{###}P<0.01$ (Control vs CP 200); $^{ns}P>0.05$ (PLN 5 + CP 200 vs CP 200); *P<0.05 (PLN 10 + CP 200 vs CP 200), and ***P<0.001 (PP 10 + CP 200 versus CP 200), applying ANOVA (one way) through Tukey's test.

c. Catalase

In rats treated with CP 200, the catalase level was found to be significantly decreased ($^{###}P<0.001$). When treated with PLN 5 and PLN 10, the level of catalase increased non-significantly and significantly ($^{ns}P>0.05$ and *P<0.05). Catalase levels significantly increased in the propranolol-treated group (**P<0.01) (Figure 6).

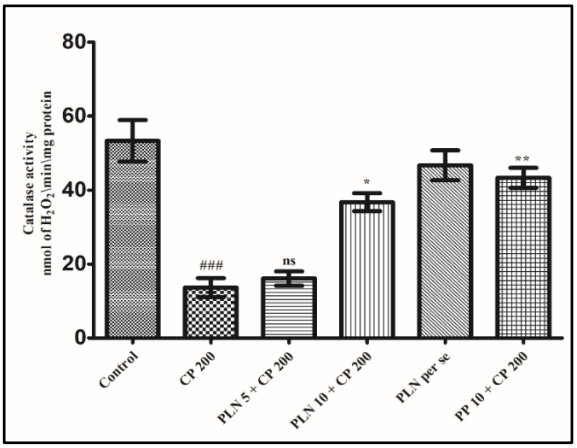

Figure 6: Results are mean±SEM & n=6 viewing $^{###}$P<0.01 (Control vs CP 200); ^{ns}P>0.05 (PLN 5 + CP 200 vs CP 200); *P<0.05 (PLN 10 + CP 200 vs CP 200), and **P<0.01 (PP 10 + CP 200 versus CP 200), applying ANOVA (one way) through Tukey's test.

d. Superoxide dismutase (SOD)

In rats treated with CP 200, the level of SOD was discovered to be noticeably low ($^{###}$P<0.001). When treated with PLN 5 and PLN 10, the level of SOD increased non-significantly and significantly (^{ns}P>0.05 and *P<0.05). Significantly more SOD levels were found in the propranolol treatments group. (**P<0.01) (Figure 7).

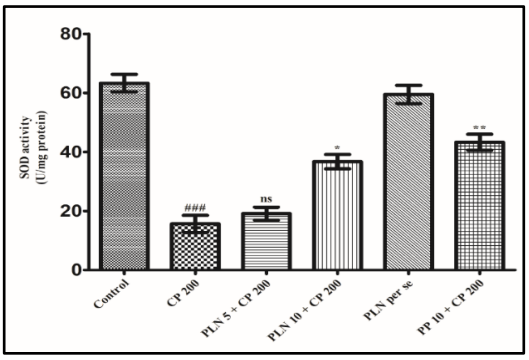

Figure 7: Results are mean±SEM & n=6 viewing $^{###}$P<0.01 (Control vs CP 200); ^{ns}P>0.05 (PLN 5 + CP 200 vs CP 200); *P<0.05 (PLN 10 + CP 200 vs CP 200), and **P<0.01 (PP 10 + CP 200 versus CP 200), applying ANOVA (one way) through Tukey's test**.**

Table 8: Biochemical estimation in heart tissue of different groups

Groups	TBARS (nmol of MDA/mg protein)	GSH (µmol of GSH/mg of protein)	CAT (nmol of H_2O_2\min\ mg protein)	SOD (U/per mg of protein)
Control	3.127±0.037	21.112±0.50	52.49±0.350	61.77±0.066
CP 200	22.771±0.045$^{\#\#\#}$	4.913±0.27$^{\#\#\#}$	17.246±0.143$^{\#\#\#}$	18.194±0.021$^{\#\#\#}$
PLN 5 + CP 200	18.183±0.045^{ns}	6.087±0.21^{ns}	18.51±0.232^{ns}	19.283±0.032^{ns}
PLN 10 + CP 200	13.588±0.032*	12.038±0.53*	37.91±0.224*	37.486±0.034*
PLN *perse*	4.525±0.020	20.076±0.58	45.29±0.293	58.490±0.032
PP 10 + CP 200	8.271±0.049***	14.096±0.23***	42.49±0.309**	46.541±0.074**

Biochemical estimation of serum parameters in dissimilar groups. Data represent mean ± SEM of six rats per group. Data were calculated by applying ANOVA (one way) through Tukey's test showing $^{\#\#\#}p < 0.05$ with control vs CP.

3.3. Body Weight Measurement

PLN 5 and PLN 10 were administered to Wistar rats for a total of 14 days after CP 200-induced cardiotoxicity. For a 14-day comparison research, vehicle and propranolol were also given. On days zero, seven, and fourteen, the body weight was measured. There is no discernible difference between any of the groups after compression (Figure 8).

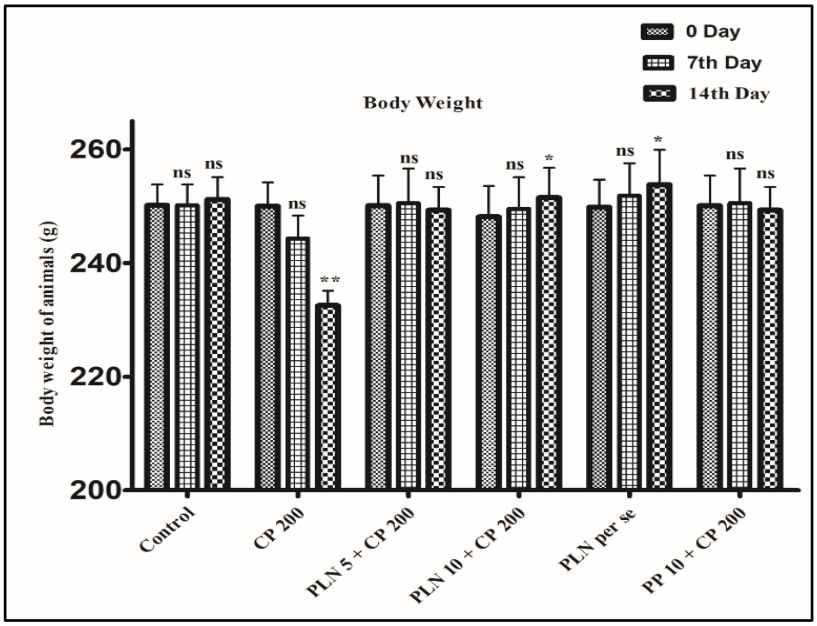

Figure 8: Results are mean±SEM & n=6 viewing $^{ns}P>0.05$ (0^{th} day vs 7^{th} and 14^{th} day in Control, CP 200, PLN 5 + CP 200, PLN 10 + CP 200, PLN *per se* and PP 10 + CP 200 group); $^{**}P<0.01$ (0^{th} day vs 14^{th} day in CP 200 group); and $^{*}P<0.05$ (0^{th} day vs 14^{th} day in PLN 10 + CP 200 and PLN *per se* group); applying ANOVA (one way) through Tukey's test.

Table 9: Body weight of animals

S. No	Groups	Body weight (g)		
		Day 0^{th} (Mean ± SEM)	**Day 7^{th} (Mean ± SEM)**	**Day 14^{th} (Mean ± SEM)**
1.	**Control**	250.16 ± 0.6	250.16 ± 0.8	251.16 ± 0.6
2.	**CP 200**	250.78 ± $0.4^{\#}$	244.33 ± $0.6^{\#}$	232.50 ± $0.9^{\#}$
3.	**PLN 5 + CP 200**	250.09 ± 0.9*	250.54 ± 0.3*	249.30 ± 1.0*
4.	**PLN 10 + CP 200**	248.16 ± 0.6	249.16 ± 0.5	251.16 ± 0.6
5.	**PLN *perse***	249.16 ± 0.6	251.84 ± 0.8	253.80 ± 0.9
6.	**PP 10 + CP 200**	250.09 ± 0.4*	250.33 ± 0.6*	249.50 ±

				0.3*

Body weight measurement parameter in different groups was performed. Data represent mean ± SEM of six rats per group. Data were analyzed by applying ANOVA (one way) through Tukey's test showing $^{###}p < 0.05$ with control vs CP.

3.4. Histological examination

A microscopic image of the healthy control group shows how the cardiac muscles are typically shaped. Inflammatory cells had infiltrated in concentrated areas in the CP-induced group. In the CP-induced group, myocardial tissue loss has increased the space between heart muscles. There are less inflammatory cells and less myofibril damage in the PLN 5 + CP 200 group treatment group. In contrast to PLN 5 + CP 200, the PLN 10 + CP 200 group has less inflammatory cells and myofibril damage. A microscopic portion of the PLN per se group demonstrates intact myocardial tissue without cardiac muscle fibre degradation. There are no inflammatory cells in the section. The cardiac muscles in the PP 10 + CP 200 group are arranged normally (Figure 9).

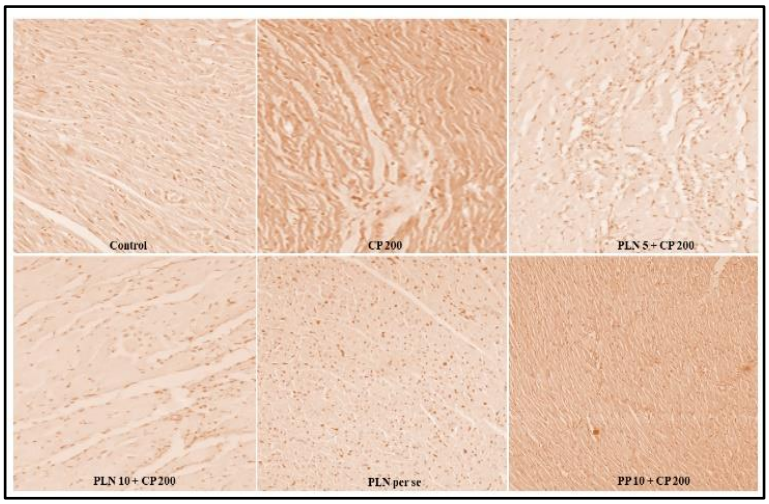

Figure 9: In cardiac tissue, CP 200 caused histological alterations that were subsequently corrected by PLN 5, PLN 10, and PP 10 (scale bar: 100 micrometre). After receiving CP 200, there was indications of myocardial tissue loss, which increased the distance between cardiac muscles, as well as localised infiltration of inflammatory cells. Treatment with PLN 10 and PP 10 successfully reduced these histological abnormalities; however, PLN 5 treatment was found to be less effective.

DISCUSSION

The current study set out to examine the cardiotoxic impacts of CP at a dosage of 200 mg/kg i.p. as well as the curative effects of PLN at doses of 5 and 10 mg/kg p.o. Cardiotoxicity is indicated by an increase in oxidative stress, calcium overload, the formation of ROS and RNS, inflammation, apoptosis, and several other histological abnormalities. Superoxide and peroxide ions are dealt with by reduced GSH, SOD, and CAT; otherwise, normal cardiomyocytes would experience oxidative stress. When we administered CP at a dose of 200 mg/kg, we discovered comparable increases in lipid peroxidation and decreases in the antioxidant enzymes SOD & CAT, establishing cardiotoxicity. We found that dosing with PLN 10 & PP 10 mg/kg caused these levels to return to normal, demonstrating cardiac protection from an increase in antioxidant status. This study supported earlier findings and illustrated how these drugs have antioxidant properties [29]. However, following treatment with PLN 5 mg/kg, the level of these measurements did not change appreciably.

Ascorbic acid, tocopherol, uric acid, and carotenoids are examples of non-specific antioxidants that are involved in TAC [30]. TAC reduces in Wistar rats that have been given CP but TAC increases after receiving test (PLN 5 and 10 mg/kg) and propranolol. The inclusion of flavonoids and phenols is hypothesised to be the cause of both formulations' strong effects.

Lipid peroxidation, mitochondrial DNA oxidative damage, and mitochondrial respiratory chain oxidative damage are all brought on by ROS. Lipid peroxidation results in the production of malondialdehyde, and the quantity of these compounds can be determined using thiobarbituric acid [31] [32]. A key metric for determining the degree of lipid peroxidation brought on by ROS is TBARS. When given as a preventative medicine, PLN 5 and 10 mg/kg greatly reduced the amount of lipid peroxidation brought on by CP, which explains why it restored the body's antioxidant levels. In terms of body weight measurements, the hazardous medication CP greatly reduced the weight of animals on days 0, 7, and 14, whereas -phellandrene significantly increased the weight of animals on days 7 and 14. CK-MB or LDH may leak from the heart into the blood as a result of damage to the myocardium's membranes. One indication of myocardial damage is this. Any medication that returns the levels of these markers to normal demonstrates cardiac protection. In the current study, the levels of these markers (CK-MB and LDH) considerably increased when rats were given CP 200 mg/kg, i.p. The levels dramatically returned to normal when we gave the rats PLN and PP at doses of 10 mg/kg and 10 mg/kg, respectively. Therefore, these molecular markers

demonstrated that the membranes were unharmed and that the heart was safe, which is consistent with earlier findings [33]. Histopathology is one of the primary indications of cellular and ultrastructural damage using H and E staining. Rats given CP underwent myofibrillar disintegration, localized pyknosis, and vacuole development [34].

CONCLUSION

The preliminary research suggested that Wistar albino rats might be protected from CP-induced toxicity by α -phellandrene at the dosage of 10 mg/kg. Treatment with CP resulted in substantial biochemical, histological, and ultrastructural cardiac abnormalities. The injection of CP raised the MDA level mechanistically. In addition, CP therapy also decreased the functioning of antioxidant enzymes such as SOD, CAT, and GSH. Additionally, administering CP damaged histology tissue. The antioxidant enzymes SOD, CAT, and GSH, as well as TBARS, were identified in significantly increased concentrations in the cardiac organs of rats treated with α -phellandrene. The treatment also restored the normal histological pattern in the rodents. The administration of α -phellandrene, on the other hand, demonstrated significant cardioprotective effects in Wistar albino rats due to its profound antioxidant and anti-inflammatory properties, leading one to the conclusion that the treatment of CP caused notable oxidative stress &cardiac dysfunction. As a result, the present study indicated that -phellandrene might be a viable and promising drug for treating CP-induced cardiotoxicity.

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Conflict of Interest

There were no conceivable conflicts of interest disclosed by the authors.

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