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Penta Herbs Forte: The Effect of PHF Extract on Superoxide Dismutase Activity in Hypoxic Sprague Dawley Rat's Heart

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Abstract

Hypoxia is a condition in which tissues receive insufficient oxygen, leading to increased production of reactive oxygen species (ROS) and triggering oxidative stress that can cause heart damage. Penta Herbs Forte (PHF), a combination of Blumea balsamifera, Curcuma xanthorrhiza, Zingiber officinale var. rubrum, Phyllanthus niruri, and Andrographis paniculata, contains bioactive compounds with antioxidant properties that may help strengthen the body's defense against oxidative injury. This study aimed to evaluate the effect of PHF extract on the activity of the antioxidant enzyme Superoxide Dismutase (SOD) in the hearts of hypoxia-induced Sprague Dawley rats. The research included in vitro assessments (total phenolic content, BSLT toxicity, and FRAP assays) and in vivo experiments involving eight groups of rats receiving PHF extract with different hypoxia exposure durations (0, 1, 7, and 14 days). Heart tissues were processed into homogenates, and SOD activity was measure by spectrophotometry, followed by statistical analysis using the Mann-Whitney and Kruskal-Wallis tests. Although PHF groups showed higher SOD activity, significant differences between control and treated groups were observed only at 1-day hypoxia. These findings suggest that PHF may help protect the heart from hypoxia-related oxidative stress by enhancing antioxidant enzyme activity.

Keywords: Penta Herbs Forte, Heart, SOD, Superoxide Dismutase

1. Introduction

Hypoxia is a condition in which tissues receive an inadequate supply of oxygen, triggering an increase in the production of reactive oxygen species (ROS) and causing oxidative stress.[1] This condition can affect various organs, including the heart, which is highly dependent on oxygen supply to keep its metabolic function. Oxidative stress in the heart plays a role in cell damage, contractility disorders, and the pathogenesis of cardiovascular disease.[2] At the molecular level, hypoxia disrupts the balance between ROS production and the ability of endogenous antioxidant systems, such as Superoxide Dismutase (SOD), to neutralize free radicals.[3] Superoxide dismutase (SOD) serves as the primary enzymatic defense by catalyzing the dismutation of superoxide anions into hydrogen peroxide. Consequently, a diminution in SOD activity may compromise the antioxidant capacity of the cell, thereby exacerbating oxidative injury within the myocardial tissue due to the unregulated accumulation of reactive oxygen species (ROS).[4]

Penta Herbs Forte (PHF) is a polyherbal formulation comprising five distinct botanical species: *Blumea balsamifera* (Sembung), *Curcuma xanthorrhiza* (Javanese Turmeric), *Zingiber officinale var. rubrum* (Red Ginger), *Phyllanthus niruri* (Meniran), and *Andrographis paniculata* (Creat). This combination is characterized by a dense profile of bioactive secondary metabolites, including flavonoids, phenolics, curcuminoids, gingerols, and andrographolides. These phytochemicals are theorized to act synergistically to augment systemic antioxidant capacity, thereby conferring a cytoprotective shield against the deleterious effects of hypoxia-induced oxidative stress. [6]

However, despite the well-documented antioxidant properties of individual herbal components, limited evidence is available regarding the combined effect of Penta Herbs Forte (PHF) on cardiac antioxidant defense, particularly under hypoxic conditions. Most previous studies have focused on single-compound antioxidants or short-term cellular models, while data on whole-herbal formulations and their effects on cardiac antioxidant enzymes during hypoxia remain scarce. Therefore, this study aimed to investigate the effect of PHF administration on superoxide dismutase (SOD) activity in the hearts of Sprague Dawley rats exposed to different durations of hypoxia, thereby providing insight into its potential role in early oxidative stress modulation.

2. Method

This experimental study comprised in vitro, in vivo, and bioassay evaluations. The in vitro analyses included the determination of total phenolic content, toxicity assessment using the Brine Shrimp Lethality Test (BSLT), and antioxidant activity assays using the FRAP method. For the in vivo experiments, Penta Herbs Forte (PHF) extract was administered to male Sprague Dawley rats under normoxic conditions and hypoxic conditions for 1, 7, and 14 days. Bioassay testing was conducted as part of the toxicity evaluation using the BSLT method to determine the toxicity profile of the PHF extract. Sample size was calculated using the Federer formula:[7]

$$(t-1)(n-1) \geq 15$$

Description:
n=sample size for each intervention
t=number of interventions

Based on this approach, four rats were included per experimental group. All experimental rats were male rats aged 10-12 weeks with body weights ranging from 200-250 grams. Rats were divided into control (without PHF administration) and treatment (with PHF administration) groups, resulting in eight experimental groups:

1. Normoxia without PHF
2. 1-day hypoxia without PHF
3. 7-day hypoxia without PHF
4. 14-day hypoxia without PHF
5. Normoxia with PHF
6. 1-day hypoxia with PHF
7. 7-day hypoxia with PHF
8. 14-day hypoxia with PHF

Each group consisted of four male rats. Hypoxia was induced using a gas mixture of 10% O_2 and 90% N_2 at atmospheric pressure in a hypoxia chamber, a method widely used to simulate systemic hypoxic conditions in rodents.[8] Previous studies using similar setups have demonstrated stable hypoxic exposure in rats with 10% O_2 concentrations.[8] In the present study, successful hypoxia induction was supported by physiological and biochemical responses such as reduced physical activity and alterations in the oxygen enzyme activity, particularly a decrease in SOD levels with increasing duration of hypoxia exposure.

Soda lime was also connected to the hypoxia chamber to capture CO_2. The fan in the chamber had to be kept running at all times. Food and water for the rats were provided in the chamber. The rats were then placed inside. The gas was turned on almost to maximum, then the regulator was turned to between 1 and 2, ensuring that there were bubbles in the soda lime.

The treatment group received Penta Herbs Forte (PHF) extract at a dose of 100 mg/kg body weight, administered orally twice daily (morning and afternoon). The PHF solution was prepared by dissolving 0.2 g of each plant extract in a small amount of Tween as a solvent, followed by the addition of distilled water to a final volume of 10 mL. The extracts were prepared separately and then combined before administration. One milliliter of the combined extract solution was

used for four administrations. The control group received the same feeding procedure without PHF administration. All experimental procedures involving animals were approved by the Ethics Committee of the Faculty of Medicine, Trisakti University (Ethical Clearance No. 003/KER/FK/09/2024).

Production of Penta Herbs Forte Extract

The five plant materials were cleaned, air-dried at room temperature, and ground into a fine powder. Each powdered plant material (75 g) was macerated in ethanol following standard extraction procedures until complete extraction was achieved. The extracts were filtered and concentrated by solvent evaporation to obtain crude extracts, which were stored at low temperature in a refrigerator until further use.

Measurement of Phenolic Content

Quantification of phenolic constituents was performed using the Folin Ciocalteu method.[9] PHF extract (0.3 g) was reconstituted in a water methanol mixture and reacted with the Folin Ciocalteu reagent. The solution was then neutralized with 20% sodium bicarbonate and subjected to a dark incubation period at room temperature. The absorbance of the resulting blue-colored complex was measured at 765 nm via spectrophotometry. All measurements were conducted in triplicate, and results were expressed as gallic acid equivalents (GAE**).**

Toxicity Test (Brine Shrimp Lethality Test)

Toxicity evaluation was performed using the brine shrimp lethality test (BSLT) following standard procedures.[10] Cysts of *Artemia salina* were incubated for hatching in artificial seawater under conditions of continuous aeration and constant illumination. Upon reaching the nauplii stage, the larvae were subjected to varying concentrations of the PHF extract. The experimental units were incubated for 24 hours, after which the mortality rate was determined by quantifying the surviving versus non-surviving larvae. The median lethal concentration LC_{50} was subsequently calculated using probit analysis to evaluate the acute toxicity profile of the extract:

$$Mortality\ (\%) = \frac{Total\ of\ dead\ larvae}{Total\ larva\ (live\ and\ dead)} \times 100$$

FRAP Assay

Antioxidant capacity was determined via the FRAP assay using a freshly reconstituted working reagent.[11] This reagent consisted of a stabilized mixture of acetate buffer, TPTZ solution, and ferric chloride. Form at an acidic pH, producing a deep blue color proportional to the antioxidant concentration in the PHF extract.

The Ferric Reducing Antioxidant Power (FRAP) of the PHF extract was evaluated by solubilizing the extract in ethanol and preparing a graded concentration series (5, 10, 15, and 20 ug/mL. An aliquot of each concentration was reacted with the freshly prepared FRAP reagent and incubated at 37^{o} C for 10 minutes to facilitate the reduction process. The resulting chromogenic change was quantified by measuring the absorbance at 594 nm using a visible spectrophotometer. Trolox 10 - 30 ug/m served as the reference standard, and the results were expressed as the capacity to reduce ferric Fe^{3+} to ferrous Fe^{2+} ions:

$$Inhibition(\%) = \frac{Abs.Control - Abs.Sample}{Abs.Control} \times 100$$

Preparation of Rat Heart Homogenate

Myocardial tissue specimens (0.1 g) were subjected to mechanical homogenization in a 0.1 M phosphate buffer solution (pH 7.2). The homogenates were subsequently centrifuged at 10,000 rpm for 10 minutes.

Superoxide Dismutase (SOD) Activity Assay

The supernatant obtained from heart homogenates was used for SOD activity measurement. Reagents were added according to the SOD Assay kit Solarbio® life science protocol.[12] Samples were incubated at 37°C for 30 minutes, and absorbance was measured at 560 nm. Percentage inhibition was calculated using the following equation:

$$Inhibition = \frac{\Delta AB - \Delta AT}{\Delta AB} \times 100\%$$

AB = blank absorbance
AT = test absorbance

SOD activity was calculated using the formula:

$$SOD\ Activity\ (u/g\ mass) = \frac{10 \times P}{1 - P} \div W \times F$$

P = percentage inhibition
W = sample weight
F = number of dilutions in the sample

Statistical Analysis

In light of the restricted sample size and the non-Gaussian distribution of the dataset, non-parametric inferential statistics were employed. Inter-group variances were initially assessed using the Kruskal–Wallis H-test, with subsequent Mann–Whitney U-tests utilized for post-hoc pairwise comparisons where statistically appropriate. All computational analyses and high-resolution data visualizations were executed via GraphPad Prism software (v10.0, La Jolla, CA, USA). Descriptive data are expressed as the median and interquartile range (IQR). Notably, effect size estimations were intentionally omitted; given the limited sample size, such metrics could yield unstable or unreliable estimates, potentially misrepresenting the magnitude of the observed effects.

3. Result

Total Phenolic Content

Quantitative analysis of phenolic constituents in the PHF extract was performed via the Folin–Ciocalteu method. Extrapolation from the standard gallic acid curve (Figure 1) yielded a TPC value of $108.02 (mg GAE/g DW). These results establish the baseline antioxidant potential for subsequent *in vivo* hypoxic challenges.

FRAP Assay

The ferric reducing antioxidant power (FRAP) assay demonstrated that PHF extract exhibited reducing activity in a concentration-dependent manner. The EC_{50} value obtained from linear regression analysis was 11.10 µg/mL, indicating a strong ferric reducing capacity of the extract (Table 1, Figure 2).

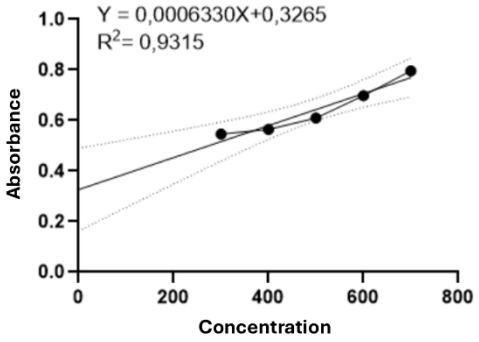

Figure 1. Absorbance value against the concentration of standard gallic acid solution

Table 1. FRAP Activity of PHF Extract

Concentration (µg/mL)	Inhibition (%)	EC50 (µg/mL)
10	48.087	11.101
15	55.607	
20	59.052	
25	66.899	
30	70.769	

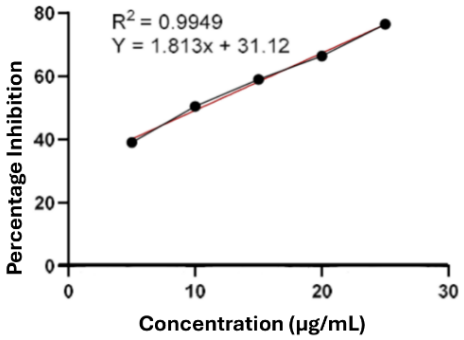

Figure 2. Percentage Inhibition Value against FRAP Concentration of PHF Extract

Table 2. Percentage Mortality Value and LC50 Value of PHF Extract determined by BSLT

Concentration (µg/mL)	Log Concentration	Live Larvae	Dead Larvae	Mortality (%)	LC50
67.5	1.83	18	2	4	239.641
125	2.10	14	6	21.053	
250	2.40	10	10	52.941	
500	2.70	5	15	84.615	
1000	3.00	1	19	98.113	

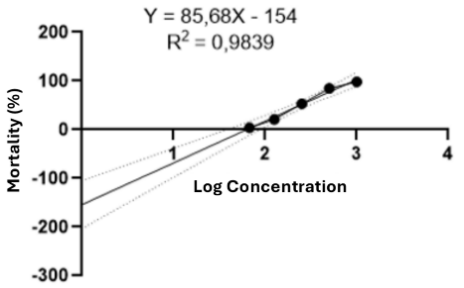

Figure 3. Percentage Mortality Value against BSLT Concentration Log of PHF Extract

Brine Shrimp Lethality Test (BSLT)

The toxicity profile of PHF extract was evaluated using the Brine Shrimp Lethality Test. The extract exhibited an LC_{50} value of 239.64 µg/mL, indicating moderate toxicity according to commonly used toxicity classification standards (Table 2, Figure 3).

Effect of Hypoxia on Heart SOD Activity in Control Rats

In control rats (without PHF administration), heart superoxide dismutase (SOD) activity showed a decreasing trend with increasing duration of hypoxic exposure. Median SOD activity declined from normoxia to 14 days of hypoxia; however, statistical analysis revealed no significant difference among groups (Kruskal–Wallis test, p = 0.109) (Table 3, Figure 4.).

Effect of PHF Administration on Heart SOD Activity under Hypoxia

In PHF-treated rats, heart SOD activity also decreased with prolonged hypoxic exposure. However, statistical analysis demonstrated a significant difference among treatment groups (Kruskal–Wallis test, p = 0.0002). Post hoc analysis showed significant differences between the normoxia group and the 7-day and 14-day hypoxia groups, as well as between the 1-day hypoxia group and the 7-day and 14-day hypoxia groups (Table 4, Figure 5).

Comparison of Heart SOD Activity between Control and PHF-Treated Rats

Comparison between control and PHF-treated groups revealed a significant difference in heart SOD activity in the 1-day hypoxia group (p = 0.0286). No significant differences were observed between control and treatment groups under normoxic conditions or after 7 and 14 days of hypoxia (Figure 6).

Table 3. Heart superoxide dismutase (SOD) activity in control rats under normoxic and hypoxic conditions

Hypoxia Treatment	SOD Activity (U/g mass, median [IQR])
Normoxia	4927.06 (4121.78-5865.89)
1-Day Hypoxia	3941.32 (3707.58-4173.09)
7-Day Hypoxia	3463.1 (3133.88-3818.45)
14-Day Hypoxia	3206.02 (2741.8-3787.62)

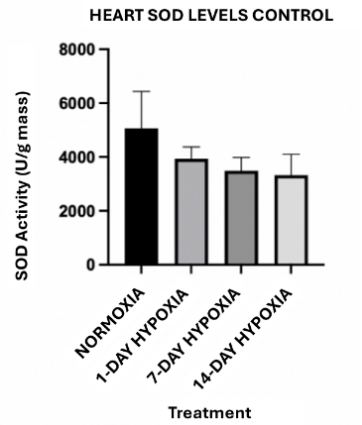

Figure 4. Heart superoxide dismutase (SOD) activity in control rats under normoxic and hypoxic conditions

Table 4. Heart superoxide dismutase (SOD) activity in PHF-treated rats under normoxic and hypoxic conditions

Hypoxia Treatment	SOD Activity (U/g mass, median [IQR])
Normoxia	6918.84 (6037.82-7881.04)
1-Day Hypoxia	6101.56 (6007.77-6174.55)
7-Day Hypoxia	3638.68 (3602.67-3809.39
14-Day Hypoxia	3172.22 (3039.28-3456.25)

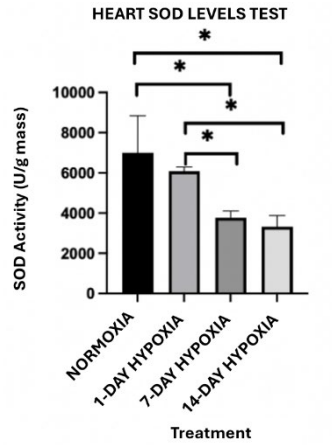

Figure 5. Heart superoxide dismutase (SOD) activity in PHF-treated rats under normoxic and hypoxic conditions

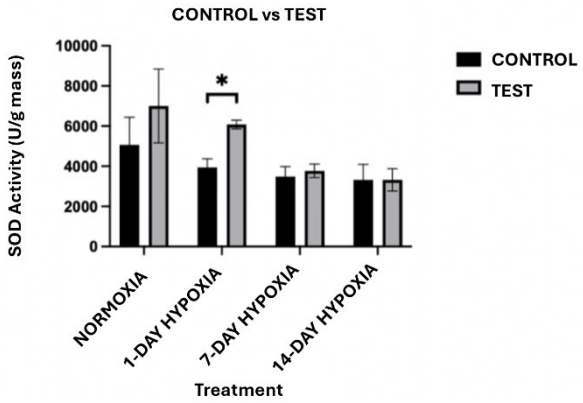

Figure 6. Comparison of heart superoxide dismutase (SOD) activity between control and PHF-treated rats under hypoxic conditions

4. Discussion

Penta Herbs Forte (PHF) extract showed a high total phenolic content of 108,02 mg GAE/g DW, indicating that this extract is rich in antioxidant compounds that can act as electron donors to neutralize free radicals.[13] This strong phenolic content is the basis for why PHF's antioxidant activity remained consistently good in all subsequent tests.[14] Heryanto et al. reported a total phenolic content of 62.68 mg GAE/g DW in Blumea balsamifera leaves extracted with 70% ethanol.[15] Sani et al. reported a higher phenolic content of 178.79 mg GAE/g in Andrographis paniculata extracted with 50% ethanol.[16] Mahmudati et al. reported a phenolic content of 12.25 mg GAE/g in red ginger (Zingiber officinale var. rubrum) prepared as an infusion.[17] Asyhar et al. reported a total phenolic content of 3.65 mg GAE/g DW in Curcuma xanthorrhiza collected from Jambi.[18,19] In addition, DA'i et al. reported that Phyllanthus niruri exhibited a phenolic content of 81.59 mg GAE/g.[19]

The antioxidant activity of PHF can therefore be attributed not only to its overall phenolic content but also to the presence of phenolic constituents such as flavonoids, phenolic acids, and curcuminoids, which exert antioxidant effects primarily through hydrogen atom donation, electron transfer, and metal-chelating mechanisms.[6]

The FRAP EC_{50} value of 11,101 µg/mL indicates strong antioxidant activity through

its ability to reduce Fe^{3+} ions to Fe^{2+}. This high reduction capacity shows that PHF not only acts as a scavenger but is also capable of maintaining cellular redox balance, especially under oxidative stress conditions caused by hypoxia.[20]

BSLT toxicity testing showed an LC_{50} value of 239.64 µg/mL, which is categorized as moderately toxic.[21] This value indicates that PHF is still safe to use within the research dosage range and does not pose a high toxicity risk. Thus, strong antioxidant activity is not accompanied by significant toxic effects, so PHF has a safety profile that can be considered for further research.

Taken together, the in vitro assays provide an initial indication of the antioxidant potential of PHF. To determine whether this potential is reflected at the biological level, the effects of PHF were further evaluated in vivo using SOD activity as an indicator of oxidative stress under hypoxic conditions. In the present study, SOD activity in the PHF-treated group was higher than that of the control group; however, statistically significant differences were observed only during acute hypoxic exposure (1 day). The findings suggest that the surge in reactive oxygen species (ROS) during sustained hypoxia surpassed the compensatory capacity of PHF, rendering the inter-group differences non-significant. Such results illuminate the challenges of in vivo applications, where the bioavailability of phytochemicals is often moderated by complex metabolic pathways and tissue-specific delivery. These factors collectively dictate the actual antioxidant potential within a living system, often diverging from results observed in controlled *in vitro* environments. [22]

The diminished effect of PHF during prolonged hypoxia may also be associated with the overwhelming generation of reactive oxygen species that surpasses endogenous antioxidant defenses, even in the presence of exogenous antioxidants. Similar findings have been reported in previous studies, where herbal antioxidants demonstrated greater efficacy during early or acute oxidative stress compared to chronic conditions.[23] Therefore, the reduced protective effect of PHF under prolonged hypoxic conditions may reflect a time-dependent response, in which persistent oxidative stress, mitochondrial dysfunction, and excessive ROS production limit the capacity of both endogenous and exogenous antioxidants to maintain redox homeostasis. Similar observations have been reported in previous studies indicating that antioxidant interventions, including herbal antioxidants, are generally more effective during the early or acute phase of oxidative stress, whereas prolonged or chronic conditions are associated with diminished responsiveness due to overwhelming ROS generation and altered cellular adaptation mechanism.[23,24]

Several limitations should be acknowledged. First, the relatively small sample size limited the statistical power and may have reduced the ability to detect subtle differences between groups. Furthermore, the geographical scope was confined to a single institution, SMA Negeri 1 Sekayu, which may restrict the generalizability of the findings to broader or more diverse student populations in different socio-economic contexts. Second, the assessment of oxidative stress was limited to SOD activity, without evaluation of other oxidative biomarkers such as malondialdehyde, catalase, glutathione peroxidase, or histopathological changes. These limitations may restrict the interpretation of the antioxidant effects of PHF. Future studies with larger sample sizes and additional oxidative stress biomarkers are warranted to further elucidate the antioxidant and cardioprotective effects of PHF, particularly under prolonged hypoxic conditions.

5. Conclusion

This study demonstrates that Penta Herbs Forte (PHF) exhibits notable antioxidant activity in vitro, as indicated by high total phenolic content and low IC_{50} values in FRAP assays. These findings confirm the strong radical-scavenging capacity of PHF under controlled experimental conditions. However, such in vitro antioxidant potential does not fully translate into equivalent biological effects in vivo. In the in vivo model, SOD activity in the PHF-treated groups was higher than in the control group under both normoxic and hypoxic conditions; however, statistically significant differences were observed only during acute hypoxia. As the duration of hypoxic exposure increased, the protective effect of PHF diminished, suggesting that its antioxidant capacity may be insufficient to counter prolonged oxidative stress. The discrepancy between in vitro and in vivo findings may be attributed to factors such as limited bioavailability, metabolic transformation of active compounds, and the complexity of oxidative stress regulation in biological systems. Moreover, the absence of additional cardiac injury markers, such as malondialdehyde levels, creatine kinase-MB, or histopathological evaluation, limits the interpretation of cardioprotective effects. Overall, the present study suggests that PHF possesses antioxidant potential under acute hypoxic conditions, but its effectiveness in long-term oxidative stress remains limited. Further studies incorporating comprehensive biochemical and histological assessments are required to clarify the therapeutic relevance of PHF in cardiovascular protection.

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