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Phyllanthin from *Phyllanthus amarus* protects the myocardium during pressure overload-induced cardiac hypertrophy by inhibiting the angiotensin-converting enzyme.

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Keywords: angiotensin-converting enzyme; aortic stenosis; cardiac hypertrophy; collagen-I; Phyllanthin; *Phyllanthus amarus*; pressure overload.

Abstract. Ischemic heart disease results from obstruction of blood flow and leads to myocardial infarction. Various lignans of herbal origin have been shown to protect against cardiotoxicity. The present study aimed to assess the potential of phyllanthin, identified from a standardized methanolic extract of *Phyllanthus amarus* (PAME), against pressure overload-induced cardiac hypertrophy in experimental rats. Lignan was identified in PAME using HPLC. Ligating the abdominal aorta induced cardiac hypertrophy in Wistar rats (220-240g). Then they were treated with (n=15, each) either distilled water (10 mL/kg, aortic stenosis control), lisinopril (15 mg/kg), or PAME (50, 100 and 200 mg/kg) for 28 days. Lignan compounds were identified using UV spectra in PAME, and HPLC analysis showed the presence of phyllanthin at 25.30 retention time with an area of 70.22%. Treatment with PAME (100 and 200 mg/kg) significantly and dose-dependently (p<0.01 and p<0.001) ameliorated AS-induced elevation in absolute and relative heart weights, increased serum biomarker levels, and alterations in electrocardiographic and hemodynamic functions. PAME effectively inhibited AS-induced oxide-nitrosative stress dose-dependently (p<0.01 and p<0.001). Up-regulated mRNA expression of cardiac angiotensin-converting enzyme (ACE) and Collagen-I were also markedly inhibited (p<0.01 and p<0.001) by PAME. Furthermore, PAME significantly reduced (p<0.01 and p<0.001) pressure overload-induced alterations in cardiac histopathology. In conclusion, phyllanthin identified from *P. amarus* ameliorated pressure overload-induced cardiac hypertrophy by inhibiting ACE and collagen-I formation pathways to alleviate hypertension and fibrosis. These findings collectively suggest that *P. amarus* represents promising therapy for managing ischemic heart diseases.

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La filantina de la *Phyllanthus amarus* protegió el miocardio durante la hipertrofia cardíaca inducida por sobrecarga de presión mediante la inhibición de la enzima convertidora de angiotensina.

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Palabras clave: Enzima convertidora de angiotensina; estenosis aórtica; hipertrofia cardíaca; colágeno-I; Filantina; *Phyllanthus amarus*; sobrecarga de presión.

Resumen. La cardiopatía isquémica es el resultado de la obstrucción del flujo sanguíneo del corazón y conduce al infarto de miocardio. Se ha demostrado que varios lignanos de origen herbario protegen contra la cardiotoxicidad. El presente estudio tuvo como objetivo evaluar el potencial de la filantina, identificada a partir de un extracto metanólico estandarizado de *Phyllanthus amarus* (PAME), contra la hipertrofia cardíaca inducida por sobrecarga de presión en ratas experimentales. El lignano se identificó en PAME mediante HPLC. La ligadura de la aorta abdominal indujo hipertrofia cardíaca en ratas Wistar (220-240 g). Luego se las trató con (n = 15, cada una) agua destilada (10 ml/kg, control de estenosis aórtica), lisinopril (15 mg/kg) o PAME (50, 100 y 200 mg/kg) durante 28 días. Los compuestos de lignano se identificaron utilizando espectros UV en PAME, y el análisis de HPLC mostró la presencia de filantina en un tiempo de retención de 25,30 con un área de 70,22%. El tratamiento con PAME (100 y 200 mg/kg) mejoró significativamente y de manera dosis-dependiente ($p<0,01$ y $p<0,001$) la elevación inducida por AS en los pesos cardíacos absolutos y relativos, aumentó los niveles de biomarcadores séricos y las alteraciones en las funciones electrocardiográficas y hemodinámicas. PAME inhibió eficazmente el estrés óxido-nitrosativo inducido por AS de manera dosis-dependiente ($p<0,01$ y $p<0,001$). La expresión de ARNm regulada al alza de la enzima convertidora de angiotensina cardíaca (ECA) y el colágeno-I también fueron inhibidos notablemente ($p<0,01$ y $p<0,001$) por PAME. PAME redujo significativamente ($p<0,01$ y $p<0,001$) las alteraciones inducidas por sobrecarga de presión en la histopatología cardíaca. En conclusión, la filantina identificada en *P. amarus* mejoró la hipertrofia cardíaca inducida por sobrecarga de presión al inhibir las vías de formación de la ECA y del colágeno-I para aliviar la hipertensión y la fibrosis. Estos hallazgos en conjunto sugieren que *P. amarus* ofrece una terapia prometedora para el manejo de las enfermedades cardíacas isquémicas.

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INTRODUCTION

Ischemic heart disease (IHD) is the most prevalent cardiovascular disease (CVD) and is characterized by the accumulation of plaques within the walls of the coronary arteries, resulting in a decreased flow of blood to the cardiac tissue [1]. This obstruction of blood flow leads to acute coronary syndromes such as unstable angina or myocardial infarction [2]. IHD significantly impacts long-term global development and, as per

the World Health Organization reports, an annual toll of 17.7 million lives lost to IHD, imposing a pervasive social and economic burden globally [3].

Treatment interventions to manage IHD include antiplatelet agents (such as aspirin or clopidogrel) to reduce the risk of thrombosis, statins (such as lovastatin, atorvastatin, simvastatin, and rosuvastatin) to reduce the risk of atherosclerosis, beta-blockers (such as timolol, metoprolol, atenolol, and propranolol) to decrease heart rate and blood pressure, calcium channel blockers (such as amlodipine, nicardipine, diltiazem, verapamil) to enhance blood flow to the cardiac tissue, angiotensin-converting enzyme (ACE) inhibitors (lisinopril), or angiotensin receptor blockers to lower blood pressure and improve heart function [4]. Despite extensive exploration of these treatment regimens, their side effects, such as toxicity to various organs, limit their availability as definitive therapeutic or prophylactic interventions for managing IHD [5]. However, the cost of treatment and its efficacy in a fraction of patients limit its implications for optimizing patient outcomes and improving quality of life. Thus, for the effective management of IHD, a multidisciplinary approach is needed.

Among the diverse species of medicinal plants, the *Phyllanthus* spp. (*Euphorbiaceae*) has been used in traditional medicine for thousands of years and in Thai folk medicine for treating various ailments, including diabetes, diarrhea, hepatitis, abdominal pain, and various kidney diseases [6]. Among the *Phyllanthus* spp., *Phyllanthus amarus* Schum. & Thonn. (Euphorbiaceae) is a valuable medicinal plant, extensively distributed across tropical and subtropical zones, including Asia, Africa, the West Indies, and South America [7]. *P. amarus* has been extensively studied for its hepatoprotective, antiviral, antiulcer, antiepileptic, anti-asthmatic, antidiabetic, anti-inflammatory, anticancer, and antioxidant properties [8-12]. Pharmacological studies have reported that various bioactive compounds, including alkaloids, polyphenols, tannins, flavonoids, sterols, volatile oils, lignans, and triterpenes are responsible for this array of pharmacological activities. Phyllanthin and hypophyllanthin from *P. amarus* have been reported as inflammatory markers, including tumor necrosis factor-α (TNF-α), which exerts their antiulcer potential [12]. Furthermore, the inhibitory effect of phyllanthin on TNF-α, interleukins, heme oxygenase-1, and transforming growth factor-beta supports its anti-asthmatic properties [11]. However, its potential against IHD is yet to be evaluated. This study aimed to assess the potential of phyllanthin identified from a standardized methanolic extract of *Phyllanthus amarus* aerial parts against pressure overload-induced cardiac hypertrophy in experimental rats.

MATERIALS AND METHODS

Animals

Ninety adult Wistar rats (male, 220-240 g, 7-8 weeks, were purchased from the animal house of Qingdao Central Hospital), with the following housing considerations: temperature: 24°C±1°C, relative humidity: 45-55%, normal dark/light cycle with free access to standard pellet chow and water. The Qingdao Central Hospital approved the experimental protocol (Protocol Number: 559974002). All surgeries were performed under sodium thiopental anesthesia, and efforts were made to minimize suffering.

Preparation and identification of a standardized methanolic extract of *P. amarus*

This procedure was performed according to a previously reported method [12]. Briefly, the quantity (500 g) of air-dried powder (Mesh size-16) of the aerial parts of *P. amarus* was macerated with distilled methanol at room temperature by soaking and eventually stirring for seven days and then filtered. The filtrate was dried in a tray dryer and maintained at 40°C. A semi-solid methanolic extract of *P. amarus* (PAME) was obtained, and

colloidal silicon dioxide was added and dried in a vacuum tube dryer. Phytochemical analysis of PAME was performed to identify phyllanthin content using high-performance liquid chromatography (HPLC). Analyses were conducted using an HPLC system (Camag, Muttenz, Switzerland) with an RP C18, 5μ, 250 X 4.6 mm, and 1.5 mL/min flow rate. Acetonitrile: Buffer (40:60 v/v) was used as the mobile phase for isolation and detection. The buffer consisted of 0.136 g of potassium hydrogen phosphate and 0.5 mL of o-phosphoric acid. The optimum injection volume was 20 μL, and the detection wavelength of the detector was set to 230 nm. The autosampler temperature was maintained at 10°C, and the system pressure was 1000 psi.

Induction of pressure overload-induced cardiac hypertrophy and treatment schedule

Wistar rats were anesthetized using sodium thiopental (35 mg/kg, intraperitoneally) and the abdominal aorta above the left renal artery was exposed by cutting the mid-abdomen. Then, it was constricted using a 40 mm cannula (0.9 sizes) ligation and withdrawn after 10 min [13]. After a week of recovery, the rats were randomly assigned to various groups. The rats received the following treatments (15 rats per group): aortic stenosis control (AS, received distilled water [DW], 10 mL/kg), lisinopril (15 mg/kg), and PAME (50, 100, and 200 mg/kg). The dosages of lisinopril (15 mg/kg) and PAME (50, 100, and 200 mg/kg) were selected based on previous studies [9,12]. Other groups of age- and body-weight-matched sham rats were maintained without aortic ligation and treated with DW (10 mL/kg). Rats were treated orally with DW, lisinopril, or PAME for 28 days.

Behavioral and biochemical determination

On the 29th day, blood was collected using the retro-orbital puncture method from anesthetized rats (urethane, 1.25 g/kg, intraperitoneally), and serum (six rats per group) was separated to evaluate the parameters including creatine kinase-MB (CK-MB), lactate dehydrogenase (LDH), and alkaline phosphatase (ALP) using various reagent kits (Accurex Biomedical Pvt. Ltd., Mumbai, India).

Electrocardiographic (ECG) and hemodynamic functions (including heart rate and blood pressure viz, systolic blood pressure [SBP], diastolic blood pressure [DBP], and mean arterial blood pressure [MABP]) were estimated (six rats per group) after blood collection using an AD Instrument data-acquisition system (LabChart 7.3; AD Instrument Pvt. Ltd., Australia).

Animals were sacrificed by cervical dislocation. Cardiac tissue was isolated and perfused with cold phosphate-buffered saline to flush blood from the tissue and stored at -70°C. A previously reported method was used to determine the levels of total protein, superoxide dismutase (SOD), reduced glutathione (GSH), lipid peroxidation (MDA), and nitric oxide (NO) in cardiac tissue homogenates (six rats per group) [14].

Reverse transcription polymerase chain reaction (RT-PCR) analysis was used to determine the messenger ribonucleic acid (mRNA) expression of angiotensin-converting enzyme (ACE; forward primer: CCTGATCAACCAGGAGTTTGCAGAG, reverse primer: GCCAGCCTTCCCAGGCAAACAGCAC, base pair: 303) and collagen-I (forward primer: GAGCGGAGAGTACTGGATCG, reverse primer: GGTTCGGGCTGATGTACCAG, base pair: 218) in cardiac tissue (6 rats per group) [15,16]. β-actin was used as a reference standard (forward primer: GCCATGTACGTAGCCATC, reverse primer: GAACCGCTCATTGCCGAT, base pair: 375).

Finally, cardiac tissue from each group (three rats per group) was isolated and fixed in 10% formalin for histopathological evaluation. Briefly, cardiac tissues were cut in sections of 3-5 μm thickness by microtome and stained by hematoxylin-eosin. The samples were mount-

ed by disterene phthalate xylene (DPX). For myocardial fibers staining, Yuccafine™ Masson's trichrome staining kit (Yucca Diagnostics, India) was used. Each tissue section's photomicrographs were observed using Cell Imaging software for Life Science microscopy (Olympus Soft Imaging Solution GmbH, Munster, Germany). Microscopic scoring (0-4) of histological observations (myocardial degeneration, interstitial inflammation, and hemorrhage) was performed by an experienced histologist, unaware of the treatment groups, as described previously [15].

Statistical analysis

Data for all parameters (except histopathological findings) are expressed as mean ± standard error of the mean (SEM), and data for histopathological findings are expressed as medians (Q1, Q3). Data analysis was performed using the GraphPad Prism software (version 5.0; GraphPad, San Diego, CA, USA). Data were analyzed using one-way analysis of variance (ANOVA), and Tukey's multiple range test was used for *post hoc* analysis. A value of $p<0.05$ was considered to be statistically significant.

RESULTS

Isolation and identification of phyllanthin from PAME

PAME had a 52.78% w/w yield with glycosides, lignans, steroids, tannins, and phenolic compounds. The lignan compounds were identified by ultraviolet (UV) spectroscopy (Fig. 1A). The total run time for the HPLC column was 40 min, and phyllanthin was identified at a retention time (RT) of 25.30 min with an area of 70.22% (Fig. 1B).

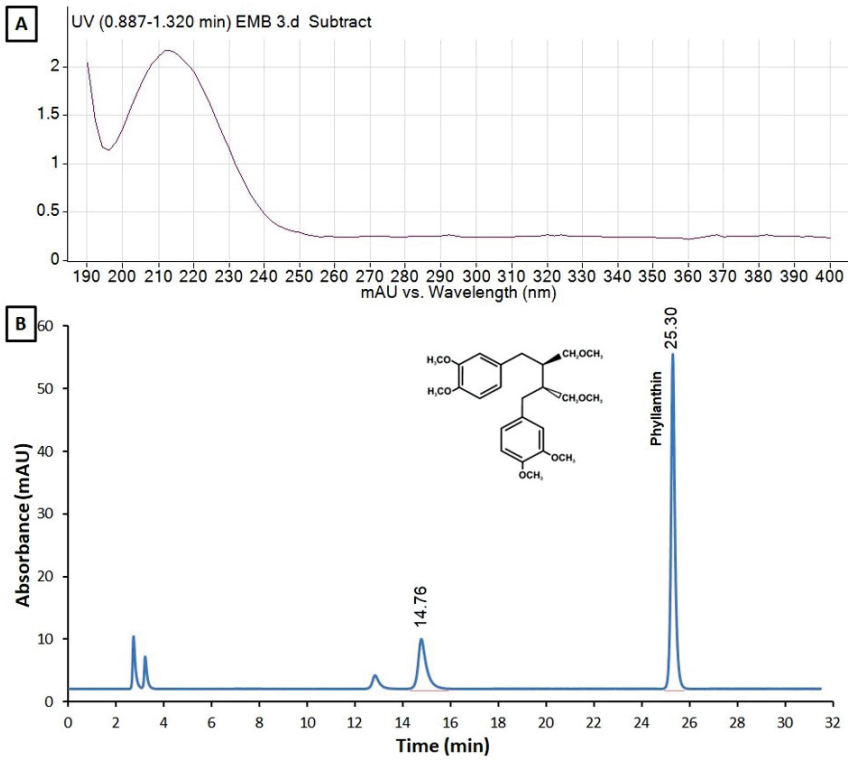


Fig. 1. A – UV spectra of standardized *P. amarus* extract; and **B** – HPLC chromatogram showing the peak of phyllanthin (RT: 25.30 min).

HPLC: High-performance liquid chromatography; mAU: milli-absorbance unit; min: minute; nm: nanomole; RT: retention time; UV: ultraviolet.

Effect of PAME on body weight and relative heart Weight

The body weights of sham rats and rats in the AS control and treatment groups did not differ significantly. Ligation of the abdominal aorta also did not cause any significant change in the body weight of AS control rats compared to sham rats. Compared to sham rats, ligation of the abdominal aorta caused a significant increase ($p<0.001$) in heart weight (absolute) and heart weight to body weight ratio (relative heart weight) in AS control rats. In contrast, treatment with lisinopril (15 mg/kg) resulted in significant attenuation ($p<0.001$) in absolute and relative heart weights compared to the AS control rats. Compared with AS control rats, PAME (100 and 200 mg/kg)-treated rats also showed a significant and dose-dependent decrease ($p<0.01$ and $p<0.001$) in absolute and relative heart weights. Administration of PAME (50 mg/kg) did not protect against AS-induced increase in cardiac weight (Table 1).

Effect of PAME on electrocardiographic and hemodynamic functions

Fig. 2 depicts AS-induced alterations in electrocardiographic recordings and their amelioration by PAME. The heart rate of AS control rats was significantly ($p<0.001$) lower than that of sham rats. In contrast, treatment with lisinopril (15 mg/kg) resulted in a significant increase ($p<0.001$) in heart rate when compared to the AS control rats. Treatment with PAME (100 and 200 mg/kg) resulted in a significant and dose-dependent increase ($p<0.01$ and $p<0.001$) in heart rate compared to that in the AS control rats (Fig. 2 and Table 2).

There was a significant ($p<0.001$) prolongation in the QRS, QT, QTc, PR, RR, and ST intervals in AS control rats compared with sham rats. However, treatment with lisinopril (15 mg/kg) significantly ($p<0.001$) inhibited the prolongation of QRS, QT, QTc, PR, RR, and ST intervals compared with AS control rats. Treatment with PAME (100 and 200 mg/kg) also resulted in a significant ($p<0.001$) decrease in QRS, QT, QTc, PR, RR, and ST intervals compared to AS control rats (Fig. 2 and Table 2).

SBP, DBP, and MABP in AS control rats were significantly ($p<0.001$) lower than those in sham rats. In contrast, treatment with lisinopril (15 mg/kg) resulted in a significant ($p<0.001$) increase in SBP, DBP, and MABP in the AS control rats. Treatment with PAME (100 and 200 mg/kg) also significantly and dose-dependently ($p<0.01$ and $p<0.001$) increased SBP, DBP, and MABP compared with the AS control rats (Table 2).

Effect of PAME on serum biochemistry

CK-MB, LDH, and ALP levels were significantly ($p<0.001$) higher in the AS control rats than in the sham rats. Treatment with lisinopril (15 mg/kg) significantly ($p<0.001$) decreased CK-MB, LDH, and ALP levels compared to AS control rats. Treatment with PAME (100 and 200 mg/kg) reduced the CK-MB, LDH and ALP significantly and dose-dependently ($p<0.001$ and $p<0.001$) compared to AS control rats. However, there was no significant decrease in CK-MB, LDH, and ALP levels in PAME (50 mg/kg)-treated rats compared to those in AS control rats (Table 1).

Effect of PAME on cardiac total protein, SOD, GSH, MDA, and NO levels

Cardiac SOD and GSH levels in the AS control rats were significantly lower ($p<0.001$) than those in the sham rats. SOD and GSH levels in the cardiac tissue of lisinopril (15 mg/kg)-treated rats were significantly higher ($p<0.001$) than those in the AS control rats. Treatment with PAME (100 and 200 mg/kg) significantly and dose-dependently attenuated ($p<0.01$ and $p<0.001$) AS-induced decreased levels of SOD and GSH compared to those in AS control rats (Table 3).

There was a significant increase ($p<0.001$) in cardiac total protein, MDA,

Table 1. Effect of PAME on pressure overload-induced alterations in absolute heart weight, relative heart weight, serum CK-MB, LDH, and ALP.

Parameters	Sham	AS control	L (15 mg/kg)	PAME (50 mg/kg)	PAME (100 mg/kg)	PAME (200 mg/kg)
Body weight (in g)	236.20 ± 4.00	241.00 ± 2.99	239.70 ± 3.90	242.80 ± 4.08	240.30 ± 4.46	240.20 ± 4.76
Heart weight (in g)	0.30 ± 0.02	0.90 ± 0.05$^{###}$	0.40 ± 0.05***	0.85 ± 0.03	0.61 ± 0.03**	0.47 ± 0.04***
Heart weight/Body weight (X10-3)	1.27 ± 0.06	3.76 ± 0.21$^{###}$	1.65 ± 0.19***	3.52 ± 0.15	2.56 ± 0.13**	1.96 ± 0.15***
Serum CK-MB (in IU/L)	1057.00 ± 56.33	2102.00 ± 66.24$^{###}$	1268.00 ± 39.52***	1952.00 ± 37.08	1658.00 ± 50.04**	1317.00 ± 50.54***
Serum LDH (in IU/L)	1356.00 ± 73.39	2733.00 ± 62.75$^{###}$	1666.00 ± 71.75***	2748.00 ± 51.49	2025.00 ± 51.74**	1601.00 ± 110.9***
ALP (in mg %)	117.60 ± 4.98	341.70 ± 5.21$^{###}$	137.20 ± 12.82***	318.20 ± 10.28	259.10 ± 7.15**	154.80 ± 11.5***

Data are expressed as mean ± SEM (six rats per group) and analyzed by one-way variance analysis followed by Tukey's multiple range test. **$p<0.01$ and ***$p<0.001$ as compared to the AS control rats, $^{###}p<0.001$ as compared to the sham rats. AS: aortic stenosis control rats; L (15): lisinopril (15 mg/kg)-treated rats; PAME (50, 100, and 200 mg/kg); *Phyllanthus amarus* methanolic extract-treated rats. The numbers in parentheses in the table header represent the doses of the respective treatments in mg/kg. ALP: alkaline Phosphatase; AS: aortic stenosis; CK-MB: creatine Kinase-MB; g: gram; IU/L: international units per liter; kg: kilogram; L: lisinopril; LDH: lactate dehydrogenase; mg: milligram; PAME: *Phyllanthus amarus* methanolic extract; SEM: standard error means.

Table 2. Effect of PAME on pressure overload-induced alterations in electrocardiographic and hemodynamic.

Parameters	Sham	AS control	L (15 mg/kg)	PAME (50 mg/kg)	PAME (100 mg/kg)	PAME (200 mg/kg)
Heart Rate (in BPM)	363.70 ± 13.70	271.00 ± 5.82$^{###}$	321.00 ± 10.64***	276.20 ± 7.80	300.70 ± 11.90**	344.5.00 ± 13.72***
QRS interval (in ms)	12.33 ± 0.67	34.17 ± 0.87$^{###}$	16.17 ± 0.54***	30.00 ± 0.93	23.33 ± 1.17***	21.33 ± 0.88***
QT Interval (in ms)	47.33 ± 2.77	92.00 ± 2.62$^{###}$	60.50 ± 3.14***	85.00 ± 3.45	69.67 ± 2.46***	64.00 ± 1.29***
QTc Interval (in ms)	130.30 ± 4.61	177.80 ± 4.74$^{###}$	143.50 ± 1.46***	168.70 ± 3.72	148.20 ± 5.26***	144.50 ± 6.16***
PR interval (in ms)	14.00 ± 0.58	29.50 ± 0.76$^{###}$	17.33 ± 0.99***	28.67 ± 0.56	24.33 ± 1.12***	22.00 ± 0.68***
RR interval (in ms)	151.70 ± 4.55	215.50 ± 4.00$^{###}$	160.80 ± 4.35***	206.70 ± 5.54	177.50 ± 5.57***	171.70 ± 5.43***
ST interval (in ms)	12.00 ± 0.58	35.50 ± 0.76$^{###}$	15.33 ± 0.99***	32.67 ± 0.56	26.33 ± 1.12***	24.00 ± 0.68***
SBP (in mmHg)	152.50 ± 3.76	106.30 ± 2.91$^{###}$	151.30 ± 4.42***	116.50 ± 1.34	131.00 ± 1.84**	137.80 ± 2.86***
DBP (in mmHg)	116.00 ± 2.99	88.33 ± 3.54$^{###}$	111.30 ± 3.75***	95.67 ± 4.57	97.17 ± 4.19**	107.50 ± 3.53***
MABP (in mmHg)	120.50 ± 2.41	93.83 ± 1.72$^{###}$	116.00 ± 1.29	101.30 ± 2.86	105.00 ± 2.63**	110.00 ± 2.00***

Data are expressed as mean ± SEM (six rats per group) and analyzed by one-way variance analysis followed by Tukey's multiple range test. **$p<0.01$ and ***$p<0.001$ as compared to the AS control rats, $^{###}p<0.001$ as compared to the sham rats. AS: aortic stenosis control rats; L (15): lisinopril (15 mg/kg)-treated rats; PAME (50, 100, and 200 mg/kg); *Phyllanthus amarus* methanolic extract-treated rats. The numbers in parentheses in the table header represent the dose of the respective treatment in mg/kg. AS: aortic stenosis; BPM: beats per minute; DBP: diastolic blood pressure; kg: kilogram; L: lisinopril; MABP: mean arterial blood pressure; mg: milligram; mmHg: millimeters of mercury; ms: millisecond; PAME: *Phyllanthus amarus* methanolic extract; SBP: systolic blood pressure; SEM: standard error means.

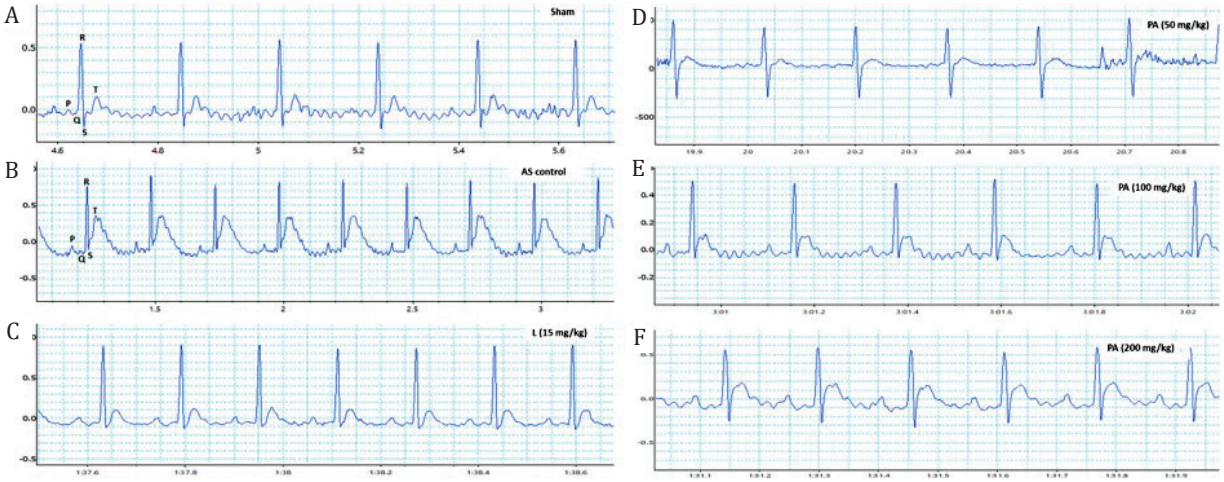


Fig. 2. Effect of PAME on pressure overload-induced alterations on electrocardiograms. A representative electrocardiographic tracing from **A** – sham rats; **B** – AS control rats; **C** – lisinopril (15 mg/kg)-treated rats; **D** – PAME (50 mg/kg)-treated rats; **E** – PAME (100 mg/kg)-treated rats; and **F** – PAME (200 mg/kg)-treated rats. AS: aortic stenosis; kg: kilogram; L: lisinopril; mg: milligram; PAME: *Phyllanthus amarus* methanolic extract.

Table 3
Effect of PAME on pressure overload-induced alterations in cardiac oxido-nitrosative stress.

Parameters	Sham	AS control	L (15 mg/kg)	PAME (50 mg/kg)	PAME (100 mg/kg)	PAME (200 mg/kg)
SOD (in U/mg of protein)	9.29 ± 0.44	4.08 ± 0.61$^{###}$	6.66 ± 0.62***	4.08 ± 0.68	5.60 ± 0.61***	6.25 ± 0.87***
GSH (in μg/mg protein)	0.35 ± 0.02	0.22 ± 0.01$^{###}$	0.34 ± 0.02***	0.25 ± 0.02	0.26 ± 0.02**	0.36 ± 0.02***
MDA (in nmol/L/mg of protein)	2.46 ± 0.32	7.17 ± 0.29$^{###}$	3.47 ± 0.29***	6.56 ± 0.35	4.67 ± 0.30**	3.35 ± 0.24***
NO (in μg/mg of protein)	212.90 ± 15.22	604.20 ± 14.52$^{###}$	307.60 ± 10.31***	552.60 ± 7.66	493.20 ± 17.84**	349.90 ± 6.36***
Total protein (in mg/mL of tissue)	24.32 ± 3.06	60.35 ± 3.47$^{###}$	33.76 ± 3.57***	56.89 ± 2.71	49.98 ± 3.15**	38.14 ± 2.60***

Data are expressed as mean ± SEM (six rats per group) and analyzed by one-way variance analysis followed by Tukey's multiple range test. **$p<0.01$ and ***$p<0.001$ as compared to the AS control rats, $^{###}p<0.001$ as compared to the sham rats. AS: aortic stenosis control rats; L (15): lisinopril (15 mg/kg)-treated rats; PAME (50, 100, and 200 mg/kg); *Phyllanthus amarus* methanolic extract-treated rats. The numbers in parentheses in the table header represent the dose of the respective treatment in mg/kg. μg: microgram; AS: aortic stenosis; GSH: glutathione peroxidase; kg: kilogram; L: lisinopril; MDA: malondialdehyde; mg: milligram; mL: milliliter; nmol: nanomole; NO: nitric oxide; PAME: *Phyllanthus amarus* methanolic extract; SEM: standard error means; SOD: superoxide dismutase.

and NO levels in AS control rats compared to sham rats. Administration of lisinopril (15 mg/kg) significantly ($p<0.001$) decreased total protein, MDA, and NO levels in cardiac tissue compared with those in AS control rats. Treatment with PAME (100 and 200 mg/kg) also significantly and dose-dependently ($p<0.01$ and $p<0.001$, respectively) decreased the cardiac total protein, MDA, and NO levels compared to AS control rats (Table 3).

Effect of PAME on cardiac ACE and collagen-I mRNA expressions

Compared with sham rats, cardiac ACE and collagen-I mRNA expressions were sig-

nificantly upregulated in AS control rats. Compared to AS control rats, ACE and collagen-I mRNA expression in the cardiac tissue of lisinopril (15 mg/kg)-treated rats was significantly downregulated ($p<0.001$). Treatment with PAME (50 mg/kg) failed to significantly downregulate ACE and collagen-I mRNA expression compared to AS control rats. However, administration of PAME (100 and 200 mg/kg) significantly and dose-dependently ($p<0.01$ and $p<0.001$) downregulated ACE and collagen-I mRNA expression compared to AS control rats (Fig. 3).

Effect of PAME on pressure overload-induced alterations in cardiac histopathology

Histopathological observations of the heart from sham rats revealed a well-maintained architecture with sham myocardial fibers and muscle bundles with well-defined boundaries and mild infiltration of neutrophils (Fig. 4A). Hearts from AS control rats showed significant ($p<0.001$) myocardial degeneration, congestion, edema, and infiltration of inflammatory cells with a disorganized arrangement of muscle bundles with no well-defined boundaries (Fig. 4B). Administration of lisinopril (15 mg/kg) protected against AS-induced myocardial damage, as reflected by a significant ($p<0.001$) reduction in myocardial necrosis, inflammatory infiltration, and congestion without any edema (Fig.4C). Heart sections from PAME (50 mg/kg)-treated rats showed severe myocardial necrosis, inflammatory cell infiltration, congestion, and edema (Fig. 4D). However, administration of PAME (100 and 200 mg/kg) significantly ($p<0.001$) reduced AS-induced myocardial aberrations, as reflected by the presence of mild to moderate myocardial necrosis, inflammatory cell infiltration, congestion, and edema (Fig. 4E and 4F). (Fig. 4G).

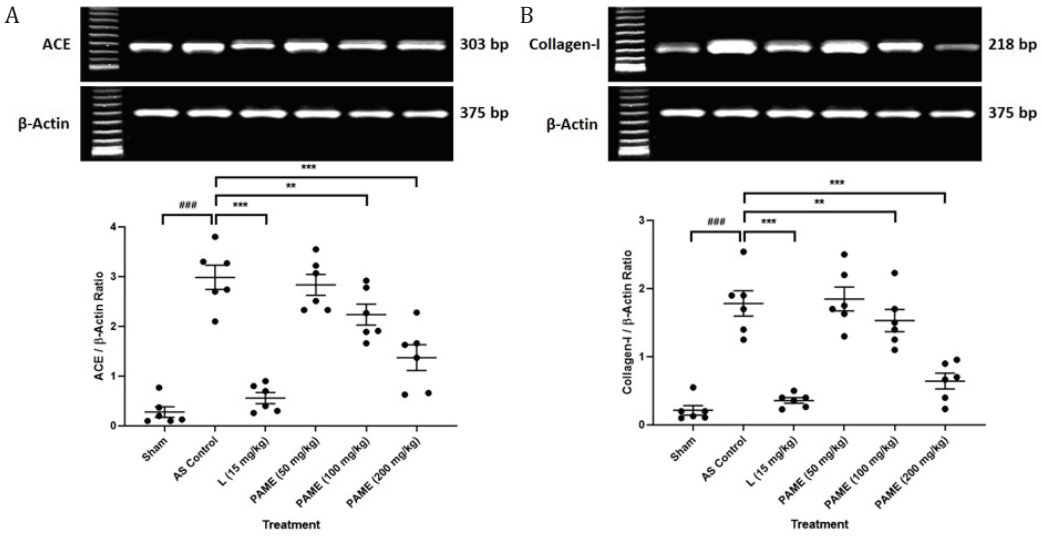
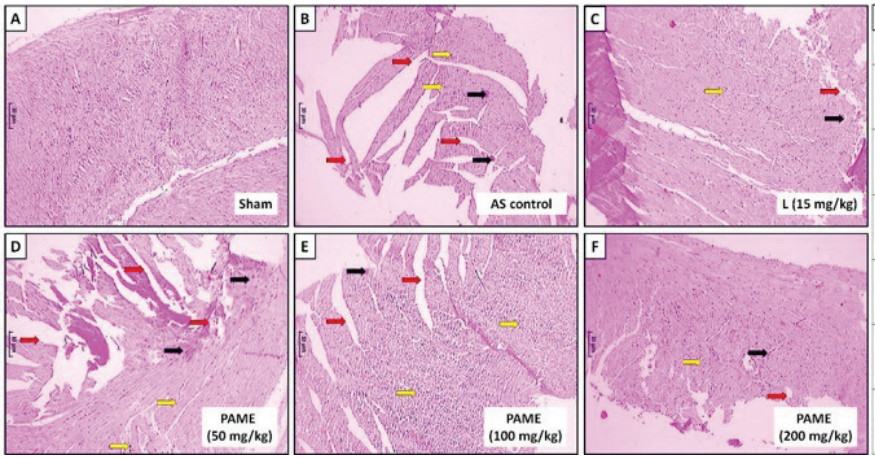


Fig. 3. A – Effect of PAME on pressure overload-induced alterations in cardiac ACE mRNA expression; and **B** – Effect of PAME on pressure overload-induced alterations in cardiac collagen-I mRNA expression.

Data are expressed as mean ± SEM (six rats per group) and analyzed by one-way variance analysis followed by Tukey's multiple range test. **p<0.01 and ***p<0.001 as compared to the AS control rats, ###p<0.001 as compared to the sham rats. L (15): lisinopril (15 mg/kg)-treated rats; PAME (50, 100, and 200 mg/kg); *Phyllanthus amarus* methanolic extract-treated rats. The numbers in parentheses on the x-axis represent the doses of the respective treatments in mg/kg. ACE: angiotensin-converting enzyme; AS: aortic stenosis; bp: base pair; kg: kilogram; L: lisinopril; mg: milligram; mRNA: messenger ribonucleic acid; PAME: *Phyllanthus amarus* methanolic extract; SEM: standard error means.



G

Treatment	Myocardial degeneration	Interstitial inflammation	Interstitial hemorrhage
Sham	0 (1, 0)	1 (1, 0)	0 (1, 0)
AS Control	4 (4, 3)###	4 (4, 3)###	4 (4, 3)###
L (15 mg/kg)	0 (1, 0)***	1 (1, 0)***	1 (1, 0)***
PAME (50 mg/kg)	4 (4, 3)	3 (4, 3)	3 (4, 3)
PAME (100 mg/kg)	2 (3, 2) **	2 (2, 1)**	1 (2, 1)**
PAME (200 mg/kg)	1 (2, 1)***	1 (1, 0)***	2 (2, 1)***

Fig. 4. Effect of PAME on pressure overload-induced alterations in cardiac histopathology. Representative photomicrographs of heart sections from **A** – sham rats; **B** – AS control rats; **C** – lisinopril (15 mg/kg)-treated rats; **D** – PAME (50 mg/kg)-treated rats; **E** – PAME (100 mg/kg)-treated rats; **F** – PAME (200 mg/kg)-treated rats; and **G** – quantitative representation of the histological scores.

The sections were stained with hematoxylin-eosin, and images were captured at 40X. Data are expressed as the median (Q1, Q3) (three rats per group) and were analyzed using the non-parametric test. **p<0.01 and ***p<0.001 as compared to the AS control rats, ###p<0.001 as compared to the sham rats. Microscopic changes in cardiac histopathology include myocardial degeneration (red arrows), interstitial inflammation (yellow arrows), and interstitial hemorrhage (black arrows). The numbers in parentheses represent the doses of the respective treatments in mg/kg.

AS: aortic stenosis; kg: kilogram; L: lisinopril; mg: milligram; PAME: *Phyllanthus amarus* methanolic extract; Q1: first quadrant; Q3: third quadrant; SEM: standard error means.

DISCUSSION

This study investigated the potential of phyllanthin isolated from *P. amarus* to prevent pressure overload-induced cardiac hypertrophy using various *in vivo* and *ex vivo* parameters in experimental rats. This study employed a comprehensive set of methodologies to assess the impact of *P. amarus* on a spectrum of parameters, ranging from serum biochemistry and electrocardiographic function to molecular markers and histopathological alterations. Assessment of serum LDH, CK-MB, AST, ALT, and ALP levels provides insights into the systemic effects of constricted abdominal aorta and the potential mitigating role of *P. amarus* [12]. Concurrently, evaluating electrocardiographic and hemodynamic parameters allows for gauging the functional impact of *P. amarus* on pressure overload-induced cardiac alterations. This study investigated the levels of SOD, GSH, MDA, and nitric oxide in the cardiac tissue homogenates to unravel the molecular underpinnings [12]. The quantification of cardiac markers, such as ACE and collagen-I mRNA expressions, sheds light on the specific influence of *P. amarus* on the molecular pathways implicated in aortic stenosis-induced cardiotoxicity. Finally, histopathological evaluation of cardiac tissues elucidated the morphological changes induced by aortic stenosis and the efficacy conferred by *P. amarus*. By addressing these knowledge gaps, our study provides robust evidence that phyllanthin from *P. amarus* confers cardioprotective efficacy against pressure overload-induced cardiac hypertrophy, and can be considered as an alternative and complementary therapeutic strategy for managing ischemic heart diseases.

Current circulating biomarkers used to detect myocardial damage are classified as (a) biomarkers with elevated levels directly in the blood circulation due to systemic reactions after the myocardial toxicity events

viz. interleukins (IL-1β, IL-6), growth factors (Insulin-like Growth Factor-1, and vascular endothelial growth factor), (b) biomarkers originating from damaged myocardial tissues that are ultimately released into the blood circulation, such as LDH and CK-MB; and (c) biomarkers with abnormal serum levels before the occurrence of myocardial infarction event viz. ALP, AST, glucose, heparanase, copeptin [17,18]. Specific biomarkers directly involved in myocardial injury were investigated in the current study, including ALP, CK-MB, and LDH. According to previous research, patients with myocardial damage showed increased ALP, CK-MB, and LDH levels, suggesting their importance during IHD [19]. In the current study, stenotic rats showed elevated serum levels of ALP, CK-MB, and LDH; however, PAME treatment effectively attenuated these elevations, suggesting its cardioprotective potential.

Oxidative stress is critical in chronic inflammatory conditions, such as diabetes, cancer, cardiovascular diseases, neurodegenerative diseases, and infections [20,21]. The imbalance between pro-oxidants and antioxidants disrupts tissue homeostasis, causing the overproduction of harmful reactive oxygen and nitrogen species and leading to cell toxicity [22]. Numerous studies have documented the crucial role of oxidative stress in pressure overload-induced cardiac hypertrophy [1,13]. A redox imbalance was observed, as measured by increased levels of MDA and nitric oxide, along with a reduction in GSH and SOD activity [23,24]. GSH is an important intracellular antioxidant system pivotal in neutralizing lipid peroxides via glutathione peroxidase (GPx)-mediated inactivation, generating glutathione disulphide as a byproduct [24,25].

Moreover, GSH is crucial for conjugation with glutathione S-transferase (GST) to detoxify reactive species from lipid peroxidation and other xenobiotics [26,27]. Consequently, GSH depletion compromises cellular integrity, induces macromolecular damage, and fosters the accumulation of its oxidized form, further contributing to electrical and contractile dysfunction. A sudden influx of blood into the cardiac tissue precipitates cardiac GSH depletion, perpetuating the continual generation of oxygen-free radicals. Similarly, SOD plays a pivotal role in counteracting aortic stenosis-induced oxidative stress [12,28]. Superoxide radicals generated at the injury site may modulate SOD levels, potentially fostering superoxide anion accumulation and the consequent myocardial damage[29]. The current findings demonstrate that rats with aortic stenosis exhibit elevated MDA and nitric oxide activities and reduced SOD and GSH activities in their cardiac tissues. However, pretreatment with the *P. amarus* extract effectively restored these imbalances by regulating cardiac oxidative stress markers. Lignans have been shown to inhibit oxidative stress [30]. In addition, extensive research has highlighted the antioxidant potential of *P. amarus* in both *in vitro* and *in vivo* studies [8]. Other studies have highlighted the considerable antioxidant capacity of *P. amarus* extract against renal oxidative stress markers induced by streptozotocin in diabetic rats [9] and its ability to protect rat liver mitochondria from oxidative damage [31]. Moreover, the methanolic extract of *P. amarus* showed antioxidant properties against cyclophosphamide-induced toxicity in mice by augmenting cellular GSH and GST levels [32]. These results emphasize and confirm the promising antioxidant efficacy of *P. amarus*, suggesting its potential against stenosis-induced cardiac hypertrophy, which may be attributed to the presence of its major bioactive lignan, phyllanthin.

Mammalian homeostasis is maintained by the renin-angiotensin system, which mainly comprises renin, Ang II, angiotensin-1 (AT1) receptors, angiotensinogen, and ACE [33]. Clinical and experimental studies have established a link between angiotensin-converting enzyme inhibitors and blood pressure regulation [34]. Additionally, mounting evidence suggests that the binding of Ang II to AT1 receptors initiates ROS generation, which stimulates inflammation influx in cardiac tissue, and their synergistic action results in cardiac damage during ventricular hypertrophy [35]. Accordingly, researchers

have demonstrated that the administration of ACE inhibitors to hypertensive patients significantly decreases systemic vascular resistance, thus reducing the risk of cardiac failure and IHD [36]. Furthermore, reduced blood flow to the cardiac tissue causes a significant drop in hydrostatic pressure in the afferent arteriole, a major factor in the release of renin [37].

Moreover, long-term occlusion of the cardiac aorta causes increased expression of renin cells in the renal tissue, which are further released into the systemic circulation, where they interact with angiotensinogen. Renin-induced cleavage of angiotensinogen to AT1 and its further conversion to Ang II by ACE is responsible for increased blood pressure. In the present study, increased cardiac ACE expression caused a significant elevation in blood pressure in AS control rats. However, PAME treatment might counteract ACE activation, thereby protecting against cardiac hypertrophy.

Extensive research has suggested that *Phyllanthus niruri* is effective against pulmonary tuberculosis [38-40], vaginal candidiasis [41], urolithiasis [42], and shockwave lithotripsy for renal lithiasis [43] in various randomized controlled trials. This validation supports using *Phyllanthus* in treating hepatitis and other chronic ailments. Clinical studies have highlighted the efficacy of *P. amarus* in the management of acute viral hepatitis [44,45]. Thus, based on the findings of the present investigation, *P. amarus* should be considered further to determine its clinical efficacy in managing ischemic heart diseases.

Our investigation revealed that phyllanthin identified from *P. amarus* showed cardioprotective effects against pressure overload-induced cardiac hypertrophy, likely through mechanisms involving (a) ameliorating the alterations in electrocardiographic and hemodynamic parameters and serum biochemical markers (CK-MB, LDH, and ALP), (b) antioxidant effects by modulating the alteration in the cardiac oxide-nitrosative stress markers, (c) inhibiting ACE and collagen-I formation pathways to ameliorate hypertension and fibrosis, and (d) preserving the histological integrity of cardiac tissue against AS-induced damage. These findings suggest that P. amarus is a promising therapeutic agent for managing ischemic heart diseases.

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Data availability

The raw data underlying this article will be shared with the corresponding author upon reasonable request.

Ethical statements

The Qingdao Central Hospital approved the experimental protocol (Protocol Number: 559974002). All surgeries were performed under sodium thiopental anesthesia, and efforts were made to minimize suffering.

Conflict of interest

The authors declare that they have no conflicts of interest.

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Author contributions

Each author has made significant contributions to the development of this manuscript. C.Z. conceived and designed the evaluation, performed parts of the statistical analysis, and drafted the manuscript; Z.L. performed data acquisition and drafted the manuscript. Y.G.: Performed parts of the statistical analysis and drafted the manuscript. All authors read and approved the final version of this manuscript.

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