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Combinatorial protective effect of *Cinnamomum verum* and Stingless Bee Honey against oxidative stress in isoproterenol-induced cardiotoxicity in Wistar rats

Arul Mozhi Padmanabhan¹ and Victor Arokia Doss^{1*}

Abstract

Background Cardiac hypertrophy (CH) is a prevalent consequence of cardiovascular disease, necessitating treatment with synthetic medications that often have adverse effects. Natural products, like *Cinnamomum verum* (CV) and Stingless Bee Honey (SBH), rich in phenolic compounds, hold promise as safer alternatives. In this study, we investigated the potential cardioprotective effects of CV bark and honey produced by Stingless bee in male Wistar rats. In a 7-day controlled study, rats were randomly assigned into seven treatment groups, receiving either saline (Group I), isoproterenol (ISO) (Group II) or ISO combined with drug interventions of Losartan (Group III), CV (Group IV) and SBH (Group V) along with several combined regimens (Group VI & VII). Biochemical assays were conducted to measure antioxidant enzyme activities (Superoxide Dismutase, Catalase, Glutathione Peroxidase), non-enzymatic antioxidants (Vitamin C, Vitamin E, Reduced Glutathione), inflammatory markers, and oxidative stress levels. Histopathological analysis was also performed. stress markers in cardiac tissues, shedding light on potential therapeutic interventions.

Results ISO induces myocardial oxidative stress, characterized by reduced antioxidants, increased oxidative stress and inflammatory markers with elevated collagen content. Our results show that co-administration of CV + SBH effectively attenuated ISO-induced myocardial oxidative stress, outperforming individual CV or SBH administration in restoring antioxidant levels and normalizing oxidative stress and inflammatory markers.

Conclusions The results from this study underscore the importance of advancing research on the combination of CV and SBH, which hold significant promise as synergistic therapeutic agents in the treatment of complex diseases, potentially informing future healthcare policies and clinical practices.

Keywords Cardiac hypertrophy, *Cinnamomum verum*, Stingless Bee Honey, Oxidative stress, Isoproterenol

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Background

Worldwide, cardiovascular disease has emerged as a leading contributor to disability and mortality [1]. Cardiac hypertrophy (CH) is an adaptive response of the heart to pathological pressure overload and is a common adverse result of cardiovascular disease. It momentarily improves cardiac function but eventually impairs it and may cause heart failure [2]. The prevalence of CH has been consistently high over the world in recent years. In the present day, CH is treated with synthetic medications such as diuretics and angiotensin-converting enzyme inhibitors (ACE-I), which ease the condition by decreasing the cardiac strain and boosting cardiac contractility, respectively [3]. Isoproterenol, a synthetic β -adrenergic agonist, induces cardiac hypertrophy by overstimulating cardiac tissue, resulting in elevated oxidative stress. This overstimulation leads to an increased production of reactive oxygen species (ROS), which contributes to myocardial damage, inflammation, and fibrosis [4]. Thus, isoproterenol is widely utilized as a model for studying hypertrophic cardiomyopathy and oxidative stress mechanisms. Drugs have improved clinical outcomes in recent years, but they also come with adverse effects that limit their utility. This emphasizes the significance of finding reliable and safe alternative therapies for CH treatment.

In addition, the potency of medicinal herbs is anticipated to be comparable to that of traditional medication, but with less adverse effects that have been generally associated with allopathic drugs [5]. Studies have shown that consumption of foods and beverages rich in phenolic content is correlated with reduced incidence of heart disease. The most common plant phenolic antioxidants include flavonoid compounds, cinnamic acid derivatives, coumarins, tocopherols and polyfunctional organic acids [6].

Cinnamomum verum (CV), a member of the *Lauraceae* botanical family, exhibits considerable pharmacological characteristics, including anti-allergic, antiulcerogenic, antipyretic, and anaesthetic properties. Its bark-derived essential oil holds notable constituents like cinnamaldehyde and eugenol. Cinnamaldehyde, in particular, is associated with a range of biological activities, including peripheral vasodilation, anti-tumor effects, antifungal properties, cytotoxicity, and antimutagenicity [7, 8].

Intriguingly, Stingless Bee Honey (SBH) has been consumed relatively less though it has superior dietary and therapeutic characteristics than the most extensively used varieties of honey. Phenolic acids and flavonoids, classified as secondary constituents in plants, are transported to honey through the intermediary processes involving nectar, pollen, or propolis, facilitated by bees [9]. They are responsible for the most antioxidant activity in honey and several studies have indicated other fascinating bioactivities of honey such as antinociceptive,

immunomodulatory, and nematocidal activities. The main active mechanisms of honey that are involved in the healing of wounds are its antioxidant, antibacterial, and anti-inflammatory properties [10].

Extremely hazardous free radicals are released during auto-oxidation of Isoproterenol. Degradation of myocardial membrane-bound Polyunsaturated Fatty Acids (PUFA) by free radicals has been speculated to be the source of myocardial dysfunction and structural deterioration [11]. It's already been highlighted that reactive oxygen species (ROS) such as hydrogen peroxide, superoxide anion, hydroxyl radicals, and peroxy radicals were some of the most detrimental free radicals that can promote cardiotoxicity [12, 13]. Antioxidants are organic molecules that are intended to counteract the oxidation of certain biomolecules, thereby halting or even reversing the deleterious impacts of ROS. In cellular systems, malondialdehyde (strong reactive aldehyde) is considered as a biomarker of oxidative stress. MDA is produced as a by-product of lipid peroxidation, a process in which free radicals lead to the degradation of PUFA [14].

The nuclear factor erythroid-2 related factor 2 (Nrf2-)/heme oxygenase (HO-1) signaling pathway is a key regulatory signaling mechanism in oxidative stress responses as well as a major therapeutic target for cardiovascular disorders. Over the past several years, Nrf2 has become extensively established as a significant mediator of oxidative resistance and has been positively correlated to numerous adverse outcomes that are often driven by oxidative stress. It has also been postulated that increasing Nrf 2 gene expression is a promising drug target for cardiovascular diseases [15].

In a wide array of clinical conditions, CV and SBH have been reported to alleviate oxidative stress [16, 17], but their effects on antioxidant activity and oxidative stress markers in hypertrophied rat hearts have not been addressed. The objective of this research is to seek a naturally existing antioxidant that is capable of lowering the risk of cardiotoxicity. Furthermore, we evaluated the solitary and combinatorial effect of aqueous CV bark extracts and SBH treatment on oxidative stress and antioxidant activities in Wistar rats.

Methods

Chemicals

Most of the analytical-grade chemicals were bought from Hi-Media Laboratories Pvt. Ltd and Sisco Research Laboratories, India. Sigma-Aldrich supplied isoproterenol (isoprenaline hydrochloride) and Losartan (Losar 50) was ordered from local suppliers.

Plant collection, authentication and preparation of the extract

The *Cinnamomum verum* plant was collected in its whole and certified by the Botanical Survey of India, Southern Regional Centre, in Coimbatore, India (BSI/SRC/5/23/2019/Tech/465). Airtight containers were used to keep the dried, coarsely crushed *C. verum* barks. Honey produced by stingless bees (SBH) was purchased at an apiculturist in the Kodai Hills of Tamil Nadu.

Appropriate amounts of both CV bark powder and SBH were added alone or combination to a tube resuspended in 1 ml of sterile water. Fresh aqueous extracts of CV, SBH and CV + SBH were prepared during all treatment purposes.

Animals used

Adult male Wistar rats were procured and habituated, prior to the study. The animals were housed in a regulated environment ($25 \pm 2^\circ \text{C}$) with appropriate light/dark cycle and *ad libitum* access to food and water for 7 days.

Experimental design

Male adult Wistar rats, weighing 150–200 g, were divided into seven groups, each consisting of six animals. Over a 7-day experimental period [18], the animals received the designated treatments as per the experimental grouping as represented in Table 1. On the final day, each animal was humanely euthanized by intraperitoneal Ketamine overdosage of 100 mg/kg of body weight (BW) as per CPCSEA ethical guidelines.

Preparation of serum and tissue homogenates

Heart tissues were gently homogenized in a Teflon plunger using 0.1 M Tris-HCl Buffer. Serum and tissue homogenate were separated by centrifuging at 8000 rpm for 20 min [27].

Table 1 Induction and treatment setup

Groups	Dosage and mode of administration
I- NOR	Normal (Saline; oral.)
II- ISO	ISO (30 mg/kg/BW; i.p.) [19]
III- ISO + LOS	ISO (30 mg/kg/BW; i.p.) + LOS (50 mg/kg/BW; oral.) [19, 20]
IV- ISO + CV	ISO (30 mg/kg/BW; i.p.) + <i>C. verum</i> (500 mg/kg/BW; oral.) [19, 21]
V- ISO + SBH	ISO (30 mg/kg/BW; i.p.) + SBH (500 mg/kg/BW; oral.) [19, 22, 23]
VI- ISO + CV + SBH-1	ISO (30 mg/kg/BW; i.p.) + <i>C. verum</i> (250 mg/kg/BW; oral.) + SBH (100 mg/kg/BW; oral.) – 1 [19, 24, 25]
VII- ISO + CV + SBH-2	ISO (30 mg/kg/BW; i.p.) + <i>C. verum</i> (100 mg/kg/BW; oral.) + SBH (250 mg/kg/BW; oral.) – 2 [19, 24, 26]

Biochemical assays

Biochemical assays such as Enzymic, Non-enzymic, oxidative stress, inflammatory markers were estimated in serum and tissue homogenate samples by the standard protocols.

Antioxidant enzymes

SOD activity was measured based on its ability to inhibit pyrogallol auto-oxidation. One enzyme unit equals the amount required to achieve a 50% inhibition. The absorbance was observed at 425 nm [28]. CAT activity was estimated with hydrogen peroxide as a substrate, was recorded at an absorbance of 570 nm, with 1 unit defined as the enzyme amount that catalyses 1 mmol of H_2O_2 per minute [29]. GPx was assessed and read colorimetrically at 412 nm, one unit of GPx as the enzyme amount converting 1 mol of GSH to GSSG per minute in the presence of H_2O_2 [30]. The data were represented as unit/ml and unit/mg protein in serum and tissue samples.

Non enzymatic antioxidants

Serum vitamin C concentration was assessed using the Jagota and Dani method [31], where ascorbic acid reacts with Folin's reagent to produce a blue color, maximally absorbed at 760 nm. Vitamin E in serum was quantified according to Rosenberg method [32], with tocopherol as the reference standard. Serum levels of reduced glutathione (GSH) were measured following Sedlak and Lindsay's method [33], with absorbance recorded colorimetrically at 412 nm.

Inflammatory markers

Inflammatory markers such as ceruloplasmin [34] and myeloperoxidase [35] were assessed in the serum samples. Estimation of ceruloplasmin involved adding 0.1 ml of serum to 8 ml of 0.1 M acetate buffer (pH 5.5) and 1 ml of 0.5% p-phenylenediamine dihydrochloride. The mixture was incubated at 37°C for 1 h, followed by the addition of 1 ml of 0.5% sodium azide. After cooling at 4°C for 30 min, the resulting color was measured at 530 nm against a control. Determination of Myeloperoxidase activity was performed in an appropriate volume of samples combined with 0.75 mM H_2O_2 , 2.9 mM TMB (prepared in 14.5% DMSO), and 150 mM sodium phosphate buffer (pH 5.4). The reaction was terminated by adding 2 M H_2SO_4 , and absorbance was recorded at 450 nm over 3 min.

Oxidative stress markers

Estimation of lipid peroxidation was assessed with 1 ml of tissue homogenate was mixed with 2 ml of a TCA-TBA-HCl solution (15% TCA, 375 mg TBA, and 0.25 N HCl) and thoroughly combined. The mixture was incubated in a boiling water bath for 5 min, after which the

clear supernatant was collected, and its absorbance was measured colorimetrically at 535 nm against a blank. MDA standards ranging from 20 to 100 μ l were used for calibration [36].

Nitric oxide content was estimated in 0.4 ml sample mixed with 0.4 ml of Griess reagent (1% sulfanilamide and 0.1% N-1-naphthylethylenediamine dihydrochloride in 2.5% phosphoric acid) and incubated for 10 min in the dark at room temperature. Absorbance was then measured at 540 nm against a reagent blank, with nitrite concentration determined from a sodium nitrite standard curve (10–50 μ g/ml) [37].

According to the method of Ellman and Lysko, Total sulfhydryl content was carried out in 20 μ l sample, buffered with 2.9 ml of 0.2 M Tris-HCl (pH 8.2) containing 0.02 M EDTA. To this mixture, 0.1 ml of DTNB solution (prepared by dissolving 100 mg DTNB in 2.5 ml absolute methanol) and 3 ml of chloroform-isopropanol (3:2) were added. After incubating for 10 min, the reaction mixture was centrifuged at 3000 rpm for 5 min and the absorbance was read at 412 nm [38].

Protein carbonyl content was measured with 1 ml of cardiac tissue homogenate treated with 4 ml of 10 mM DNPH in 2.5 M HCl and incubated at room temperature for 60 min. Following this, 20% TCA was added, and the resulting mixture was washed three times with 4 ml of an ethanol-ethyl acetate solution. The protein precipitate was then separated by centrifugation, redissolved in 2 ml of 6 M guanidine HCl, and the carbonyl content was measured at an absorbance of 382 nm [39].

Determination of Advanced oxidation Protein products (AOPP) were experimented with Taylor et al. [40]. Aliquoted 10 μ L of KI and 20 μ L of glacial acetic acid into 200 μ L of the diluted sample (10% PBS), followed by spectrophotometric analysis at 340 nm against a blank solution. A standard curve was generated using 200 μ L of chloramine T standards ranging from 0 to 100 μ M.

Collagen content was assayed in cardiac tissue samples that were thoroughly mixed with 50 μ l of 2 N NaOH and hydrolyzed at 120 °C for 20 min. Once the samples had cooled to room temperature, 450 μ l of 0.056 M chloramine-T was added, followed by a 20-minute incubation. Subsequently, 1 M Ehrlich Reagent was introduced, and the absorbance was measured at 550 nm after 15 min. The hydroxyproline content in the unknown samples was quantified using a standard curve, and collagen content was calculated by multiplying the hydroxyproline content by 8.2 [41].

Gene expression of *nrf-2*

Excised cardiac tissue samples were processed for total RNA extraction using the TRIzol method (Catalog No: T9424), followed by cDNA synthesis as per the manufacturer's instructions (Catalog No: 1708890). Polymerase

chain reaction (PCR) amplification targeted *nrf-2* (forward primer: 5'- CAGTCTTCACCACCCCTGAT-3'; reverse primer: 5'- GGTA GTCTCAGCCTGCTGCT-3') with *arbp* utilized as the reference gene (forward primer: 5'- CCCTTCTCCTTCGGGCTGAT-3'; reverse primer: 5'-TGAGGCAACAGTCGGGTAGC-3') [42].

Histopathological analysis

Histological sections the left ventricular hearts were stained using Haematoxylin and Eosin. Microscopic observation at magnifications of 10x and 40x facilitated the documentation of any deviations from the standard structural characteristics.

Statistical analysis

All experiments were conducted in triplicate ($n=3$), and the results are presented as mean $\pm$ standard deviation (SD) for each experimental group. Differences between groups were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test for post-hoc analysis to assess significance between treatment groups. A p -value of <0.05 was considered statistically significant. Statistical analyses and data visualization were performed using software such as GraphPad Prism.

Results

Effect on enzymatic antioxidants

The animals treated solely with CV or SBH (Groups IV and V) demonstrated increased enzymatic antioxidant activities, reflecting a restorative trend towards normal levels observed in Group I (Fig1). This finding highlights the potential of CV and SBH as individual agents in mitigating the oxidative stress induced by ISO (Group II). Compared to losartan (Group III), the combined treatment with CV and SBH showed comparable efficacy in restoring enzymatic antioxidant activities. This synergistic effect observed in Groups VI and VII underscores the superior efficacy of the combined treatments. While CV or SBH alone partially restored antioxidant enzyme levels, their combination resulted in near-complete normalization of CAT, SOD, and GPx activities in both serum and tissue. This suggests a complementary mechanism of action between CV and SBH, potentially amplifying their individual antioxidative properties.

Effect on non-enzymatic antioxidants

Animals induced with ISO (Group II) exhibited significantly reduced non-enzymatic antioxidant serum levels such as Vitamin C and Vitamin E, compared to the control group (Group I). Treatment with aqueous extracts of CV and SBH individually showed a notable improvement in antioxidant levels. However, the combined treatment with CV and SBH (Group VI and VII) demonstrated a synergistic effect, restoring antioxidant

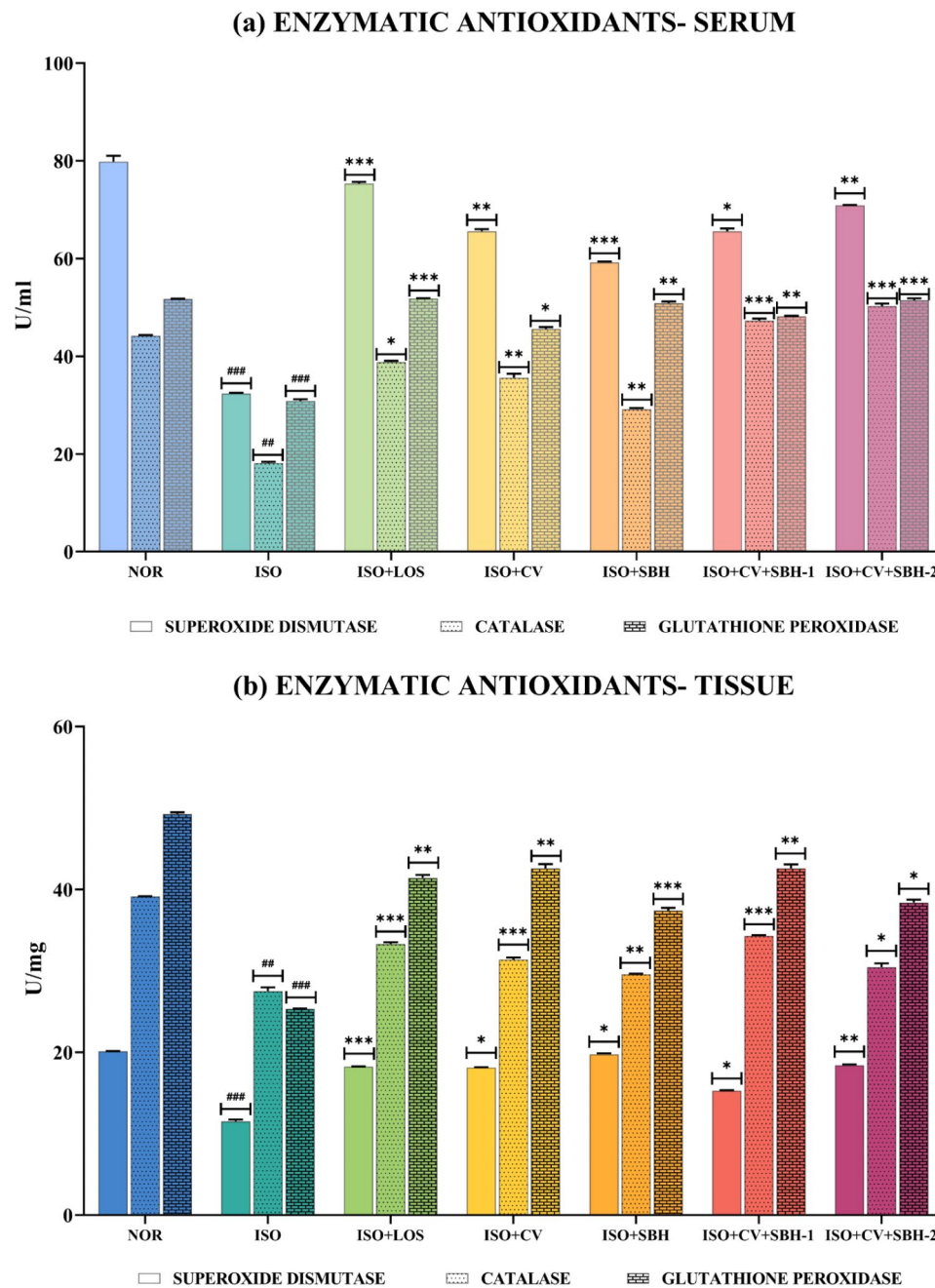


Fig. 1 Combinatorial effect of *Cinnamomum verum* and Stingless Bee Honey extract supplementation on Enzymatic antioxidant activities of Superoxide dismutase activity in (a) serum and (b) tissue in isoproterenol induced hypertrophied experimental rats. Values are mean $\pm$ SD; Statistical significance at ### $p < 0.001$, ## $p < 0.01$, # $p < 0.05$ when compared with the normal group and *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ when compared with Isoproterenol group by one-way repeated-measures ANOVA, post-hoc Dunnett's multiple comparison test. Group I-NOR-Untreated rats; Group II-ISO- Isoproterenol induced rats; Group III-ISO+LOS- Isoproterenol induced rats treated with losartan; Group IV-Isoproterenol induced rats treated with aqueous bark extract of *C. verum*; Group V-Isoproterenol induced rats treated with aqueous extract of SBH; Group VI-Isoproterenol induced rats treated with aqueous extract of both CV (high) and SBH (low); Group VII-Isoproterenol induced rats treated with aqueous extract of both CV (low) and SBH (high). NOR-Normal; ISO-Isoproterenol; LOS-Losartan; CV-*C. verum* and SBH-Stingless Bee Honey

levels more effectively than either treatment alone. This combined treatment outperformed individual treatments by achieving levels comparable to the untreated and Losartan groups. Myocardial antioxidant status, after

isoproterenol administration was evaluated by measuring intracellular non-enzymatic antioxidant levels (Fig2).

The serum levels of reduced glutathione were higher in the experimental groups treated with LOS, CV + SBH-1, and CV + SBH-2 (Groups III, VI and V), approaching

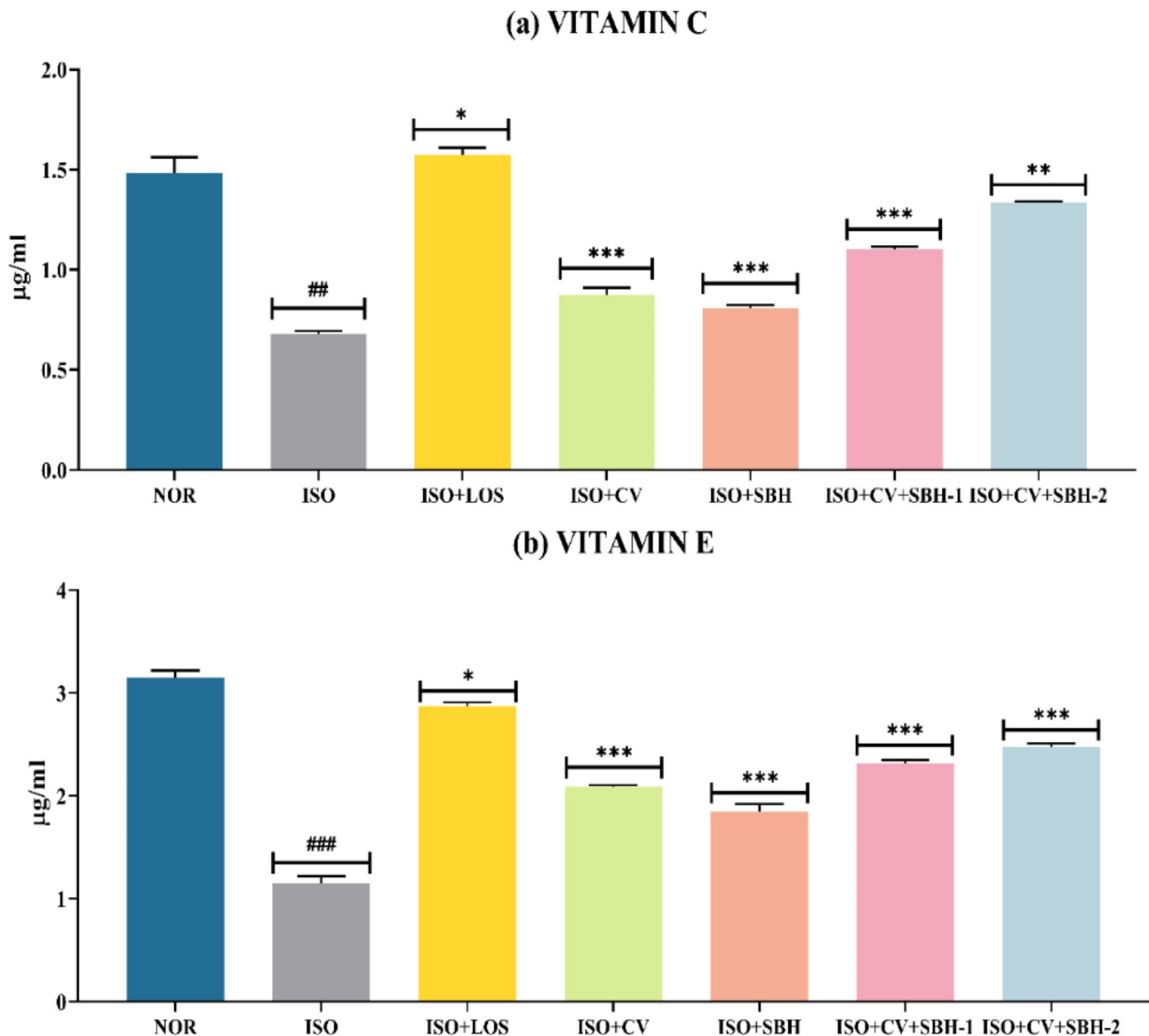


Fig. 2 Combinatorial effect of *Cinnamomum verum* and Stingless Bee Honey extract supplementation on serum levels of (a) Vitamin C and (b) Vitamin E in isoproterenol induced hypertrophied experimental rats. Values are mean $\pm$ SD; Statistical significance at ### p < 0.001, ** p < 0.01, * p < 0.05 when compared with Isoproterenol group by one-way repeated-measures ANOVA, post-hoc Dunnett's multiple comparison test. Group I-NOR-Untreated rats; Group II-ISO- Isoproterenol induced rats; Group III-ISO+LOS- Isoproterenol induced rats treated with losartan; Group IV-Isoproterenol induced rats treated with aqueous bark extract of *C. verum*, Group V-Isoproterenol induced rats treated with aqueous extract of SBH; Group VI-Isoproterenol induced rats treated with aqueous extract of both CV (high) and SBH (low); Group VII-Isoproterenol induced rats treated with aqueous extract of both CV (low) and SBH (high). NOR-Normal; ISO-Isoproterenol; LOS-Losartan; CV-*C. verum* and SBH-Stingless Bee Honey

levels observed in the control group (Group I). Notably, the combined treatment groups (CV + SBH-1 and CV + SBH-2) demonstrated superior efficacy in restoring reduced glutathione levels compared to groups treated with either CV or SBH alone (Fig3). This indicates that the synergistic effects of CV and SBH enhance the antioxidant defence more effectively than isolated treatments. Furthermore, while LOS treatment (Group III) also improved glutathione levels, the combined CV + SBH treatment showed comparable effects, emphasizing its

potential as a therapeutic alternative with antioxidant benefits that surpass those of losartan.

Effect on lipid peroxidation

ISO-treated experimental rats (Group II) exhibited a significant increase in tissue MDA levels, indicating heightened oxidative stress (Fig4). In rats co-administered CV and SBH (Groups VI and VII), lipid peroxidation derivatives declined to levels close to those in normal controls and losartan-treated groups (Groups I and III). This

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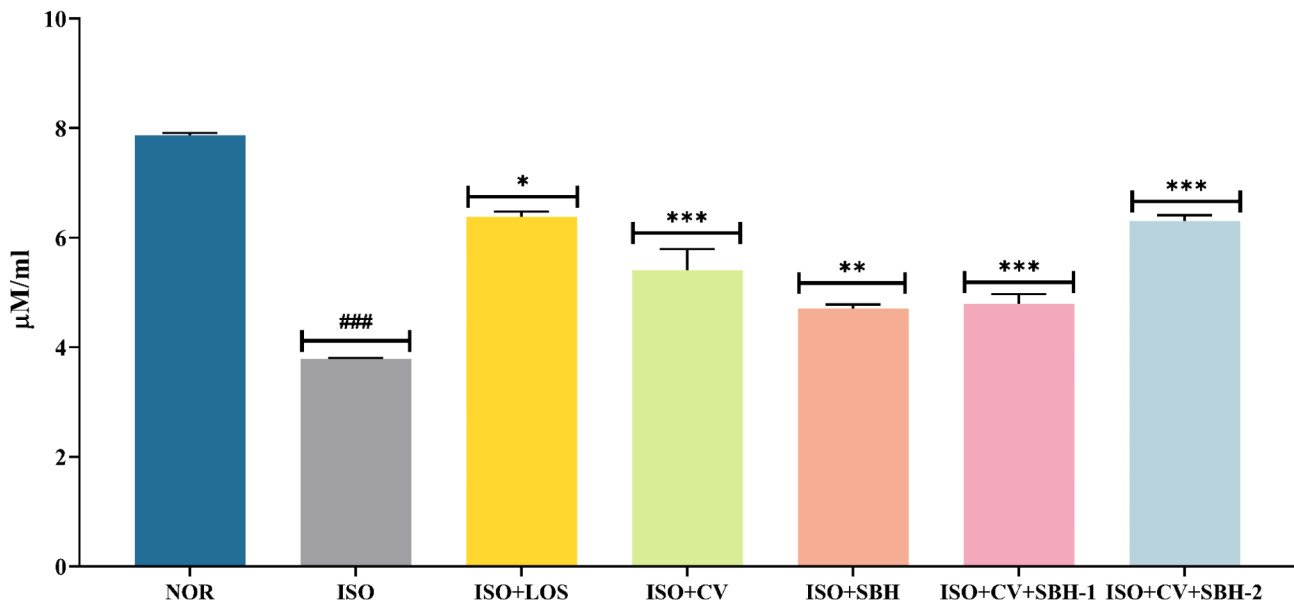


Fig. 3 Combinatorial effect of *Cinnamomum verum* and Stingless Bee Honey extract supplementation on serum reduced glutathione in isoproterenol induced hypertrophied experimental rats. Values are mean $\pm$ SD; Statistical significance at ### p < 0.001 when compared with the normal group and *** p < 0.001, ** p < 0.01, * p < 0.05 when compared with Isoproterenol group by one-way repeated-measures ANOVA, post-hoc Dunnett's multiple comparison test. Group I-NOR-Untreated rats; Group II-ISO- Isoproterenol induced rats; Group III-ISO+ LOS- Isoproterenol induced rats treated with losartan; Group IV-Isoproterenol induced rats treated with aqueous bark extract of *C. verum*, Group V-Isoproterenol induced rats treated with aqueous extract of SBH; Group VI-Isoproterenol induced rats treated with aqueous extract of both CV (high) and SBH (low); Group VII-Isoproterenol induced rats treated with aqueous extract of both CV (low) and SBH (high). NOR-Normal; ISO-Isoproterenol; LOS-Losartan; CV-*C. verum* and SBH-Stingless Bee Honey

reduction exceeded the effects of individual treatments with CV or SBH (Groups IV and V), indicating a synergistic enhancement of anti-lipid peroxidation properties in the combined therapy.

Effect on protein carbonylation and advanced oxidation protein products

In comparison to the ISO group, a decrease in PCO and AOPP was observed in the combined treatment group, with values falling below those noted in both CV and SBH administered individually. The initial administration of the combined CV and SBH regimen resulted in a significant reduction in PCO and AOPP levels compared to the Losartan-treated group (Group III). This reduction achieved statistical significance, especially when contrasted with the individual CV and SBH groups (Fig5A).

Effect on nitric oxide and total sulphydryl content

A significant reduction in nitric oxide and total Sulphydryl content was observed in the isoproterenol-treated group when compared to the Control group (Group I). Conversely, CV and SBH treatment in Groups VI and VII resulted in a statistically significant increase in both markers. The largest increases were observed in the CV and SBH-treated groups (IV and V), as depicted in Fig5B.

Effect on serum inflammatory markers such as ceruloplasmin and myeloperoxidase

Serum levels of ceruloplasmin and myeloperoxidase were significantly higher in the ISO group, as shown in Fig6. However, groups treated individually with CV or SBH (Groups IV and V) exhibited a moderate decrease in these levels. A more substantial decline was noted in the groups receiving both CV and SBH (Groups VI and VII), indicating a potent synergistic effect of the combined treatment.

Effect on collagen

In Fig7, control rats displayed baseline collagen levels, whereas ISO-induced rats (Group II) showed a marked increase in collagen accumulation. Losartan administration in isoproterenol-treated rats (Group III) reduced collagen content. In rats receiving both the combination of CV and SBH, collagen content was significantly lower than in the isoproterenol-only group. The use of either CV or SBH alone also resulted in reduced collagen levels, but these effects were less significant than when both were used together.

Effect on Nrf-2 gene expression

In hypertrophied rats (Group II), there was a pronounced decrease in *nrf-2* gene expression levels relative to both

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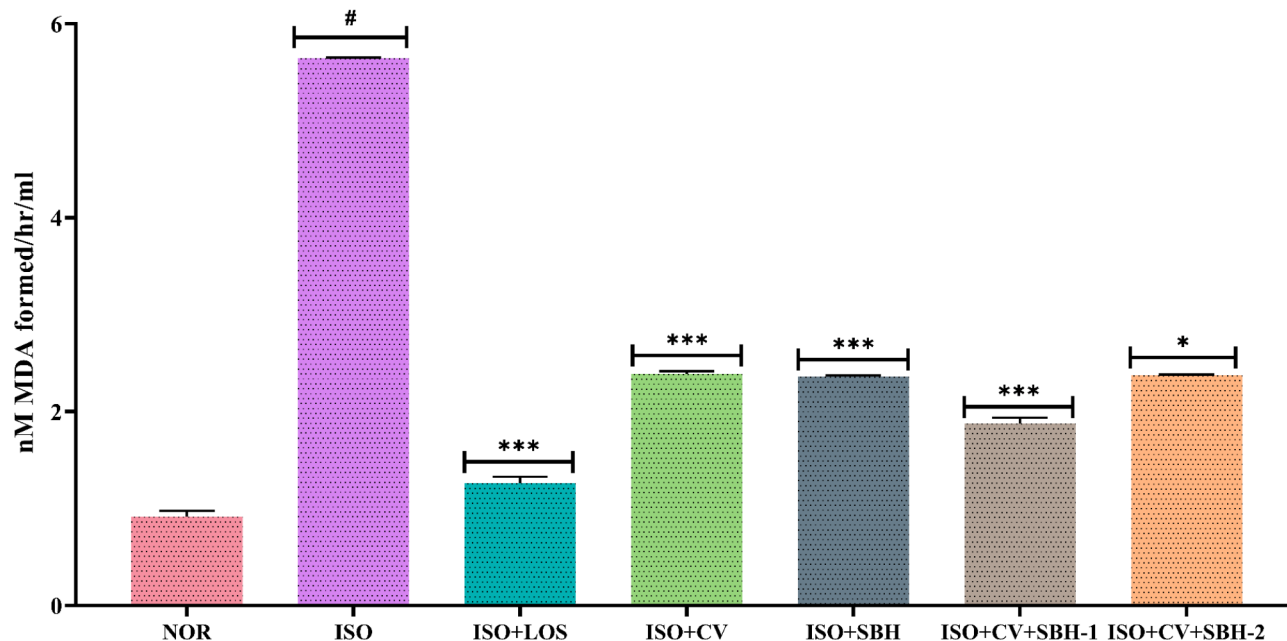


Fig. 4 Combinatorial effect of *Cinnamomum verum* and Stingless Bee Honey extract supplementation on tissue lipid peroxidation in isoproterenol induced hypertrophic experimental rats. Values are mean $\pm$ SD; Statistical significance at # $p < 0.05$ when compared with the normal group and *** $p < 0.001$, * $p < 0.05$ when compared with Isoproterenol group by one-way repeated-measures ANOVA, post-hoc Dunnett's multiple comparison test. Group I-NOR-Untreated rats; Group II-ISO- Isoproterenol induced rats; Group III-ISO + LOS- Isoproterenol induced rats treated with losartan; Group IV-Isoproterenol induced rats treated with aqueous bark extract of *C. verum*; Group V-Isoproterenol induced rats treated with aqueous extract of SBH; Group VI-Isoproterenol induced rats treated with aqueous extract of both CV (high) and SBH (low); Group VII-Isoproterenol induced rats treated with aqueous extract of both CV (low) and SBH (high). NOR-Normal; ISO-Isoproterenol; LOS-Losartan; CV-*C. verum* and SBH-Stingless Bee Honey

normal and losartan-treated rats (Group I and III). However, administration of CV and SBH, either alone or in combination (Groups IV-VII), resulted in a significant reversal of *nrf-2* gene expression levels (Fig8).

Effect on histopathological analysis

In the ISO group, the presence of severe muscle fibres necrosis, marked by fragmentation and concurrent vascular changes, is evident. In contrast, both Normal and Losartan treatment groups exhibit a normal cardiac muscle arrangement, distinguished by well-defined striations. Furthermore, in comparison to the ISO group, individual CV and SBH treated groups exhibit diminished myocardial damage and a reduction in inflammation. Conversely, within the ISO + CV + SBH-1 and 2 groups, the cardiac sections demonstrated an almost normal myocardial structure close to normal, with the only deviation being the presence of wide gaps between the cardiac fibres in certain localized regions. Cellular necrosis, myofibril degeneration, accompanied by an expansion of interstitial space were prominent features of cell necrosis induced by ISO administration (Fig9).

Discussion

Synergism in pharmacodynamics pertains to the combined effect that occurs when diverse mechanisms of actions target active constituents with cumulative therapeutic activities [43]. Few reports have demonstrated that the utilization of polyherbal combinations, consisting of two or more plant extracts, have exhibited significantly better bioactivities in comparison to their individual extracts [44].

Enzymatic antioxidants are a vital class of enzymes that actively combat harmful free radicals, protecting cells from oxidative damage. They play a crucial role in maintaining the delicate balance of reactive oxygen species (ROS) within cells, as discussed by Valko et al. [45]. Research has shown that increased oxidative stress, as seen in ISO-induced cardiac damage, correlates with decreased activity of key antioxidant enzymes like GPx, SOD, and CAT in heart tissue of ISO-treated rats [46]. This study provides empirical evidence supporting the antioxidant efficacy of CV and SBH in addressing oxidative stress, potentially mitigating membrane damage that are supported by their findings, which aligns with previous studies [47, 48].

Vitamin C holds a critical role in collagen synthesis and immune function, while also aiding in iron absorption

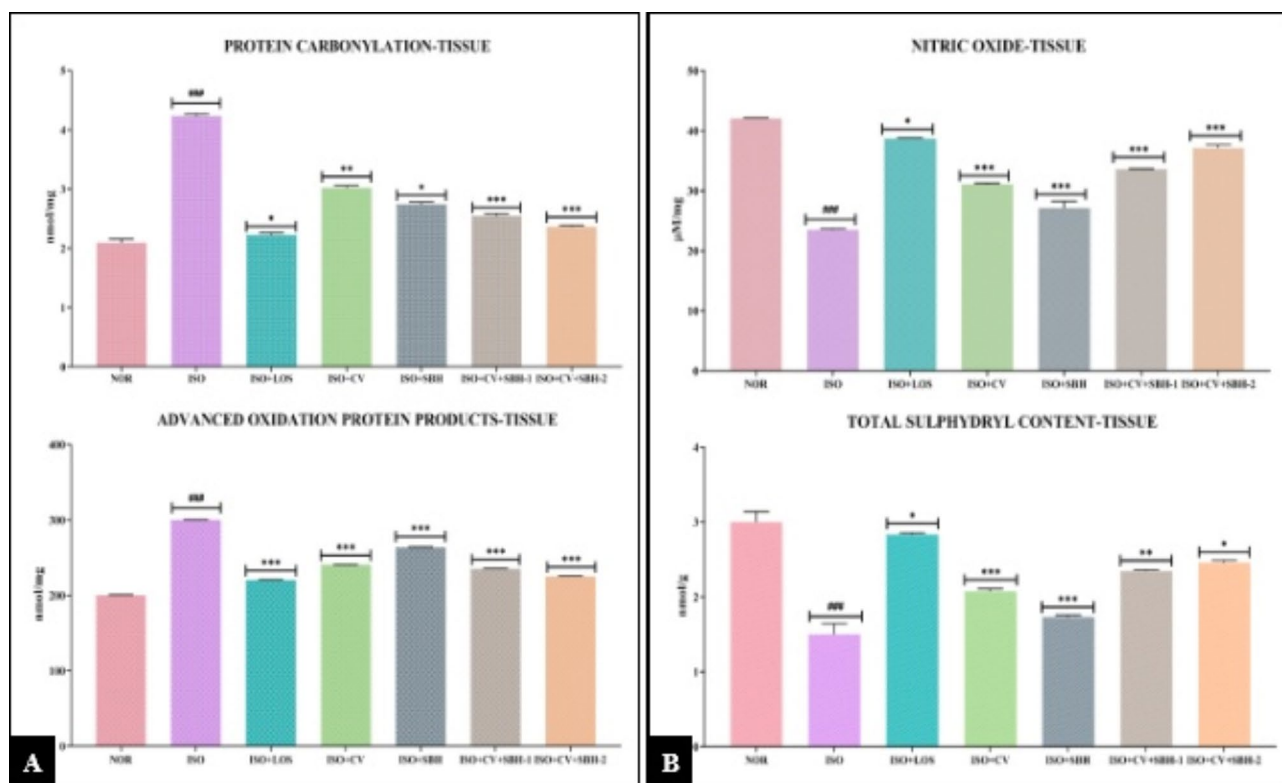


Fig. 5 Combinatorial effect of *Cinnamomum verum* and Stingless Bee Honey extract supplementation on tissue levels of **(A)** Protein carbonyls and Advanced oxidation protein products **(B)** Nitric oxide and Total Sulphydryl content in isoproterenol induced hypertrophied experimental rats. Values are mean $\pm$ SD; Statistical significance at $^{***}p < 0.001$ when compared with the normal group and $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$ when compared with Isoproterenol group by one-way repeated-measures ANOVA, post-hoc Dunnett's multiple comparison test. Group I-NOR-Untreated rats; Group II-ISO-Isoproterenol induced rats; Group III-ISO + LOS- Isoproterenol induced rats treated with losartan; Group IV-Isoproterenol induced rats treated with aqueous bark extract of *C. verum*, Group V-Isoproterenol induced rats treated with aqueous extract of SBH; Group VI-Isoproterenol induced rats treated with aqueous extract of both CV (high) and SBH (low); Group VII-Isoproterenol induced rats treated with aqueous extract of both CV (low) and SBH (high). NOR-Normal; ISO-Isoproterenol; LOS-Losartan; CV-*C. verum* and SBH-Stingless Bee Honey

and neurotransmitter production [46]. Concurrently, Rizvi et al. emphasized the significance of vitamin E in cell signalling, skin health promotion, and cardiovascular well-being. Together, these vitamins act as antioxidants, bolstering the immune system, maintaining skin integrity, and preventing oxidative damage. Tocopherols, particularly, excel in scavenging peroxy radicals, protecting cells from lipid peroxidation [49]. The use of Cinnamon and Honey treatment appeared to normalize serum vitamin C and E levels, potentially indicating a reduction in membrane damage through antioxidative pathways, consistent with the earlier research conducted by Ponnian et al. [50].

Isoproterenol generates quinones that undergo a reaction with oxygen, resulting in the production of superoxide anions [$O_2^{\cdot-}$] and H_2O_2 [51]. The elevated concentration of LPO products further highlighted the severity of cardiac membrane damage by the release of ROS by ISO could be the origin of rising cardiac markers, oxidative damage, and lipid peroxidation. The essential oil of cinnamon contains an array of antioxidant-rich

ingredients, notably (E)-cinnamaldehyde, eugenol, and linalool, that are known to hinder LPO and peroxynitrite-induced nitration [52]. Studies have shown that stingless bee honey (SBH) has a higher concentration of the polyphenols that act as antioxidants over other kinds of honey [53]. Therefore, it has been considered that routine consumption of CV and SBH strengthens the antioxidant defense mechanism against lipid peroxidation.

Protein carbonylation, an irreversible oxidative change, serves as an early sign of oxidative stress-related ailments. It occurs through direct oxidation of tryptophan, metal-catalyzed oxidation of various residues, and reactions with lipid peroxidation products. Carbonylated proteins are unrepairable by cellular enzymes and are degraded by the proteasomal system [54]. Plasma proteins that have been oxidized due to exposure to oxidation products and are transported by albumin in the blood are called Advanced Oxidation Protein Products (AOPPs). Elevated levels of AOPP in ISO-induced rats, facilitated by chronic oxidative stress, contribute to cardiac remodeling [55]. Important steps in the development of cardiomyopathy

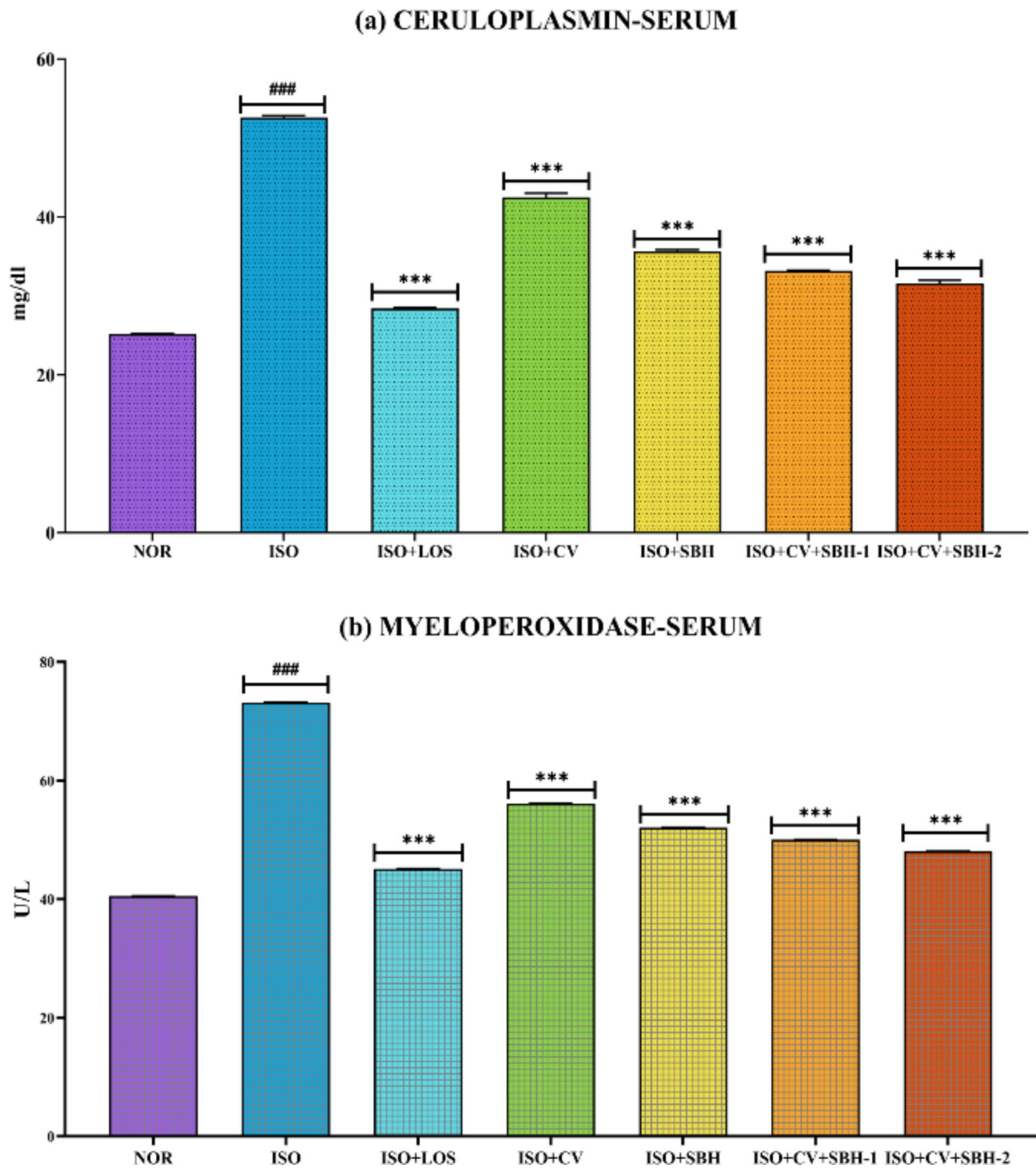


Fig. 6 Combinatorial effect of *Cinnamomum verum* and Stingless Bee Honey extract supplementation on serum levels of **(a)** ceruloplasmin and **(b)** myeloperoxidase in isoproterenol induced hypertrophied experimental rats. Values are mean $\pm$ SD; Statistical significance at $^{###}p < 0.001$ when compared with the normal group and $^{**}p < 0.01$, $^{*}p < 0.05$ when compared with Isoproterenol group by one-way repeated-measures ANOVA, post-hoc Dunnett's multiple comparison test. Group I-NOR-Untreated rats; Group II-ISO- Isoproterenol induced rats; Group III-ISO + LOS- Isoproterenol induced rats treated with losartan; Group IV-Isoproterenol induced rats treated with aqueous bark extract of *C. verum*; Group V-Isoproterenol induced rats treated with aqueous extract of SBH; Group VI-Isoproterenol induced rats treated with aqueous extract of both CV (high) and SBH (low); Group VII-Isoproterenol induced rats treated with aqueous extract of both CV (low) and SBH (high). NOR-Normal; ISO-Isoproterenol; LOS-Losartan; CV-*C. verum* and SBH-Stingless Bee Honey

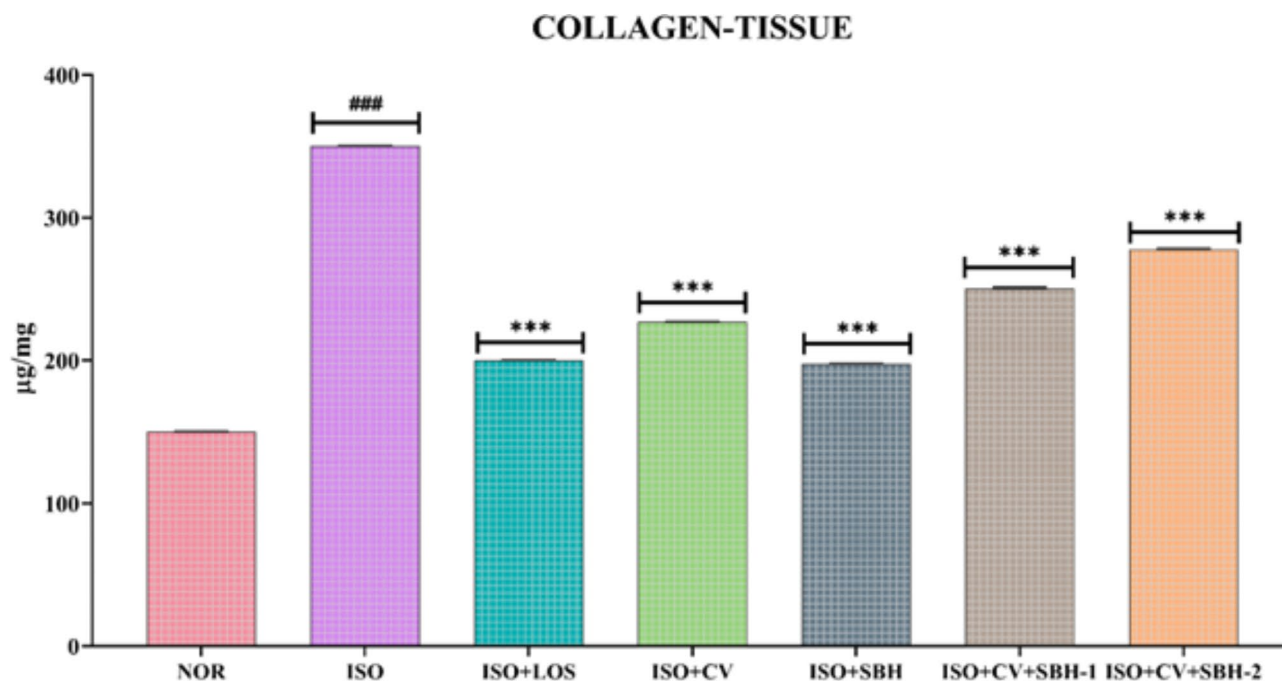


Fig. 7 Combinatorial effect of *Cinnamomum verum* and Stingless Bee Honey extract supplementation on cardiac tissue levels of Collagen in isoproterenol induced hypertrophied experimental rats. Values are mean $\pm$ SD; Statistical significance at ### $p < 0.001$ when compared with the normal group and *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ when compared with Isoproterenol group by one-way repeated-measures ANOVA, post-hoc Dunnett's multiple comparison test. Group I-NOR-Untreated rats; Group II-ISO- Isoproterenol induced rats; Group III-ISO + LOS- Isoproterenol induced rats treated with losartan; Group IV-Isoproterenol induced rats treated with aqueous bark extract of *C. verum*, Group V-Isoproterenol induced rats treated with aqueous extract of SBH; Group VI-Isoproterenol induced rats treated with aqueous extract of both CV (high) and SBH (low); Group VII-Isoproterenol induced rats treated with aqueous extract of both CV (low) and SBH (high). NOR-Normal; ISO-Isoproterenol; LOS-Losartan; CV-*C. verum* and SBH-Stingless Bee Honey

involve the activation of Receptor for Advanced Glycation End Products (RAGE) by their ligands, which promotes signal transduction and creates cellular oxidative stress by increasing superoxide levels [56]. The ability of proanthocyanidins in cinnamon extract to scavenge reactive carbonyl species to prevent AGE production has been reported by Peng et al. [57]. According to Oyagbemi et al., luteolin (a flavonoid in honey) has a strong defensive mechanism against Oxidative stress which is linked to the NF- κ B/Nrf 2 signaling pathways by reducing the protein carbonyl levels in hypertensive rats [58].

Increased mitochondrial ROS production is associated with lipid and protein structural modifications as well as impaired enzyme activity [59]. Amino acid side chains are susceptible to oxidation, which can result in either a net gain or loss of mass for the protein molecule [60].

Sulphydryl, a prevalent ligand in biological systems, undergoes oxidation in ISO-induced cardiotoxicity, particularly on proteins and thiols [61]. ISO oxidation produces o-quinones, cyclized into aminochromes, which then oxidize sulphydryl groups produces ROS that are less easily reduced in serum than intracellularly [62]. Cinnamic acid, found in cinnamon (*C. verum*) barks and Stigmasterol in Stingless Bee Honey act as antioxidants and boost the free radical trapping by suppressing the thiol oxidation [63, 64].

NO released by the constitutively produced eNOS and nNOS isoforms may be cardioprotective [65]. Energy imbalance, mitochondrial dysfunction, OS with elevated nitrite and nitrate levels are the hallmarks of cytotoxicity caused by NO production. During MI, ISO might turn on nitric oxide synthase (NOS) and favor the generation of RNS limiting endothelial response by lowering NO availability [66]. Reduced NO production or increased breakdown further diminishes NO availability [67]. Earlier reports support the anti-inflammatory effects of Cinnamaldehyde in *C. verum* and stigmasterol in SBH through the inhibition of LPS-induced iNOS production and NO release [68–70].

Ceruloplasmin is an extracellular antioxidant that catalyzes the oxidative re-incorporation of liberated iron into ferritin and thereby scavenges superoxide radicals and inhibits ferritin-dependent lipid peroxidation [71]. Activation of MPO, an inflammatory enzyme, results in the production of HOCl, increases the levels of pro-inflammatory cytokines, and promotes protein nitration. Inflammation and oxidative stress have a mutually reinforcing effect that may be particularly destructive to the heart tissues [72]. ISO-induced CH in rats exhibited lower ceruloplasmin levels, potentially due to its ferro-oxidase and copper-binding capacities being overwhelmed by free radicals. Tea polyphenols improved

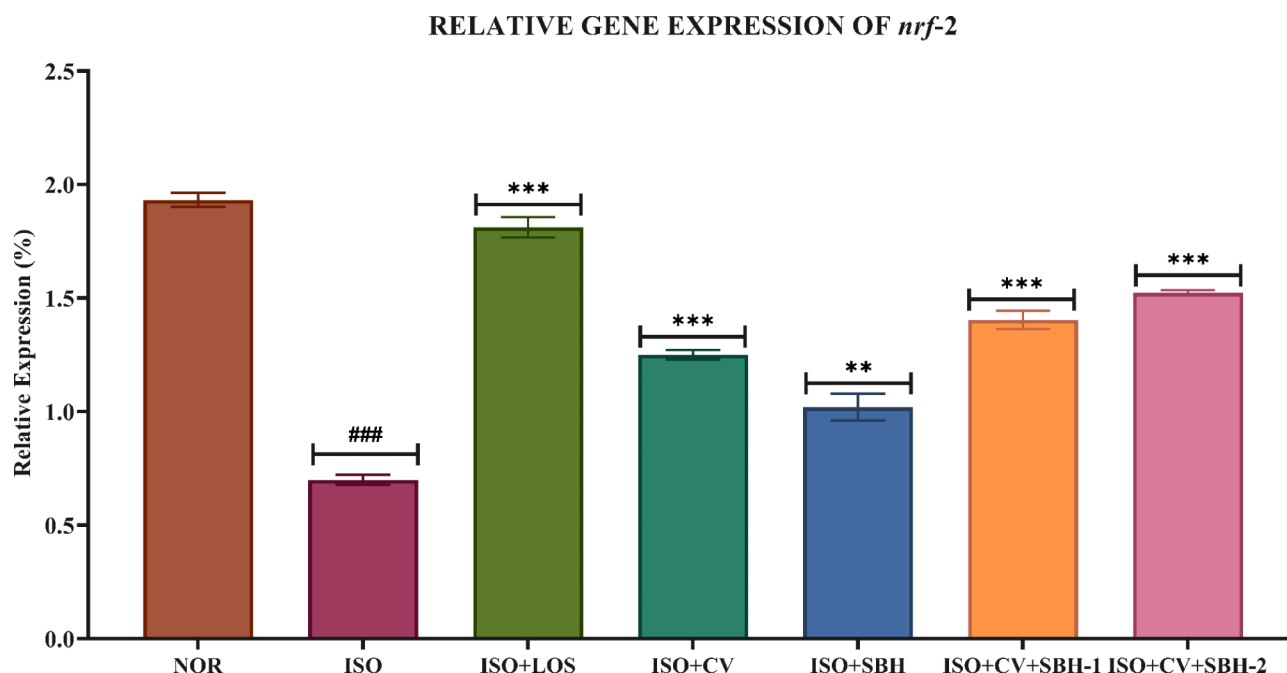


Fig. 8 Gene expression studies of *nrf2* using semi-quantitative PCR. Statistical significance at ### $p < 0.001$ when compared with the normal group and *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ when compared with Isoproterenol group by one-way repeated-measures ANOVA, post-hoc Dunnett's multiple comparison test. Group I-NOR-Untreated rats; Group II-ISO- Isoproterenol induced rats; Group III-ISO+LOS- Isoproterenol induced rats treated with losartan; Group IV-Isoproterenol induced rats treated with aqueous bark extract of *C. verum*, Group V-Isoproterenol induced rats treated with aqueous extract of SBH; Group VI-Isoproterenol induced rats treated with aqueous extract of both CV (high) and SBH (low); Group VII-Isoproterenol induced rats treated with aqueous extract of both CV (low) and SBH (high).NOR-Normal; ISO-Isoproterenol; LOS-Losartan; CV-*C. verum* and SBH-Stingless Bee Honey

hepatic iron metabolism in ceruloplasmin-deficient mice, possibly through metal-chelating effects, and flavonoid-rich plant extracts may enhance hepcidin expression, reducing tissue iron overload by stimulating Nrf-2 signaling [73]. Furthermore, *C. verum* and honey include a significant level of cinnamaldehyde (polyphenol) and Stigmasterol (phytosterol), which has an anti-inflammatory action [74, 75] and may help to downregulate the inflammatory pathway induced by ISO.

An integral component in the onset of heart failure is the remodeling of collagen tissue in the heart. One of the most striking features of cardiac fibrosis is an abnormally high concentration of matrix fibrillar collagens. ROS could effectively influence the activation of collagen that exerts the adverse effect on Extracellular matrix proteins [76]. Increased levels of matrix metalloproteinases (MMPs) are related to cardiac dysfunction, and recent research has revealed that isoproterenol plays a critical and facilitating role in collagen production in these hearts [77]. Treatment with cinnamaldehyde reversed the pro-fibrotic genes such as *col I* and *III* in left ventricular hypertrophied rats [78]. Luteolin prevented myocardial hypertrophy by inhibiting matrix protein synthesis through activation of the Nrf-2 antioxidant mechanism [79].

The regulation of genes encoding antioxidant proteins, detoxifying enzymes, and cytoprotective proteins by

Nrf-2, a transcription factor, underscores its pivotal role in modulating cellular antioxidant responses and defending against oxidative stress. The increased expression of *nrf-2* in Groups VI and VII suggests an adaptive response to oxidative stress in the left ventricle following isoproterenol administration. This upregulation signifies the activation of protective mechanisms aimed at mitigating oxidative damage by enhancing the synthesis of antioxidant enzymes and proteins. Previous research supports the notion that cinnamaldehyde and stigmasterol can induce *nrf-2* gene expression, thereby promoting cellular antioxidant defenses [80, 81].

Erejuwa and others have hypothesized that the antioxidant enzyme functions are restored by Combinatorial extract because of an increase in the activity or expression of *nrf2*, a transcription factor released from its repressor (Keap1) under oxidative or xenobiotic stress [82]. In response to cytotoxic oxidants, the released Nrf2 binds to the antioxidant response element of cytoprotective genes, inducing their expression and, in turn, the expression of free radical scavenging enzymes.

The rats under ISO administration led to observable alterations in rat cardiomyocytes including an enlargement of cell size accompanied by an increased cytoplasmic content. The intensification of pink staining between myocytes was attributed to the development of interstitial fibrosis, which was attributed to the mechanical

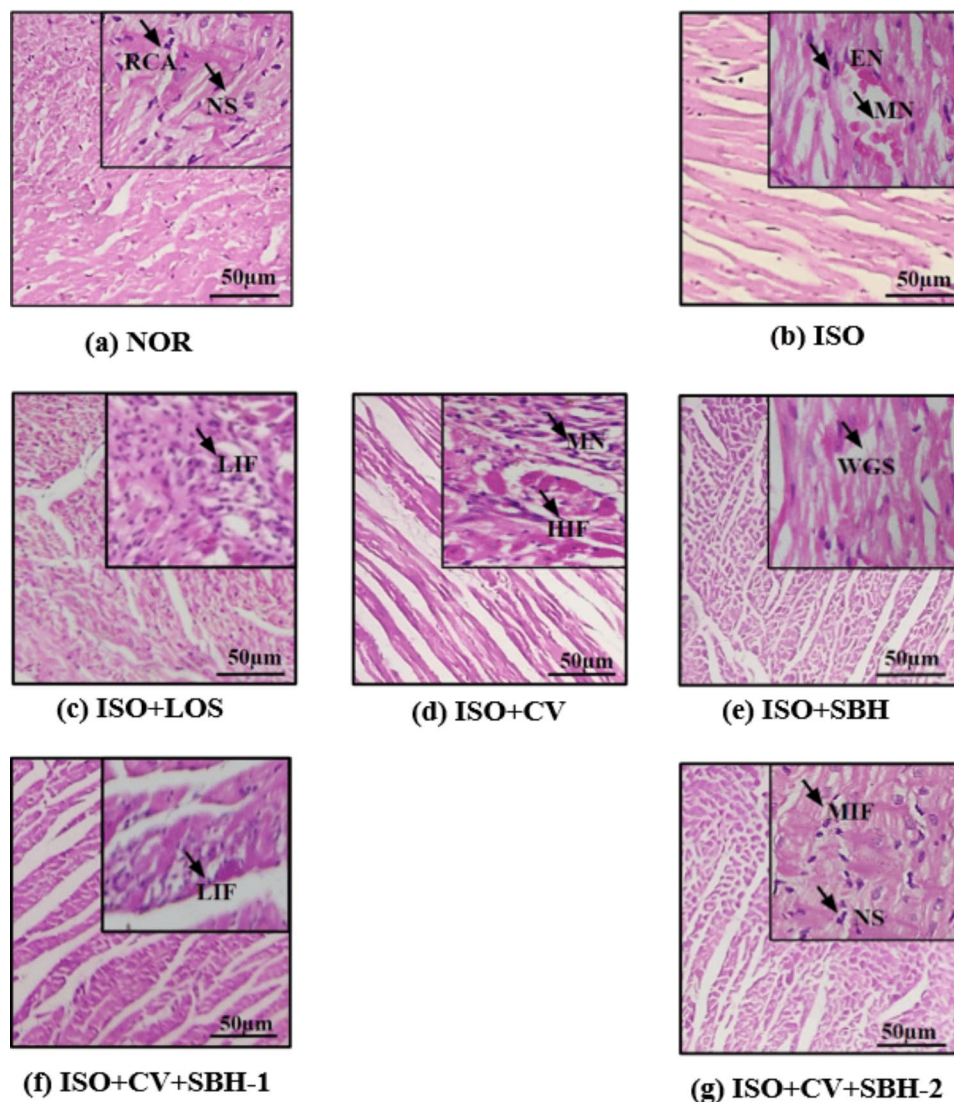


Fig. 9 Combinatorial effect of *Cinnamomum verum* and Stingless Bee Honey extract supplementation on Haematoxylin and eosin staining in isoproterenol induced hypertrophied experimental rats. Group I-NOR-Untreated rats; Group II-ISO- Isoproterenol induced rats; Group III-ISO+LOS- Isoproterenol induced rats treated with losartan; Group IV-Isoproterenol induced rats treated with aqueous bark extract of *C. verum*, Group V-Isoproterenol induced rats treated with aqueous extract of SBH; Group VI-Isoproterenol induced rats treated with aqueous extract of both CV (low) and SBH (high); Group VII-Isoproterenol induced rats treated with aqueous extract of both CV (low) and SBH (high). NOR-Normal; ISO-Isoproterenol; LOS-Losartan; CV-*C. verum* and SBH-Stingless Bee Honey. This set of histological images with a primary magnification of $\times 10$ and the insets with $40\times$ magnification. Black RCA-Regular cardiomyocyte arrangement; NS-Normal striations; MN-Myocardial necrosis; EN-Enlarged nuclei; WGS-Widened gaps between sarcoplasm; LIF- Low inflammatory infiltrates; MIF-Moderate inflammatory infiltrates; HIF-High inflammatory infiltrates

stress experienced. Furthermore, the presence of inflammatory infiltrates was a consequence of myocardial injury in response to the imposed stress. The research undertaken by Liang et al., in 2011 brought to light the disruption in the release of free oxygen radicals, causing an imbalance in myocardial redox homeostasis, consequently leading to myocardial injury [83]. Our findings align with this observation, indicating that a disruption in myocardial redox status results in myocardial injury, and this injury can be mitigated by Sulphur dioxide [84].

Our findings here corroborate ISO-induced myocardial oxidative stress by exhibiting a marked decline in myocardial antioxidant concentrations with an upsurge in myocardial oxidative stress and inflammatory markers. The efficacy of the CV and SBH combination therapy in restoring enzymatic antioxidant activities was on par with that of losartan (Group III). Losartan, a known angiotensin receptor blocker, counteracts oxidative stress indirectly by reducing hypertension-induced damage. The concurrent administration of the polyherbal combination (CV and SBH) in rats subjected to ISO treatment

resulted in an augmentation of antioxidant levels, with reciprocal modulation of oxidative and inflammatory markers almost equal to that of the normal group, affirming their cardioprotective effect.

This investigation highlighted that polyherbal combinations, when compared to individual herbal extracts, demonstrated superior efficacy in terms of bioactivity in combating oxidative stress. The synergy among various phytochemical substances within these combinations significantly increased their bioactivity. Cinnamaldehyde and stigmasterol, present in the formulated extract, demonstrated myocardial protection by suppressing oxidative stress in cardiac tissues. These findings support the use of polyherbal combinations in Ayurvedic product formulations, as they offer comprehensive, additive and synergistic treatments for complex diseases, leveraging the diverse mechanisms of individual compounds.

Conclusion

Aqueous extracts of Cinnamon and Stingless Bee Honey may be a source of dietary antioxidants, as oral consumption of *C. verum* and SBH extracts boosted antioxidant defense system performance and ameliorated the oxidative stress markers. We infer that Cinnamon barks and Stingless bee honey could turn out to be pertinent cardioprotective agents for reducing the oxidative stress on cardiac tissues. The outcome of this study also bolsters the hypothesis that the aforementioned extract formulation could be pertinent as an adjunctive treatment for metabolic conditions associated with oxidative stress.

Abbreviations

CH	Cardiac hypertrophy
ISO	Isoproterenol
LOS	Losartan
CV	<i>Cinnamomum verum</i>
SBH	Stingless Bee honey
ROS	Reactive Oxygen species
Nrf-2	Nuclear factor erythroid 2- related factor
SOD	Superoxide dismutase
CAT	Catalase
GPx	Glutathione Peroxidase
GSH	Reduced glutathione
NO	Nitric oxide
AOPP	Advanced Oxidation Protein Products

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Author contributions

DVA conceptualized the project and designed the study, AMP curated the methodology and performed the study with data interpretation; AMP drafted the manuscript, DVA revised and the final manuscript was proofread and approved by all authors.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

Following CPCSEA guidelines and institutional regulations, all animal experiments have been carried out after acquiring approval from the Institutional Animal Ethics Committee (IAEC), Ministry of Social Justice and Empowerment, Government of India (482/IAEC/2020) at PSG Institute of Medical Science and Research (PSGIMSR).

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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