RESEARCH PAPER

Phytochemical and Pharmacological Evaluation of Indian Herbal Plants for Cardioprotective Activities in Wistar Albino Rats

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Abstract; Cardiovascular disease is the leading cause of mortality worldwide, and India bears a disproportionately high burden, with higher mortality rates than in the rest of the world, and the onset of the disease occurs much earlier than in Western populations. Ischemic myocardial injuryare often linked as pathological conditions associated with oxidative stress, inflammation, and lipid dysfunction. Although conventional single-target pharmacotherapy is effective, it is limited by an inability to target these processes simultaneously, adverse-effect burden, and cost. Although most of the reported combinations are polyherbal, the formulations are not chemically standardized, and most of them are not tested for safety and efficacy in a single investigation. *Vitex negundo*, *Abutilon indicum*, and *Gloriosa superba* are three herbs that have individual pharmacological evidence based on the presence of the marker compounds agnuside, luteolin, and colchicine in each, but no previous study has brought all three together in a single standardized formulation. Therefore, the present work was undertaken to prepare and evaluate such a formulation from the root material of these three species.;

Key Words: Cardiovascular disease, mortality, Ischemic myocardial, pharmacotherapy
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Introduction: Approximately 80% of the world's population, who live in low- and middle-income countries where the availability and affordability of synthetic pharmaceuticals are limited, continue to depend on traditional methods of healing for first-line treatment. The use of plant-based medicines for the treatment of febrile illness, inflammatory conditions, metabolic disease, and cardiac ailments dates back to many centuries when their efficacy and safety have been continuously proven and practiced through ethnobotanical methods, and the World Health Organization officially recognizes traditional medicine as a valid part of the national health infrastructure if the efficacy and safety can be established scientifically[1] Specifically in India, the codified Ayurvedic, Siddha, and Unani systems of medicine have documented thousands of formulations used for cardiac, hepatic, and metabolic and inflammatory conditions, which are all based on plant extracts[2]. The use of a combination of herbs is a therapeutic technique with a history of more than 1000 years, and is known as the polyherbal approach, with the present study providing the traditional medicine basis for this approach[3]

Cardiovascular disease is at the top of the global mortality and disability lists and is one of the largest individual burdens on health systems and national economies[4]. In 2022, nearly 19.8 million people died due to cardiovascular causes, which represented one-third of all deaths globally, with acute myocardial infarction and stroke alone responsible for 85% of these fatalities, especially in lower-income countries[5]. In 2021, at least 38% (18 million) of all premature deaths (before 70 years) among adults were due to cardiovascular diseases[6]. The trend is not improving, as cardiovascular disease cases and deaths have steadily increased from 311 million and 13.1 million in 1990 to 626 million and 19.2 million in 2023, respectively[7], and projections predict that cardiovascular disease deaths will reach 35.6 million per year and cardiovascular disease prevalence will reach 1.14 billion by mid-century across all world regions, with the greatest burden expected to be in the cities, as a result of lifestyle transition and population ageing[8].The multi-target pharmacodynamic profile of natural products is inherent and is one of the key differences in the mechanisms of action of natural products compared to single-target synthetic agents in the cardiovascular setting[9] The effects of plant-derived

polyphenols, flavonoids, terpenoids, alkaloids, and saponins involve oxidative stress, inflammation, hypolipidemic, antihypertensive, antiplatelet, and endothelium protective effects, working in concert at various molecular nodes involved in cardiovascular pathogenesis[10]. For instance, a purified extract of Salvia miltiorrhiza reduced myocardial injury, inflammation, and oxidative stress through metabolomic analysis in isoproterenol-induced myocardial infarction in mice, and taxifolin, a compound found in S. miltiorrhiza, was found to activate Nrf2 and HO-1 and inhibit NF-κB and NLRP3 inflammasome in mice with isoproterenol-induced myocardial infarction[11].the present work focused on detailed phytochemical standardization of the three plant species *Vitex negundo*, *Abutilon indicum*, and *Gloriosa superba* and their collective pharmacological evaluation.

Material & Methods:

Plant Profile: Vitex negundo commonly called Nirgundi or Sambhalu in the local language is a deciduous aromatic shrub or small tree growing up to 2–5 m tall. Roots, leaves, seeds, bark, and fruit have been used in Ayurvedic, Unani, and folk medicine as anti-inflammatory, antispasmodic, antipyretic, antifungal, and anticonvulsant agents[12].plant contain flavonoids lignans, terpenoids and iridoid glycosides. Comparative chromatographic analysis of different parts of the plant showed that negundoside was relatively more abundant in small branches than in roots, and polar fractions consistently exhibited high antioxidant activity in all the assays[13]

Abutilon indicum commonly known as Atibala or Kanghi is an erect, branching, perennial herb or undershrub. Traditional uses are systematically distributed over the various parts of the plant: seeds are used as a respiratory laxative, root is used as a diuretic, leaves and bark are used as anti-inflammatory, analgesic, and antipyretic, and the whole plant is used as a cardiac tonic[14].Alkaloids, flavonoids, saponins, terpenoids, sesquiterpene lactones, phenolic acids , sterols and a number of glycosides have been identified by chemical investigation in *Abutilon indicum* [15]

Gloriosa superba is a climbing perennial herb Traditional uses throughout Ayurvedic and Unani medicine include anti-inflammatory, anti-arthritic, analgesic, and anthelmintic uses, Colchicine is the major bioactive alkaloid of G. superba, found in tuber material at 0.021-0.86% w/w, depending on the origin, harvest time, and analysis method[16]; other tropolone alkaloids are also detected, including gloriosine, colchicoside, 3-demethylcolchicine, and N-deacetylcolchicine, as well as lycorine, phenanthrene derivatives, flavonoids (quercetin, kaempferol), sterols, and various glycosides[17,18]

SOLVENTS AND CHEMICALS

Isoproterenol (agent chosen for the induction of experimental MI) was procured from Shakti Chemicals. Throughout the investigation, all other solvents and chemicals used for extraction, phytochemical screening, biochemical estimation, and preparation of histology were of analytical grade and purchased from Shakti Chemicals.

EXPERIMENTAL ANIMALS AND ETHICAL APPROVAL

Wistar albino rats (170-200 g) were purchased from the Small Animal Breeding Center, Delhi and housed in a laboratory under standard laboratory conditions of 12 h/12 h light/dark cycle, 60-70% relative humidity, and were provided with standard pellet feed and water ad libitum throughout the experimental period. All experiments conducted for this thesis were carefully evaluated and registered by the Institutional Animal Ethics Committee of NIMS Institute of Pharmacy, NIMS University, Rajasthan, Jaipur under registration number NIMSUR/IAEC-01/2023/07 dated 23/09/23 as per the standards and

guidelines of CPCSEA and NIH on care and use of laboratory animals.

INSTRUMENTS

The Soxhlet apparatus, mechanical grinder, semi-automated biochemistry autoanalyser (BIOLIS 24i), rotary vacuum evaporator, digital analytical balance, hemocytometer, and light microscope with camera for histopathological photomicrography were used during the study.

PROCESSING OF PLANT MATERIAL

The roots of the three plants were washed under running tap water to remove the adhering soil and debris, shade-dried at room temperature, and ground with a mechanical grinder to a fine powder to improve the surface area for extraction, as per the standard practice in particle size reduction before Soxhlet extraction[19]. Each plant was powdered, and the powdered material was stored in individual plant boxes, which were airtight, until required for extraction.

PHYTOCHEMICAL SCREENING

Each ethanolic extract was subjected to standard qualitative tests for flavonoids, alkaloids, glycosides, and tannins, and the percentage extractive yield of each individual extract was calculated gravimetrically on a weight-by-weight basis relative to the starting powdered material.

PREPARATION OF THE POLYHERBAL EXTRACT

The three individual ethanolic root extracts were combined in a ratio of 2:2:1 parts by weight of *V. negundo: A. indicum: G. superba*, corresponding to 40%, 40% and 20% w/w of the total extract mass, respectively, the proportions being fixed before commencement of the animal studies and held constant for every batch used in this work.

At the acute toxicity limit dose of 5,000 mg/kg, the formulation delivered 9.50 mg/kg of colchicine as a single oral administration, representing 20 and 10 times the daily colchicine exposure at the two therapeutic dose levels. This quantity lies below the reported oral median lethal dose of colchicine in rats, which exceeds 25 mg/kg, and the species is markedly more resistant to colchicine poisoning than humans; therefore, it is an imperfect model of human colchicine toxicity[20]. The margin obtained applies to single-dose administration only and does not constitute a cumulative-exposure margin, as repeated-dose toxicological evaluation has not been undertaken.

Preparation involved the accurate weighing of each dried, concentrated extract, thorough trituration to a homogeneous mass, and storage at 4 °C in an airtight amber container until use. The rationale for combining the extracts was the reported observation that polyherbal preparations may exert antioxidant and lipid-modulating effects exceeding those of their constituent extracts administered individually.

ACUTE ORAL TOXICITY STUDY

Determination of Acute Oral Toxicity (OECD Test No. 423)

The acute oral toxicity of the polyherbal extract was evaluated using the Acute Toxic Class Method of OECD Guideline 423. A limit test was conducted at 5,000 mg/kg body weight in six female Wistar albino rats allocated to two sequential steps of three animals each, alongside a concurrent vehicle control group of three animals receiving 2 mL distilled water. The exceptional upper limit dose permitted under the guideline was adopted in place of the default 2,000 mg/kg because the three constituent plants have a documented history of human traditional use with no reported lethality below 2,000 mg/kg, and on the polyherbal precedent set out in the plant profile section. Animals were fasted for 12 h before dosing with water available ad libitum, the dose volume being derived from fasted body weight at a maximum of 2 mL/100 g, and the extract was administered by gavage. Observations were recorded individually at 30 min and at 1, 2, 4, 8, 24,

and 48 h and daily thereafter to Day 14, for changes in skin and fur, eyes, mucous membranes, autonomic and central nervous system signs, gait and behavior, tremors, convulsions, salivation, diarrhea, lethargy, sleep pattern, coma, and mortality, using criteria identical for treated and control animals and applied by the same trained observer. As the study comprised a single administration with a 14-day observation period, its findings are confined to acute oral tolerability and do not extend to repeated-dose, sub-chronic, chronic, reproductive, or genotoxic safety.

CARDIOPROTECTIVE STUDY

Induction of Myocardial Infarction and Group Design

Myocardial injury was induced with isoproterenol, a non-selective beta-adrenergic agonist whose administration to rats produces reproducible catecholamine-mediated myocardial damage and is accordingly the established pharmacological model for evaluating cardioprotective agents in this species [21]. Isoproterenol was administered subcutaneously at 20 mg/kg on two consecutive days at the conclusion of the treatment period. Rats were allocated to eight groups of six animals each, comprising normal control, isoproterenol control, propranolol 10 mg/kg, the three individual root extracts each at 500 mg/kg, and the polyherbal formulation at 250 and 500 mg/kg, all treatments being administered orally once daily for 28 days preceding isoproterenol. Propranolol was selected as the reference agent on the basis of its established protective effect against catecholamine-induced cardiac injury in the rat, in which beta-adrenergic blockade preserves cardiac function following catecholamine challenge [22].

Serum Cardiac Biomarkers and Paired Marker Enzymes

Serum cardiac troponin-T, troponin-I, and CK-MB were estimated as biomarkers of myocardial necrosis. Creatine phosphokinase, lactate dehydrogenase, aspartate aminotransferase, alanine aminotransferase, and alkaline phosphatase were determined in both serum and cardiac tissue homogenate to demonstrate enzyme efflux as the concurrent rise in serum and fall in tissue activity of the same enzyme rather than as a change in one compartment alone; this paired design provides stronger evidence of membrane disruption than serum measurement alone. Serum iron and ceruloplasmin were estimated as ancillary acute-phase and copper-transport indices and are reported as such, without inference regarding any specific inflammatory signalling pathway. All estimations were performed using a semi-automated biochemistry autoanalyzer (BIOLIS 24i).

Preparation of Cardiac Tissue Homogenate

Hearts were excised immediately after sacrifice, rinsed in ice-cold saline, blotted, and weighed. A weighed portion of the ventricular tissue was homogenized in ice-cold phosphate buffer, the homogenate was centrifuged at 4 °C, and the supernatant was used for the biochemical estimations below, all procedures being conducted at 4 °C to preserve enzyme activity.

Myocardial Antioxidant Status and Lipid Peroxidation

Superoxide dismutase, catalase, and glutathione peroxidase activities were determined in the tissue supernatant following the established spectrophotometric protocols for the measurement of these antioxidant enzymes in animal tissue [23]. Superoxide dismutase activity was measured using the pyrogallol autoxidation method, and lipid peroxidation was estimated as thiobarbituric acid reactive substances, the chromogen formed between thiobarbituric acid and malondialdehyde being read at 532 nm, and the concentration derived from its molar extinction coefficient[24] . Glutathione-S-transferase activity and reduced glutathione concentration were determined using standard

spectrophotometric procedures, and all values were expressed relative to the tissue protein content.

Histopathological Examination

A portion of each heart was fixed in 10% neutral-buffered formalin, processed, sectioned, and stained with hematoxylin and eosin. Myocardial necrosis, interstitial edema with inflammatory infiltration, and myofibrillar degeneration were each graded independently on a semi-quantitative scale of 0 to 6, where 0 denotes complete absence of the lesion and 6 its most severe expression, and an overall descriptive grade of normal, mild, moderate, or severe was assigned to each group from the three component scores. A multiparametric semi-quantitative approach of this kind, in which several independent histological features are scored separately and combined, has been developed and validated as a reproducible means of assessing tissue damage in animal models [25]. All slides were scored by the same observer using the pre-defined criteria above, applied identically to every group; formal blinding of the observer to group identity was not implemented, and this limitation is stated in Section 6.12. The liver and aorta from the anti-hyperlipidaemic study were processed by the same procedure and examined qualitatively only. No semi-quantitative grading of hepatic or aortic sections was performed, and no scored hepatic or aortic histopathology dataset was reported; therefore, hepatic response in the anti-hyperlipidaemic study is represented by liver weight (Table 7.14) alone, and formal grading of hepatic steatosis and aortic intimal change is recommended as future work.

Result: Cardioprotective Activity — ISO-Induced Myocardial Infarction Model

Table 1: Effect on Heart Weight and Relative Heart Weight (ISO Model)

Group	Body weight (g)	Heart weight (g)	Relative heart weight (%)
Group I – Control	175.6±4.8	0.82±0.07	0.46
Group II – ISO induced	154.8±6.9[a]	1.56±0.09[a]	1.00[a]
Group III – Propranolol (10 mg/kg) + ISO	167.5±5.5[b]	0.95±0.06[b]	0.56[b]
Group IV – Vitex negundo alone + ISO	158.2±5.2[b]	1.15±0.08[b]	0.72[b]
Group V – Abutilon indicum alone + ISO	161.4±4.9[b]	1.22±0.10[b]	0.75[b]
Group VI – Gloriosa superba alone + ISO	159.9±5.8[b]	1.18±0.07[b]	0.73[b]
Group VII – ISO + Polyherbal Extract (250 mg/kg)	169.6±5.1[bc]	0.89±0.08[bc]	0.52[bc]
Group VIII – ISO + Polyherbal Extract (500 mg/kg)	172.7±7.6[c]	0.85±0.06[c]	0.49[c]

[a] Significantly different from control ($P < 0.05$); [b] Significantly different from ISO-induced control ($P < 0.05$); [c] Significantly different from each individual-extract group (Groups IV, V, VI) ($P < 0.05$, Tukey's HSD); [e] Significantly different between Polyherbal Extract 250 and 500 mg/kg groups ($P < 0.05$, Tukey's HSD).

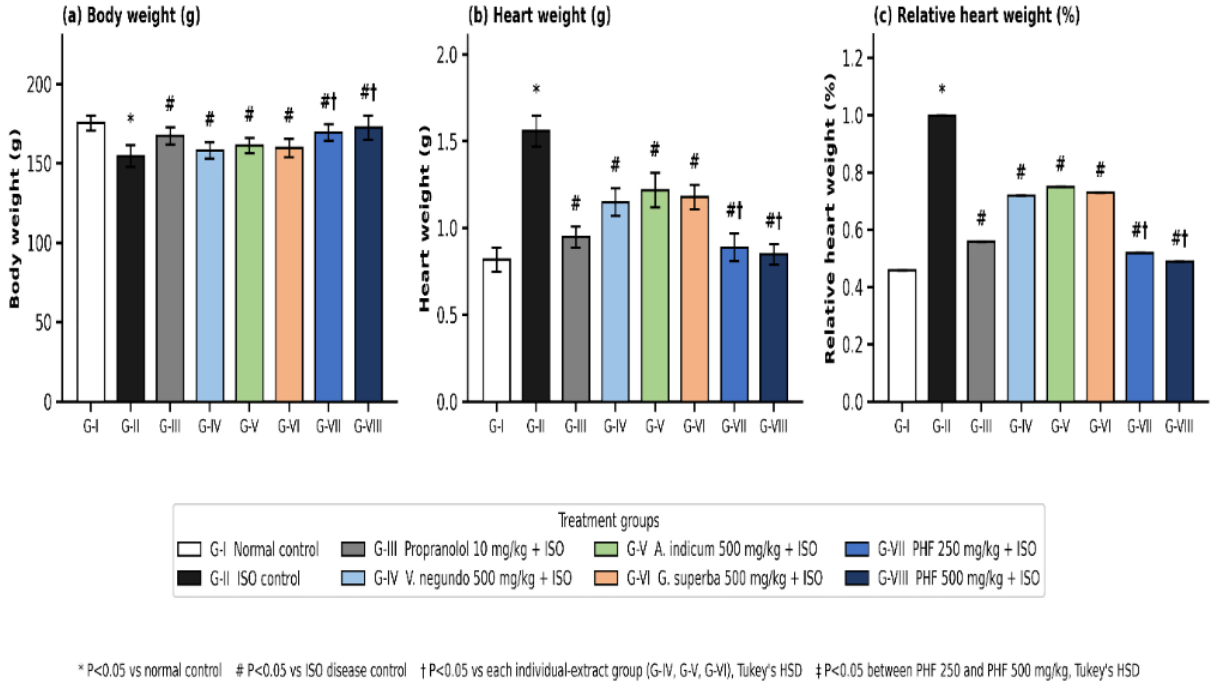

Fig. 1. Effect of individual root extracts and the polyherbal formulation on body weight, heart weight, and relative heart weight in the isoproterenol-induced myocardial infarction model. Standardized polyherbal (Group VII - PHF 250 mg/kg and Group VIII - PHF 500 mg/kg) significantly reduced the absolute and relative heart weight and significantly increased the terminal body weight when compared to each of the individual plant extract groups (Group IV - Vitex negundo, Group V - Abutilon indicum, and Group VI - Gloriosa superba) ($P < 0.05$ for all comparisons), indicating that the standardized polyherbal combination offered better protection against ISO-induced cardiac hypertrophy and edema compared to the plant extract groups used alone. Low-dose (250 mg/kg) and high-dose (500 mg/kg) polyherbal group differences in body weight and heart weight, however, were not statistically significant ($P > 0.05$), indicating that the cardioprotective effect on gross cardiac mass parameters was maximized at the lower dose and did not further increase with dose escalation.

Table 2: Effect on Cardiac Biomarkers — Troponin-T, Troponin-I, CK-MB

Group	Troponin-T (ng/ml)	Troponin-I (ng/ml)	CK-MB (IU/L)
Group I – Control	0.93±0.05	0.63±0.06	78.61±3.39
Group II – ISO induced	1.53±0.19[a]	0.94±0.10[a]	153.84±7.12[a]
Group III – Propranolol (10 mg/kg) + ISO	1.08±0.15[b]	0.71±0.07[b]	87.63±5.57[b]
Group IV – Vitex negundo alone + ISO	1.22±0.11[b]	0.81±0.08[b]	115.40±6.20[b]
Group V – Abutilon indicum alone + ISO	1.30±0.14[b]	0.85±0.09[b]	128.60±5.80[b]
Group VI – Gloriosa superba alone + ISO	1.26±0.13[b]	0.83±0.07[b]	120.25±6.15[b]
Group VII – ISO + Polyherbal Extract (250 mg/kg)	1.01±0.12[bc]	0.69±0.05[bc]	82.35±5.21[bc]

Group VIII – ISO + Polyherbal Extract (500 mg/kg)	0.92±0.08[c]	0.64±0.07[c]	79.33±4.24[c]

[a] Significantly different from control (P < 0.05); [b] Significantly different from ISO-induced control (P < 0.05); [c] Significantly different from each individual-extract group (Groups IV, V, VI) (P < 0.05, Tukey's HSD); [e] Significantly different between Polyherbal Extract 250 and 500 mg/kg groups (P < 0.05, Tukey's HSD).

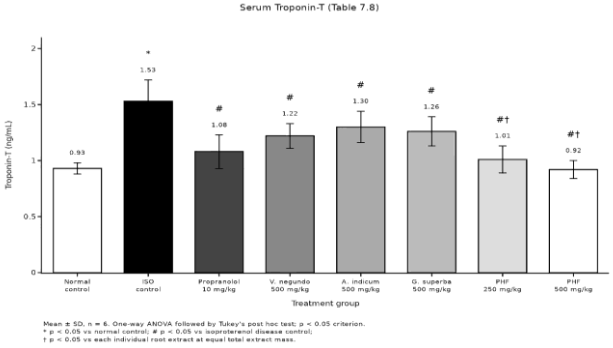

Figure 2a. Serum troponin-T across all eight groups in the isoproterenol-induced myocardial infarction model

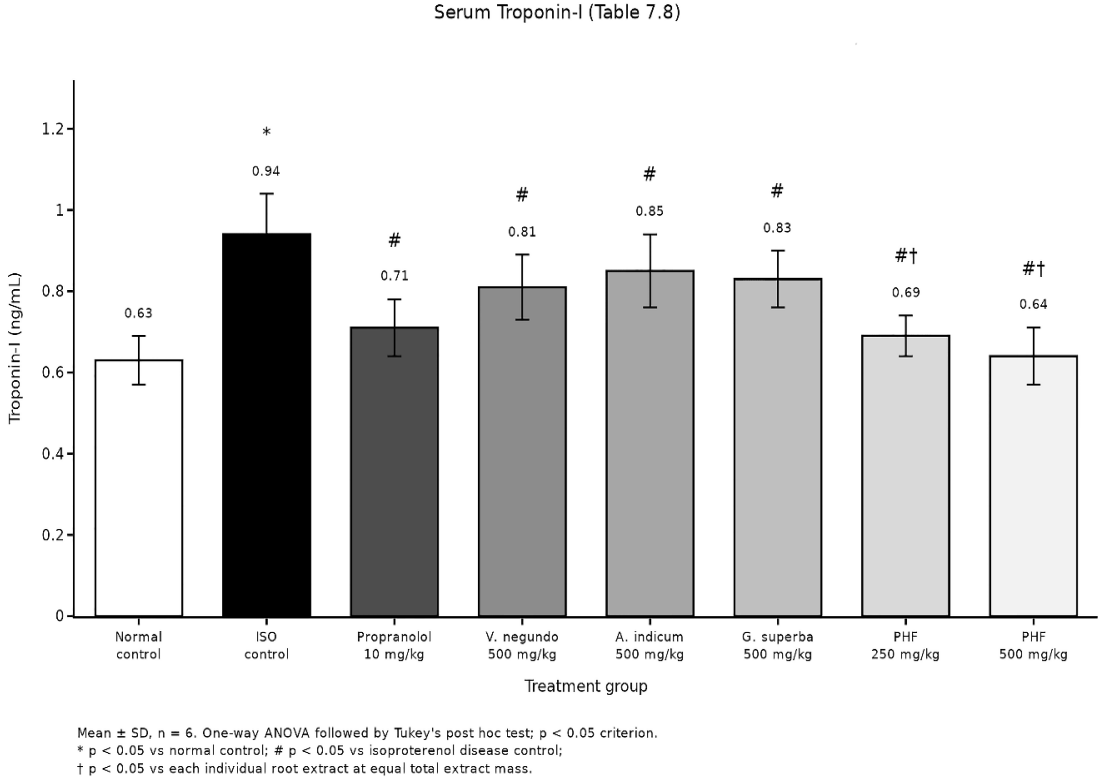

Figure 2b. Serum troponin-I in all eight groups in the isoproterenol-induced myocardial infarction model.

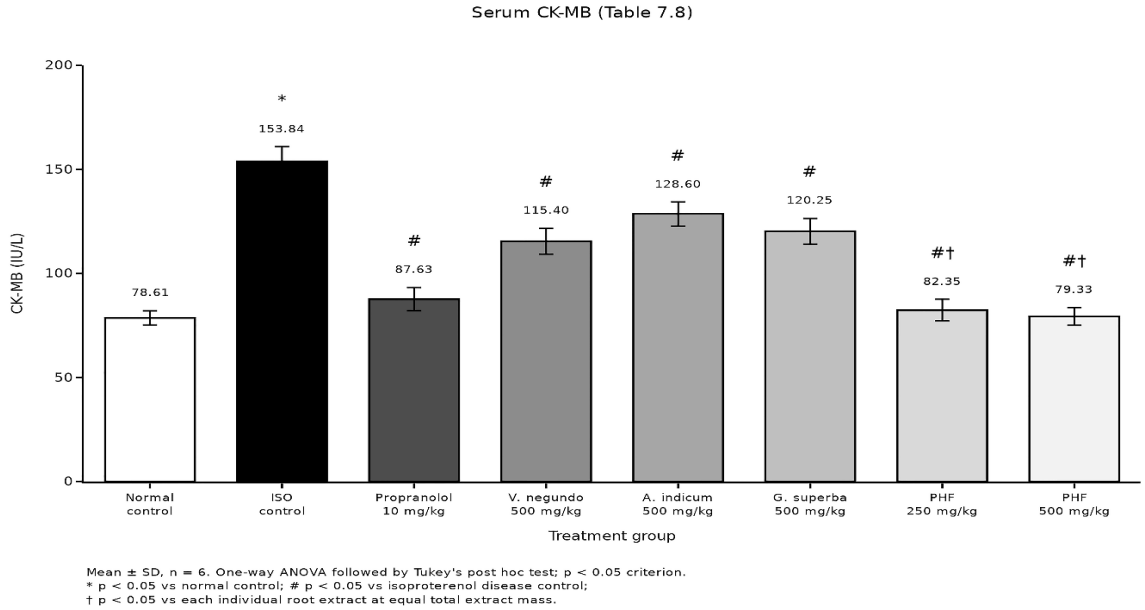

Figure 2c. Serum CK-MB levels in all eight groups in the isoproterenol-induced myocardial infarction model.

Both the polyherbal extract-treated groups showed significantly reduced serum Troponin-T, Troponin-I, and CK-MB levels compared to the three different plant extract groups ($P < 0.05$), indicating that the combined treatment was more effective in biochemical protection of the myocardium against ISO-induced myocardial injury than Vitex negundo, Abutilon indicum, and Gloriosa superba, or any of these individually. Similarly, for the three cardiac biomarkers, the difference between the lower and higher doses of the two polyherbal treatments (250 mg/kg and 500 mg/kg) was not statistically significant ($P > 0.05$), suggesting that the cardioprotective ceiling of the cardiac biomarker had already been reached with the lower dose.

Table 3: Effect on Serum and Cardiac Tissue Marker Enzymes

Group	CPK Serum (IU/L)	CPK Tissue	LDH Serum	LDH Tissue	AST Serum (IU/L)	AST Tissue	ALT Serum (IU/L)	ALT Tissue	ALP Serum	ALP Tissue
Group I – Control	132.65± 5.12	91.13± 3.52	39.54± 1.69	35.14± 1.38	26.54± 0.93	15.92± 0.41	20.88± 0.77	14.28± 0.64	27.56± 0.89	125.66 ±6.81
Group II – ISO induced	194.52± 7.09[a]	56.23± 3.04[a]	47.29± 2.06[a]	19.45± 0.97[a]	54.56± 1.26[a]	10.48± 0.31[a]	62.55± 1.28[a]	9.82±0 .50[a]	38.81± 1.16[a]	84.57± 5.27[a]
Group III – Propra	142.25± 6.32[b]	63.44± 2.47[b]	42.07± 1.47[b]	25.31± 1.02[b]	44.64± 1.04[b]	13.06± 0.45[b]	47.70± 1.02[b]	12.21± 0.55[b]	33.59± 1.07[b]	110.33 ±5.90[b]

nolol + ISO										
Group IV – Vitex negundo alone + ISO	162.45± 6.15[b]	66.25± 2.85[b]	44.35± 1.80[b]	22.15± 1.05[b]	48.20± 1.15[b]	11.85± 0.40[b]	55.10± 1.15[b]	10.95± 0.45[b]	35.40± 1.10[b]	98.50± 5.40[b]
Group V – Abutilon indicum alone + ISO	170.15± 6.40[b]	62.10± 2.70[b]	45.80± 1.95[b]	21.05± 0.95[b]	51.05± 1.25[b]	11.10± 0.35[b]	58.30± 1.20[b]	10.25± 0.40[b]	37.10± 1.15[b]	92.30± 5.20[b]
Group VI – Gloriosa superba alone + ISO	166.80± 6.25[b]	64.50± 2.80[b]	45.10± 1.85[b]	21.55± 1.00[b]	49.60± 1.20[b]	11.45± 0.38[b]	56.70± 1.18[b]	10.60± 0.42[b]	36.25± 1.12[b]	95.40± 5.30[b]
Group VII – ISO + Polyherbal (250 mg/kg)	139.72± 6.01[bc]	77.18± 2.91[b]	41.78± 1.71[b]	30.09± 1.21[b]	31.91± 0.82[bc]	14.25± 0.37[b]	32.78± 0.91[b]	13.55± 0.60[b]	30.26± 0.98[b]	120.17 ±5.54[b]
Group VIII – ISO + Polyherbal (500 mg/kg)	133.74± 5.89[c]	89.32± 3.07	38.77± 1.60	33.87± 1.19	27.63± 0.55[ce]	15.02± 0.50	24.49± 0.86	13.92± 0.49	28.75± 0.82	122.59 ±6.07

[a]Significantly different from the control ($P < 0.05$); [b]Significantly different from the ISO-induced control ($P < 0.05$); [c]Significantly different from each individual-extract group (Groups IV, V, and VI) ($P < 0.05$, Tukey's HSD); [e]Significantly different between the PEE 250 and 500 mg/kg groups ($P < 0.05$, Tukey's HSD).

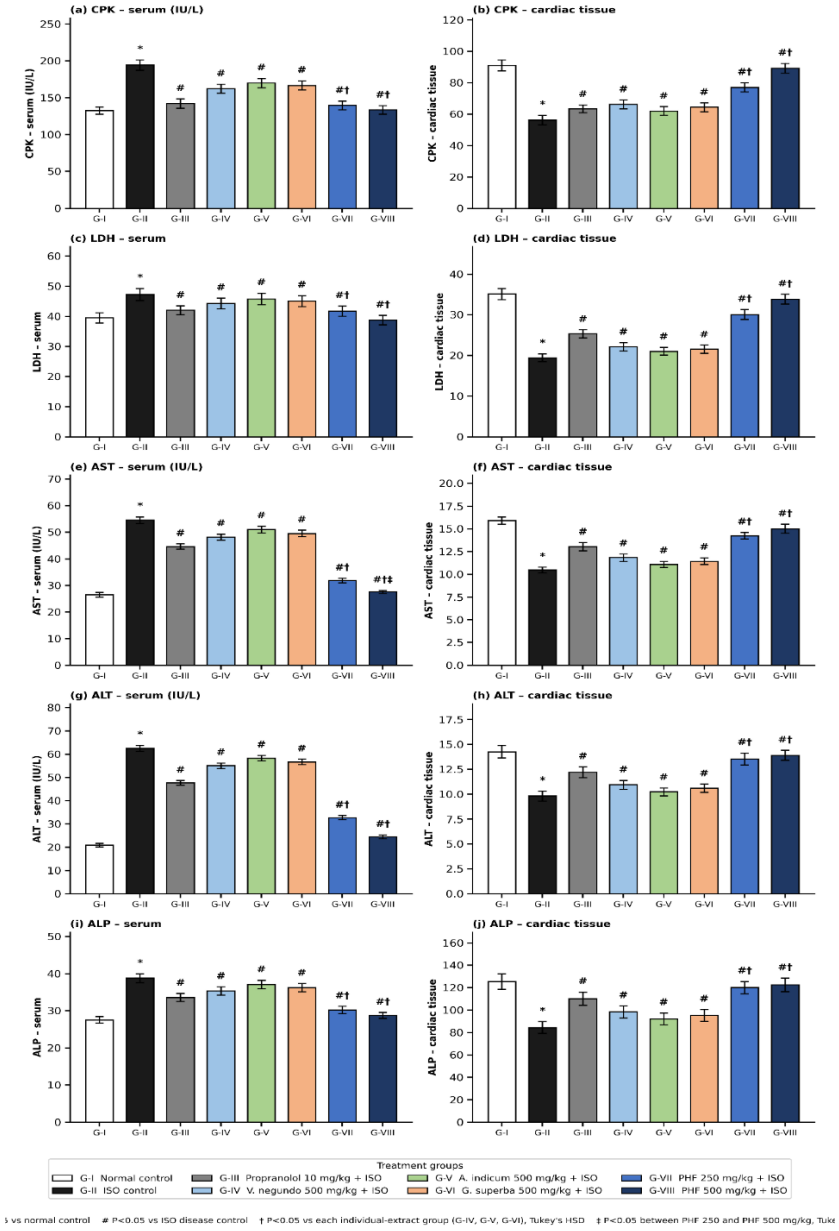

Fig. 3. Effect of the individual root extracts and the polyherbal formulation on serum and cardiac tissue marker enzymes (CPK, LDH, AST, ALT, ALP)

The polyherbal extract-treated groups had significantly greater normalization of serum CPK and AST activity than each individual extract group ($P < 0.05$), thus confirming the superiority of the polyherbal formulation over the individual herbs in reducing leakage of myocardial enzymes. In contrast to the biomarkers in Table 7.8, there was a genuine dose-dependent improvement in this particular enzyme marker (serum AST) as the high-dose (500 mg/kg) polyherbal group was significantly different from the low-dose (250 mg/kg) group ($P < 0.05$).

Table 4: Serum Iron and Ceruloplasmin

Group	Iron	Ceruloplasmin
Group I – Control	11.43±1.33	11.14±0.49
Group II – ISO induced	73.94±3.41[a]	20.08±1.09[a]
Group III – Propranolol + ISO	17.09±1.17[b]	13.03±0.07[b]
Group IV – Vitex negundo alone + ISO	38.50±2.15[b]	16.45±0.85[b]
Group V – Abutilon indicum alone + ISO	45.20±2.80[b]	17.80±0.92[b]
Group VI – Gloriosa superba alone + ISO	41.35±2.40[b]	17.10±0.88[b]
Group VII – ISO + Polyherbal (250 mg/kg)	15.43±1.83[bc]	10.79±1.03[bc]
Group VIII – ISO + Polyherbal (500 mg/kg)	12.39±0.81[ce]	12.18±1.02[ce]

[a]Significantly different from the control ($P < 0.05$); [b]Significantly different from the ISO-induced control ($P < 0.05$); [c]Significantly different from each individual-extract group (Groups IV, V, and VI) ($P < 0.05$, Tukey's HSD); [e]Significantly different between the P250 and 500 mg/kg groups ($P < 0.05$, Tukey's HSD).

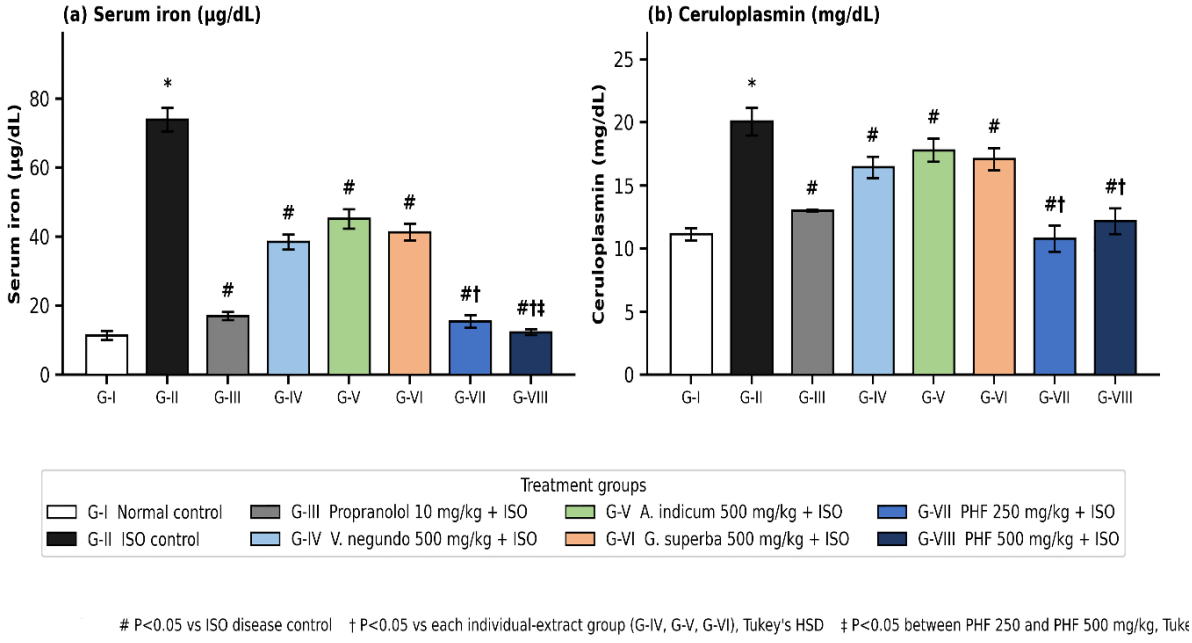

Fig. 4. Effect of individual root extracts and the polyherbal formulation on serum iron and ceruloplasmin levels

The serum values of iron and ceruloplasmin were significantly closer to those of normal controls in both polyherbal doses than in any of the individual plant extract groups when fed at the same total extract mass ($P < 0.05$), reflecting greater attenuation of the isoproterenol-induced acute-phase response by the combined formulation. A dose-related effect was observed for serum iron, which was found to be significantly different between the two doses of polyherbal (value at 500 mg/kg, 12.39 ± 0.81 µg/dL, was closer to the normal control value, 11.43 ± 1.33 µg/dL, than the value at 250 mg/kg, 15.43 ± 1.83 µg/dL). The same was true for ceruloplasmin: the value obtained at 250 mg/kg (10.79 ± 1.03 mg/dL) was slightly below the normal control value (11.14 ± 0.49 mg/dL), while the value

obtained at 500 mg/kg (12.18 ± 1.02 mg/dL) was slightly above it, both differences being small and of the order of the measurement variability. Thus, ceruloplasmin was reported as " normalized " at both dose levels, and no dose-response is supposed to exist for this parameter. The increase in serum iron after isoproterenol (11.43 ± 1.33 to 73.94 ± 3.41 μg/dL) is considered to be unusually large for an acute-phase index, and no ferritin, transferrin saturation, or hepcidin had been measured to differentiate between iron released from damaged myocardium and any acute-phase component.

Table 5 : Myocardial Antioxidant Enzyme Status

Group	SOD (U/mg protein)	CAT (U/mg protein)	GPx (U/mg protein)	GST (U/mg protein)	GSH (μg/mg protein)	MDA (nmol/mg protein)
Group I – Control	12.42±1.15	42.15±2.80	24.50±1.95	5.20±0.45	4.85±0.35	1.25±0.12
Group II – ISO induced	4.15±0.82[a]	14.55±1.60[a]	9.25±1.10[a]	1.85±0.25[a]	1.52±0.18[a]	6.75±0.48[a]
Group III – Propranolol + ISO	10.55±0.95[b]	38.20±2.15[b]	21.05±1.45[b]	4.55±0.38[b]	4.10±0.28[b]	2.15±0.22[b]
Group IV – Vitex negundo alone + ISO	6.85±0.75[b]	22.45±1.90[b]	14.30±1.25[b]	2.95±0.30[b]	2.55±0.20[b]	3.26±0.35[b]
Group V – Abutilon indicum alone + ISO	6.15±0.68[b]	19.80±1.85[b]	12.55±1.15[b]	2.50±0.28[b]	2.25±0.19[b]	3.85±0.42[b]
Group VI – Gloriosa superba alone + ISO	6.50±0.72[b]	21.10±1.75[b]	13.80±1.30[b]	2.75±0.32[b]	2.45±0.22[b]	4.16±0.38[b]
Group VII – ISO + Polyherbal (250 mg/kg)	9.85±1.05[bc]	34.50±2.45[b]	19.45±1.65[b]	4.15±0.42[b]	3.85±0.32[b]	2.55±0.28[bc]
Group VIII – ISO + Polyherbal (500 mg/kg)	11.25±1.10[bce]	39.75±2.60[b]	23.15±1.80[b]	4.90±0.48[b]	4.45±0.35[b]	1.85±0.20[bce]

[a]Significantly different from the control ($P < 0.05$); [b]Significantly different from the ISO-induced control ($P < 0.05$); [c]Significantly different between each individual-extract group (Groups IV, V, VI) ($P < 0.05$, Tukey's HSD); [e]Significantly different

between the Polyherbal Extract 250 and 500 mg/kg groups ($P < 0.05$, Tukey's HSD).

Figure 7.11 Myocardial antioxidant status and lipid peroxidation — isoproterenol model (mean ± SD, n = 6)

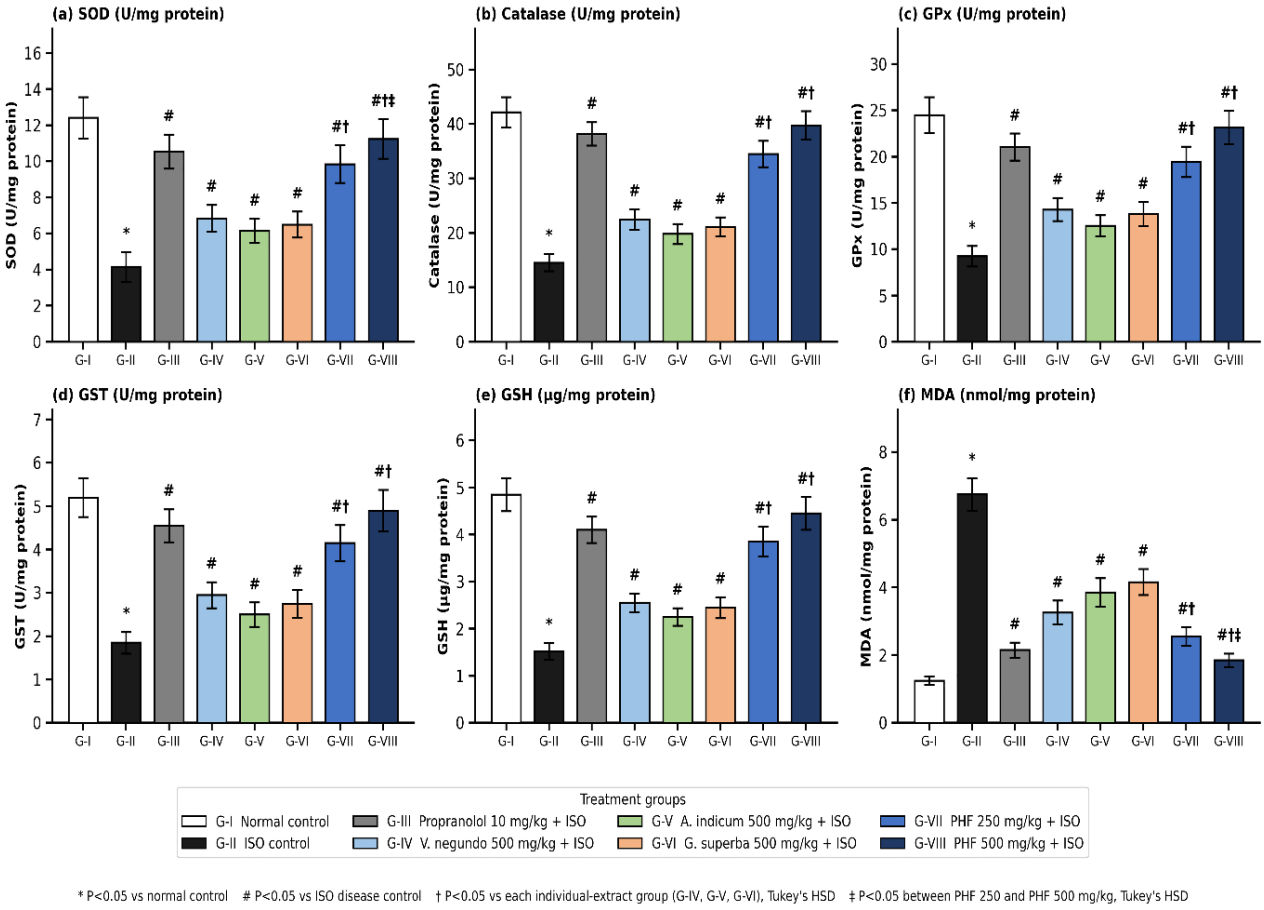

Fig. 5. Effect of the individual root extracts and the polyherbal formulation on myocardial antioxidant enzyme status and lipid peroxidation

The polyherbal groups significantly restored SOD activity and reduced MDA levels compared with each individual plant extract group ($P < 0.05$); therefore, it was concluded that the tri-plant group was more effective in restoring the antioxidant capacity of the myocardium and limiting lipid peroxidation than any individual plant extract. A dose-dependent effect was also noted between the 250 mg/kg and 500 mg/kg polyherbal groups for both SOD ($P < 0.05$) and MDA ($P < 0.05$); that is, the antioxidant-related effects of the formulation were not only present in the 250 mg/kg group but continued to improve with dose.

Table 6 : Histopathological Scoring of Cardiac Tissue (ISO Model)

Group	Myocardial necrosis (score)	Edema/inflammation (score)	Myofibrillar degeneration (score)	Overall histopathology grade
Group I – Control	0.00±0.00	0.00±0.00	0.00±0.00	Normal
Group II – ISO induced	4.83±0.40[a]	5.50±0.54[a]	4.66±0.51[a]	Severe

Group III – Propranolol + ISO	2.16±0.40[b]	2.00±0.00[b]	2.16±0.40[b]	Mild
Group IV – Vitex negundo alone + ISO	3.50±0.54[b]	4.16±0.40[b]	3.63±0.56[b]	Moderate
Group V – Abutilon indicum alone + ISO	3.73±0.50[b]	3.50±0.62[b]	3.69±0.58[b]	Moderate
Group VI – Gloriosa superba alone + ISO	3.76±0.59[b]	3.49±0.58[b]	3.62±0.53[b]	Moderate
Group VII – ISO + Polyherbal (250 mg/kg)	1.50±0.54[bc]	1.33±0.51[b]	1.50±0.54[b]	Mild
Group VIII – ISO + Polyherbal (500 mg/kg)	1.00±0.00[bce]	0.83±0.40[b]	1.00±0.00[b]	Mild

[a] Significantly different from control ($P < 0.05$); [b] Significantly different from ISO-induced control ($P < 0.05$); [c] Significantly different from each individual-extract group (Groups IV, V, VI) ($P < 0.05$, Tukey's HSD); [e] Significantly different between Polyherbal Extract 250 and 500 mg/kg groups ($P < 0.05$, Tukey's HSD).

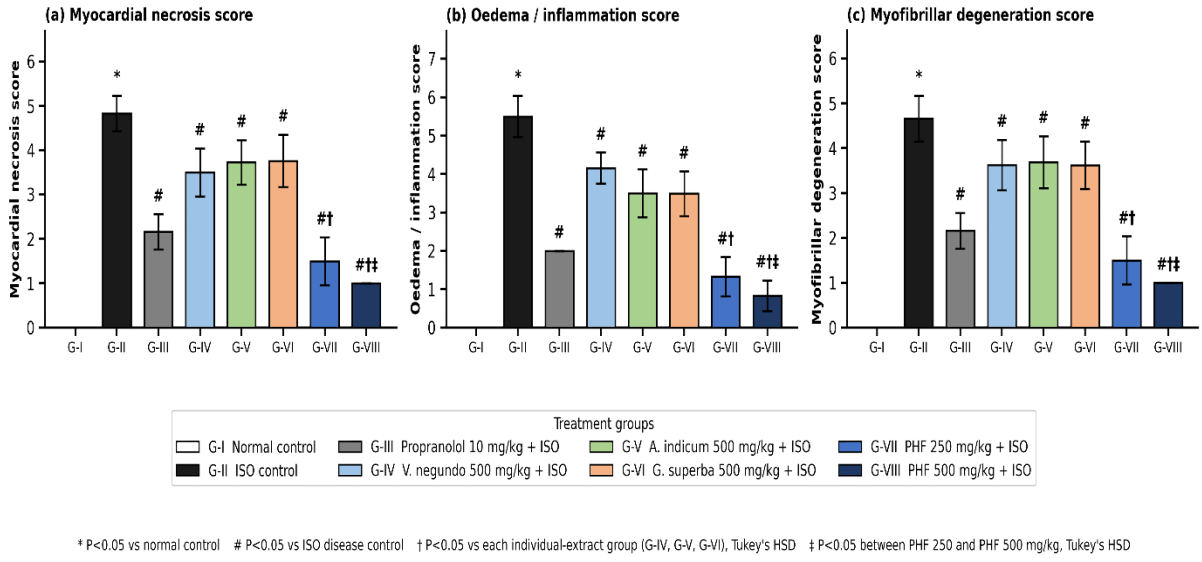

Fig. 6a. Histopathological scoring of cardiac tissue in the isoproterenol-induced myocardial infarction model

The results showed that the myocardial necrosis scores for the polyherbal extract-treated groups were significantly reduced compared to each individual plant extract-treated group ($P < 0.05$), which was consistent with the biochemical results that the polyherbal formulation was more effective in protecting against myocardial injury induced by ISO

than Vitex negundo, Abutilon indicum, or Gloriosa superba alone. A real dose-dependent histoprotective effect was also observed as the high-dose polyherbal group exhibited a significantly reduced necrosis score compared to the low-dose group ($P < 0.05$) (Table 7.11).

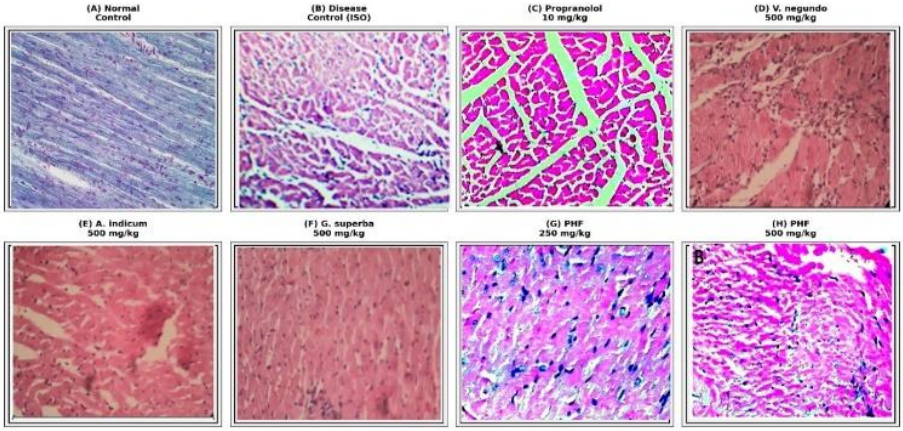

Fig. 6b: Representative photomicrographs of myocardial histopathology (H&E, 400×) in the isoproterenol-induced myocardial infarction model. (A) Group I – Normal Control; (B) Group II – ISO-induced Disease Control; (C) Group III – Propranolol 10 mg/kg + ISO; (D) Group IV – *Vitex negundo* 500 mg/kg + ISO; (E) Group V – *Abutilon indicum* 500 mg/kg + ISO; (F) Group VI – *Gloriosa superba* 500 mg/kg + ISO; (G) Group VII – PHF 250 mg/kg + ISO; (H) Group VIII – PHF 500 mg/kg + ISO. Histological grading was done according to the semi-quantitative scoring criteria for cardiomyocyte injury.

The biochemical evidence of cardioprotection reported in Tables 7.8-7.11 was confirmed by histopathological examination of myocardial sections. All three parameters scored 0.00 ± 0.00 and an overall histopathology grade of "Normal" in sections from Group I (Normal Control) without any evidence of myocardial necrosis, interstitial oedema/inflammatory infiltration or myofibrillar degeneration. In Group II (Disease Control), isoproterenol caused severe myocardial injury, as indicated by necrosis, oedema/inflammation, and myofibrillar degeneration scores of 4.83 ± 0.40, 5.50 ± 0.54 and 4.66 ± 0.51, respectively ($P < 0.05$ vs. control) and an overall grade of "Severe." This injury was significantly reduced by pre-treatment with the reference agent propranolol (Group III), resulting in the three scores being 2.16 ± 0.40, 2.00 ± 0.00 and 2.16 ± 0.40, respectively ($P < 0.05$ vs. ISO-induced control), which equates to an overall grade of "Mild." The 500 mg/kg treatments with the three individual plant extracts, *Vitex negundo* (Group IV), *Abutilon indicum* (Group V) and *Gloriosa superba* (Group VI), each had significant but only partial histoprotection as compared to their corresponding disease controls, with necrosis scores of 3.50 ± 0.54, 3.73 ± 0.50 and 3.76 ± 0.59, respectively ($P < 0.05$ vs. disease controls, all of which were "Moderate").

The polyherbal formulation (PHF) showed a dose-dependent histoprotective effect, which was higher than that of all the individual extracts. Necrosis, oedema/inflammation, and myofibrillar degeneration scores were 1.50 ± 0.54, 1.33 ± 0.51, and 1.50 ± 0.54, respectively, at 250 mg/kg (Group VII), which was considered "Mild" ($P < 0.05$ vs. ISO-induced control and vs. each individual-extract group). In the 500 mg/kg (Group VIII), scores decreased to 1.00 ± 0.00, 0.83 ± 0.40, and 1.00 ± 0.00, respectively, which was significantly lower than the 250 mg/kg PHF dose ($P < 0.05$) and all individual-extract groups ($P < 0.05$), and were still

in the "Mild" category. This dose-dependent trend was observed in both PHF groups, and this finding is consistent with the biochemical data of cardioprotection against isoproterenol-induced myocardial injury, as reported in Tables 7.8-7.11, which further consolidates the evidence of superior histological protection offered by the standardized polyherbal combination than any of its three constituent plant extracts administered alone.

Discussion & Conclusion: Despite cardiovascular disease being the leading cause of death worldwide, India has an age-standardized mortality rate higher than the global average, and the first cardiovascular event in India occurs approximately 10 years earlier than the first cardiovascular event in Western populations. While Vitex negundo, Abutilon indicum, and Gloriosa superba have been studied individually, no prior work has combined all three into a single formulation of extracts with chemically standardized quantities and assessed their acute oral toxicity and cardioprotective activities using a common batch of extracts and statistical framework. The present work aimed to fill this gap,

There are three findings that address the question asked. First, there was no mortality or treatment-related organ, haematological, or persistent biochemical change at 10 times the highest therapeutic dose (HTD) subsequently studied (Tables 7.1-7.6). Second, in both disease models, it reversed the induced phenotype in all categories of endpoint measured, including necrosis biomarkers, membrane-integrity enzymes, myocardial redox status, tissue architecture, and the complete lipid profile (Tables 7.7-7.14), and did so to a significantly greater degree than did each of the three single-extract treatments administered at the same total extract mass in each model, despite delivering only the equivalent of two-fifths, two-fifths and one-fifth of the single-extract dose of each species. Third, there was not a uniform dose-response, with increases from 250 to 500 mg/kg resulting in significant improvement in some endpoints and no change in others, suggesting a plateau at the lower dose for some of the parameters.

The main implication for the field is methodological rather than pharmacological: a polyherbal formulation that can be characterized with regard to its markers in the actual product administered, and whose advantage over the constituent herbs can be established at the same amount of extract load, can be audited and reproduced when most of the polyherbal literature cannot. In this formulation, the results warrant further preclinical development, but not beyond that. The priorities that arise from the limitations above are, in the order of logical priority, independent quantitative re-assay and stability testing of the finished blend; isobolographic or combination-index analysis across a matrix of ratios, with ratio titration, to determine whether the interaction is additive or synergistic and whether 2:2:1 is optimal; direct molecular assay in myocardial tissue from the same model, to convert the present biochemical and histological evidence into mechanism; formal grading of hepatic steatosis and aortic intimal change; repeated-dose and colchicine-specific toxicology with a male-rat acute toxicity arm and sex-disaggregated efficacy analysis; and, finally, pharmacokinetic bridging to establish a human-equivalent dose. Only then would the clinical evaluation be a defensible proposition. Based on the present evidence, the standardized 2:2:1 ratio root-extract formulation of V. negundo, A. indicum, and G. superba is a chemically traceable and acutely well-tolerated formulation that is cardioprotective in isoproterenol-induced myocardial injury. It is more effective than any of its individual extract components at the same total extract load, thus providing a rational preclinical basis for its further development.

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