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Effect of Stress-WIN, a novel polyherbal formulation, on DOCA-salt-induced hypertensive Wistar rats

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Abstract

Hypertension is a multifactorial disorder and one of the most important risk factors and a leading cause of stroke, heart disease, and end-organ damage. Hypertension leads to the production of high levels of pro-inflammatory cytokines and cholesterol, which increase thrombogenesis and fibrosis, ultimately resulting in chronic inflammation. Chronic inflammation causes endothelial dysfunction by producing excess reactive oxygen species (ROS) through pro-inflammatory cytokines such as interleukine-1β (IL-1β) and tumor necrosis factor-α (TNF-α). Further, the high level of nitric oxide (NO) and malonaldehyde (MDA) increases the oxidative stress, which worsens the liver's production of SGOT and SGPT and glomerular filtration of the kidney. In this study, an Ayurvedic polyherbal formulation (StressWIN) was shown to be a potent therapeutic drug for the treatment of deoxycorticosterone (DOCA)-salt-induced hypertension in Wistar rats. This study showed that administration of Stress-WIN significantly reduced the expression of pro-inflammatory cytokines (IL-1β and TNF-α) and also reduced the level of ROS, NO, MDA, SGOT, and SGPT in the serum of DOCA-salt-induced hypertension in Wistar rats. Furthermore, Stress-WIN treatment exhibited reduced cholesterol levels and glutathione concentration. Histological analysis showed that infiltration of immune cells was reversed by Stress-WIN A treatment (500 mg/kg and 1000 mg/kg). Furthermore, Stress-WIN A (2000 mg/kg) administration attenuated DOCA-salt-mediated morphological changes in the kidney and the heart of the Wistar rat. These promising outcomes underscore the potential of Stress-WIN as a viable alternative or adjunct therapy for hypertension, warranting further clinical investigations.

Highlights

- Hypertension is a major global concern leading to chronic inflammation and detrimental for vital organs.
- Chronic inflammation due to hypertension attenuated by the oral administration of *Ayuvedic* polyherbal formulation Stress-WIN.
- Results suggest Stress-WIN as a promising therapeutic or adjunct for managing hypertension.

Keywords Hypertension, *Ayurvedic* polyherbal formulation, Stress-WIN, Malonaldehyde, Pro-inflammatory cytokines

1 Introduction

Hypertension, one of the most global public health concerns, is a risk factor for cardiovascular diseases, stroke, and fatal death [1, 2]. According to the Global Report on Hypertension in 2023, a World Health Organisation (WHO) survey, the number of affected people has doubled from 650 million in 2010 to 1.3 billion in 2019 worldwide [2]. In 2019–2020, the National Family Health Survey (NFHS-5) stated that in India, hypertension prevalence was 24% in males and 21% in women, up from 19 and 17%, respectively, in the previous year [3–6]. Hypertension is often called a 'silent killer' due to its asymptomatic nature; unless their blood pressure is measured, most people will be unaware of their condition until they have a clinical complication such as myocardial infarction, stroke, or renal failure. Various approaches have been suggested to treat hypertension, including angiotensin-converting enzyme (ACE) inhibitors, angiotensin-II receptor blockers, diuretics, and calcium channel blocking agents [4–6]. However, the effectiveness and safety of these drugs and their side effects for long-term use have not been fully evaluated. As a result, extensive research and development are required to discover safer, creative, and cost-effective hypertension prevention and treatment therapies.

In our present study, we aim to determine the protective role of polyherbal formulation (Stress-WIN) on the renal and cardiac function of hypertensive rats. Ayurveda employs natural components to eradicate the source of the ailment by restoring balance while also promoting a healthy lifestyle to avoid imbalance from recurring. The *Ayurvedic* literature *'Sarangdhara Samhita'* highlighted the concept of combining multiple herbs in a particular ratio to achieve greater therapeutic efficacy [7]. Many studies were conducted to evaluate the effect of polyherbal formulation on several diseases [8, 9]. Three well-known herbs used in *Ayurvedic* medicine are *Ashwagandha* (*Withania somnifera* (L.) Dunal), *Pushkarmoola* (*Inula racemosa* Hook. f.), and *Jatamansi* (*Nardostachya jatamansi* (D.Don) DC. [10–12]. Studies have shown that various parts of *Ashwagandha* exhibited protective effects by exerting its anti-stress, anti-ulcerative, and antidiabetic effects [13–16]. Moreover, it shows cardioprotective effects and positive impacts on the male and female reproductive systems [17–19]. *Pushkarmoola* has anti-inflammatory and diuretic properties and is also a potent agent against cardiac diseases like ischemic heart disease [20, 21]. *Jatamansi* has been shown to have anti-depressant, anti-fungal, anti-microbial, hepato-protective, and neuroprotective effects [15, 22]. In our previous work [23], we evaluated the toxicity of the polyherbal formulation (Stress-WIN) in Wistar rats and found that the *Ayurvedic* formulation is safe at a range from 500 to 2000 mg/kg body weight. Deoxycorticosterone (DOCA)-salt-mediated hypertensive model is a widely used model for displaying peripheral effects, ranging from renal to cardiovascular alterations [24]. It causes oxidative stress, inflammation, and fibrosis by activating transcription factors and pro-inflammatory cytokines [25]. Therefore, based on the above information, the current study was designed to characterize the protective effects of polyherbal formulation on DOCA-salt-induced hypertension in Wistar rats.

Table 1 Qualitative analysis of bioactive fraction isolated from column chromatography

Mobile phase for elution	Extract	Total fractions	Bioactive fractions	Test	Compounds
80:20 (chloroform: methanol)	Extract of ethyl acetate	22	17–19	Kellar Kiliani test	Glycosides
	Extract of ethyl acetate	22	21–24	Ferric chloride test	Salicylic acid
	Extract of chloroform	21	10–14	Mayer's test Wagner's test Dragendorff's reagent test	Alkaloids
60:40 (chloroform: methanol)	Extract of n-hexane	18	9–11	Salkowski test	STEROL

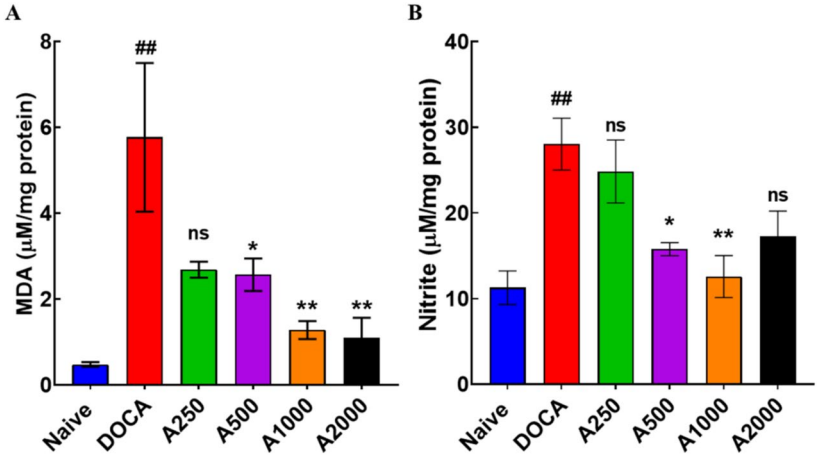

Fig. 1 Effect of PHF on DOCA-salt induced oxidative-nitrosative stress in heart tissues of rats. **A** MDA and **B** Nitrite: DOCA-salt administration significantly increased the MDA and nitrite levels in the heart of rats, which were significantly decreased with Stress-WIN A (500 and 1000 mg/kg) and Stress-WIN A (2000 mg/kg) treatment. Data were presented as mean ± SEM. (n = 3), ##p < 0.05 represents significance compared to the Naïve group. * p < 0.05 and ** p < 0.01 indicate statistical significance as compared to the DOCA rats

2 Results

Out of 61 fractions, 15 fractions showed a compound of interest given in Table 1.

2.1 Stress-WIN attenuates oxidative-nitrosative stress in heart and kidney tissues of DOCA-salt-induced hypertensive rats

Hypertension promotes oxidative stress, resulting in excess ROS and reactive nitrogen species (RNS). DOCA-salt-induced hypertensive rats exhibited increased oxidative stress. Rats treated with DOCA-salt showed significantly higher levels of nitric oxide ($p < 0.01$) and malonaldehyde (MDA) in their heart and kidney tissues compared to the healthy control group (Figs. 1 and 2). Different doses of Stress-WIN A (500 and 1000 mg/kg, 2000 mg/kg) significantly reduced the MDA and nitric oxide levels in the heart and kidneys of hypertensive animals as compared with the control group. However, the MDA and nitric oxide levels of the control group were not completely lowered. These findings indicate that Stress-WIN can have a therapeutic effect on reducing MDA and nitric oxide levels in DOCA-salt-induced hypertensive Wistar rats (Figs. 1 and 2).

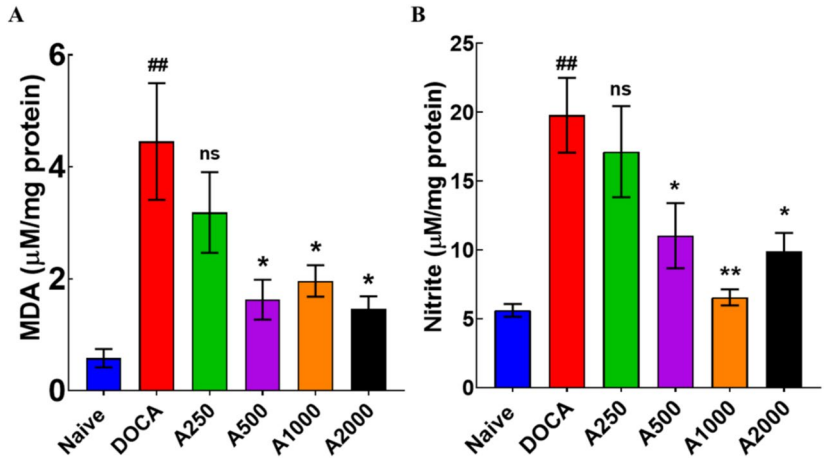

Fig. 2 Effect of PHF on DOCA-salt induced oxido-nitrosative stress in kidney tissues of rats (A) MDA and **B** Nitrite: DOCA-salt administration significantly increased the MDA and nitrite levels in kidneys of rats, which were significantly decreased with Stress-WIN A (500 and 1000 mg/kg *p.o.*) and Stress-WIN A (2000 mg/kg) treatment. Data were presented as mean ± SEM. (n = 3), ##p < 0.05 represents significance compared to the Naïve group. * $p < 0.05$ and ** $p < 0.01$ indicate statistical significance as compared to the DOCA rats

2.2 Stress-WIN decreased TNF-α and IL1β expression in the heart and kidney of hypertensive rats

Studies suggest that pro-inflammatory cytokines play a critical role in the development and progression of hypertension. This study found a significant increase in the protein expressions of TNF-α ($p < 0.001$) and IL-1β ($p < 0.001$) in the heart tissues of hypertensive Wistar rats. Stress-WIN A treatment leads to a significant decrease in protein expression of TNF-α (500 and 1000 mg/kg,$p < 0.001$) and IL-1β (500 mg/kg, $p < 0.05$ and 1000 mg/kg, $p < 0.01$) in the heart tissues of hypertensive Wistar rats. Simultaneously, there was a significant increase in the protein expressions of TNF-α ($p < 0.001$) and IL-1β ($p < 0.01$) in kidney tissues of DOCA-salt-treated Wistar rats. Interestingly, we also observed a significant reduction in the TNF-α (1000 mg/kg, $p < 0.01$) and IL-1β (1000 mg/kg, $p < 0.05$) protein expression in kidney tissues of Stress-WIN-treated hypertensive Wistar rats as compared to vehicle-treated rats. Moreover, treatment with Stress-WIN A with the dose of 2000 mg/kg BW also leads to significantly down regulation in the expression of TNF-α in the heart ($p < 0.001$) and kidney ($p < 0.05$) as well as IL-1β in the kidney ($p < 0.01$) tissues of DOCA-salt induced hypertensive of Wistar rat (Fig. 3).

2.3 Stress-WIN reduces DOCA-salt-mediated morphological changes in the kidney and heart in hypertensive rats

Histopathological examination of kidney tissues in the control group revealed normal glomerulus and tubule structure, with little or no cell infiltration, whereas DOCA-salt-treated animals showed tubule atrophy, necrosis, and glomerular congestion. Stress-WIN A, with a dose of 500 mg/kg and 1000 mg/kg, decreased the DOCA-salt-induced changes in the kidney. A low dose of Stress-WIN A (250 mg/kg) didn't reverse the histological structure in the kidneys of hypertensive Wistar rats. Similarly, the infiltration of cells of the DOCA-salt group was significantly higher than that of the control group in the ventricular wall of the heart (Fig. 4). Histological analysis showed that infiltration was reversed by Stress-WIN A treatment (500 mg/kg and 1000 mg/kg). Furthermore,

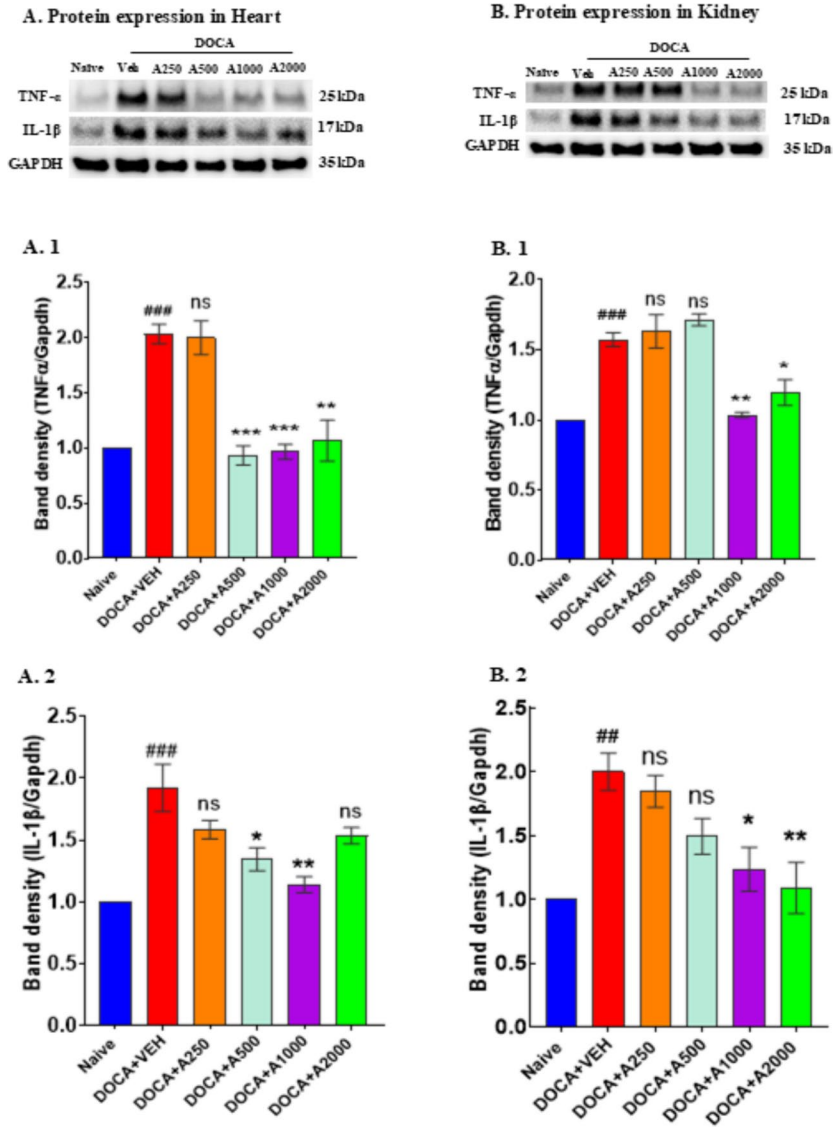

Fig. 3 Effect of PHF on DOCA-salt induced inflammation in heart and kidney of rats (**A**) TNF-α & IL-1β blots (heart): Representative blots of TNF-α & IL-1β protein expressions in the heart of rats. **A1** TNF-α protein expression (heart): DOCA-salt administration significantly increased the protein expression of TNF-α in the heart of rats, which was attenuated by the treatment with Stress-WIN A (500 and 1000 mg/kg) and Stress-WIN A (2000 mg/kg). **A2** IL-1β protein expression (heart): DOCA-salt administration significantly increased the protein expression of IL-1β in the heart of rats, which was attenuated by the treatment with Stress-WIN A (500 and 1000 mg/kg *p.o.*). **B** TNF-α & IL-1β blots (kidney): Representative blots of TNF-α & IL-1β protein expressions in the kidney of rats. **B1** TNF-α protein expression (kidney), **B2** IL-1β protein expression (kidney): DOCA-salt administration significantly increased the protein expressions of TNF-α & IL-1β in the kidneys of rats, which were attenuated by the treatment with Stress-WIN A (1000 mg/kg) and Stress-WIN A (2000 mg/kg). Data were presented as mean ± SEM. (n = 3), ##$p < 0.05$, ###$p < 0.001$ represents significance compared to Naïve group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ indicate statistical significance as compared to the DOCA rats

Stress-WIN A with a dose of 2000 mg/kg administration attenuated DOCA-salt-mediated morphological changes in the kidney and the heart (Fig. 5).

2.4 Effect of Stress-WIN on metabolic parameters of hypertensive Wistar rats

This study found that DOCA-salt-induced hypertensive rats showed a similar pattern of SGPT and SGOT compared to the control group. Stress-WIN A at lower dose (250 mg/

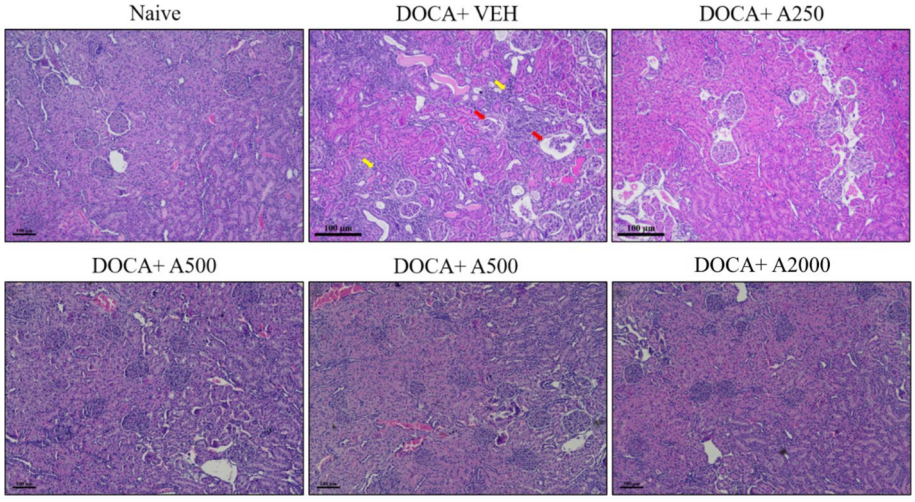

Fig. 4 Protective effect of PHF on DOCA-salt mediated changes in the kidney of hypertensive rats. DOCA-salt induced damage in the kidneys of hypertensive rats was reversed by Stress-WIN A (500 mg/kg, 1000 mg/kg) and Stress-WIN A (2000 mg/kg) treatment. Red arrows represent degeneration of the glomerulus, and yellow arrows indicate infiltration of cells

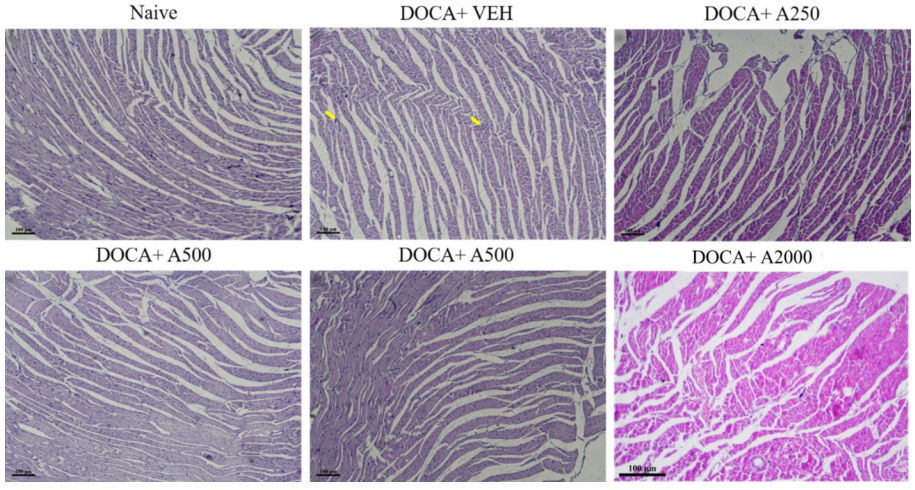

Fig. 5 Protective effect of PHF on DOCA-salt mediated changes in the kidney of hypertensive rats. DOCA-salt induced infiltration of cells in the hearts of hypertensive rats was reduced by Stress-WIN A (500 mg/kg, 1000 mg/kg) and Stress-WIN A (2000 mg/kg) treatment. Yellow arrows represent the infiltration of cells

kg) reduced significantly ($p < 0.05$) the level of SGPT, but surprisingly, increased in Stress-WIN A (500 mg/kg, 1000 mg/kg and 2000 mg/kg treated groups (Fig. 6). SGOT levels were significantly increased ($p < 0.05$) in Stress-WIN A (500 mg/kg, 1000 mg/kg and 2000 mg/kg) treated groups (Fig. 6). Blood glucose and urea levels, both were increased in hypertensive animals compared to control ones, while blood glucose levels in Stress-WIN A (250 mg/kg, 500 mg/kg) groups were declined. Higher doses of Stress-WIN A (1000 mg/kg, 2000 mg/kg) groups showed non-significant changes in blood glucose levels. All the groups of Stress-WIN A (250 mg/kg, 500 mg/kg, 1000 mg/kg, 2000 mg/kg) exhibited non-significant changes compared to the control group (Fig. 7). HDL and triglyceride levels in blood in all the groups had non-significant changes compared to the control group, while cholesterol level was significantly decreased ($p < 0.05$)

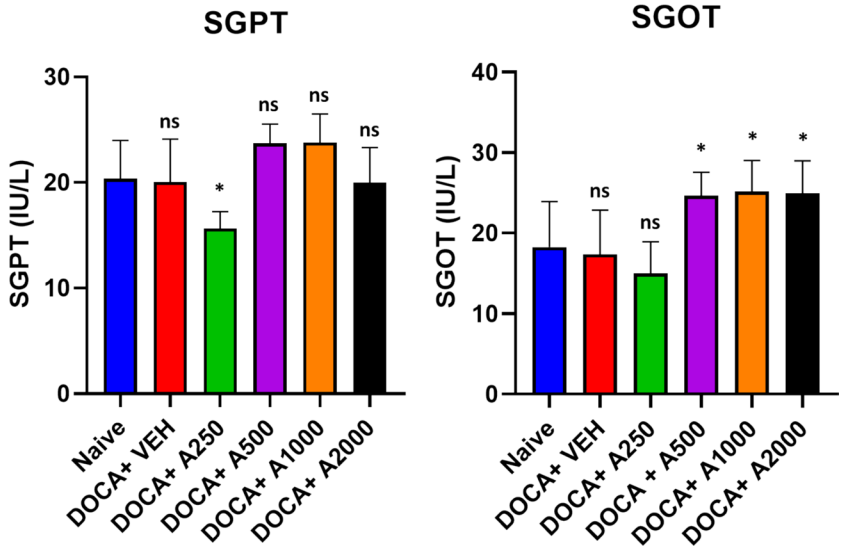

Fig. 6 Effect of PHF on SGPT and SGOT levels of DOCA-salt induced hypertensive rats. Data were presented as mean ± SEM, *$p < 0.05$ represents significance compared to the Naïve group

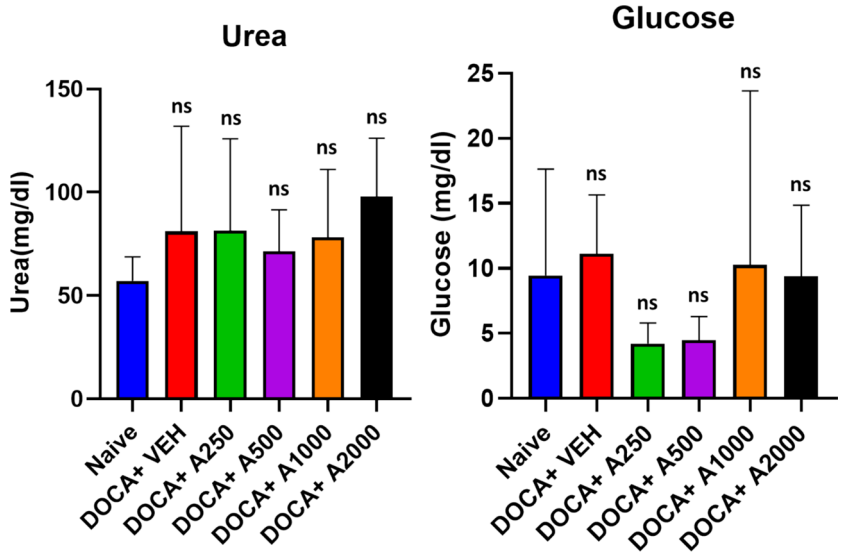

Fig. 7 Effect of PHF on blood glucose and urea levels of DOCA-salt induced hypertensive rats. Data were presented as mean ± SEM. *$p < 0.05$ represents significance compared to the Naïve group

in the Stress-WIN A (1000 mg/kg and 2000 mg/kg) treated group compared to the control group (Fig. 8).

2.5 Metabolite profiling of Stress-WIN polyherbal formulation by GC–MS and NMR

In the GC–MS chromatogram, the methanolic extract of polyherbal formulation (Stress-WIN) showed various peaks that were recognized by comparing their mass spectra along with their analogues reported in the NIST library. Two components, Salicylic acid (282), and Piperine (285) were detected in polyherbal formulation (Stress-WIN) which was confirmed by GC–MS and Co-TLC with the reference available (Fig. 9). The fraction number 17 to 19 of hexane extract show characteristic of β-sitosterol were confirmed by NMR spectroscopic analysis by given data. β-sitosterol: White needles; 1H NMR

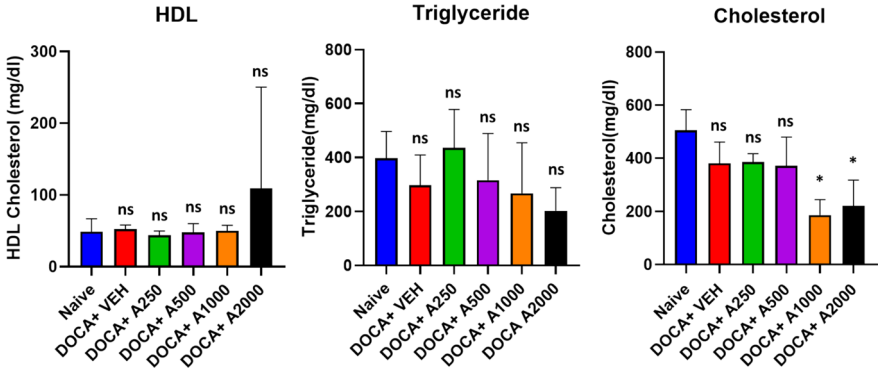

Fig. 8 Effect of PHF on HDL, triglyceride, and cholesterol levels of DOCA-salt induced hypertensive rats. Data were presented as mean ± SEM. *$p < 0.05$ represents significance compared to the Naïve group

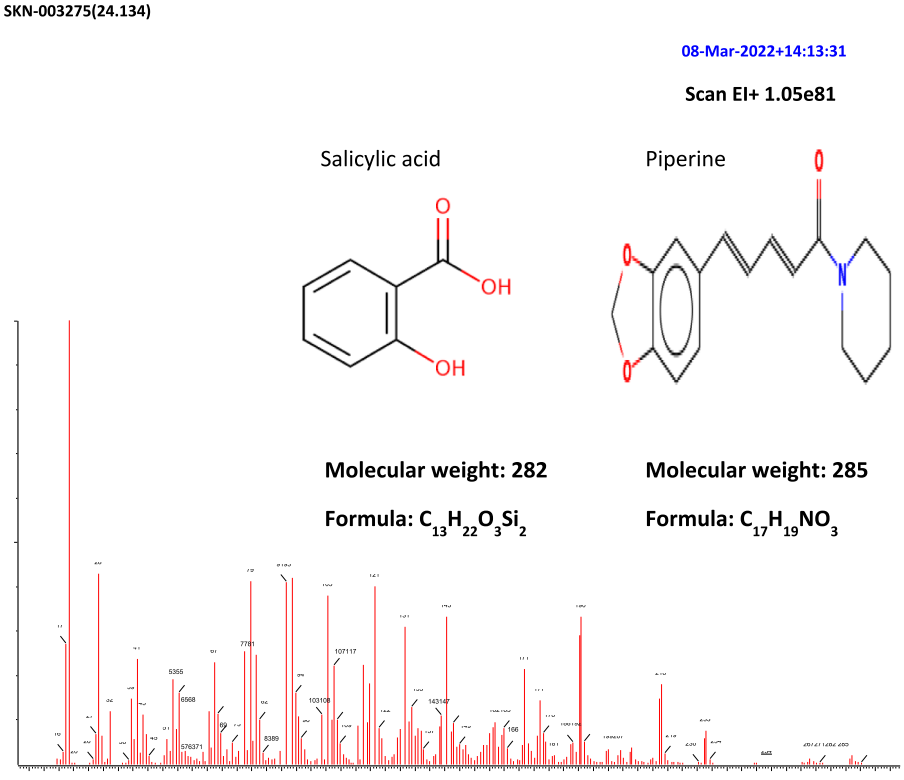

Fig. 9 GC–MS chromatogram of PHF Stress-WIN. Data were presented, Stress-WIN extract showing the peak intensities of various phytoconstituents with Major bioactive compounds

(500 MHz, CDCl3): δ 5.39–5.31 (1 H, m), δ 3.52 (1 H, ddd), δ 2.42–2.13 (2 H, m), δ 1.98 (2 H, tdd), δ 1.89–1.77 (3 H, m), δ 1.74–1.38 (11 H, m), δ 1.33–1.22 (5 H, m), δ 1.21–1.03 (6 H, m), δ 1.03–0.96 (4 H, m), δ 0.93 (4 H, t), δ 0.87–0.74 (8 H, m), δ 0.75–0.64 (3 H, m); 13C NMR (125 MHz, CDCl3): δ 140.9, 121.9, 72.0, 56.9, 56.2, 50.3, 46.0, 42.5, 39.9, 37.4, 36.7, 36.3, 34.1, 32.1, 31.8, 29.9, 29.3, 28.4, 26.2, 24.5, 23.2, 21.2, 20.0, 19.5, 19.2, 18.9, 12.1, 12.0.

Here, 1H NMR showed peaks at δ 0.75–0.64 (methyl), δ 3.52 (1 H, 3 α-H) and δ 5.39–5.31 (1H,m, C5-H) (500 MHz, $CDCl_3$) while 13 C NMR showed characteristic peaks of β-sitosterol in above data when compared with NMR data of [26, 27] (Supplementary

data Fig. 1). The fraction number 12 to 14 of ethyl acetate extract show characteristic of Apigenin were confirmed by NMR spectroscopic analysis by given data.

1H NMR (500 MHz, DMSO) δ 12.96 (s, 1H), δ 10.49 (br, 2H), δ 7.99–7.87 (m, 2H), δ 6.96–6.91 (m, 2H), δ 6.78 (s, 1H), δ 6.50 (dd, 1H), δ 6.20 (t, 1H); 13C NMR (125 MHz, DMSO) δ 182.2, 164.6, 164.2, 161.9, 161.6, 157.8, 128.9, 121.6, 116.4, 104.2, 103.3, 99.3, 94.4.

Here, 1H NMR showed peaks at 7.99–7.87 and 6.96–6.9 (6 H, ring system), 6.50 (dd, 1H, meta position on other ring), and 6.20 (t, 1H) (500 MHz, $CDCl_3$) while 13 C NMR showed characteristic peaks of Apigenin in above data when compared with NMR data of [27, 28] (Supplementary data Fig. 2).

2.6 Gene enrichment analysis

The number of common target genes with hypertension for salicylic acid, apigenin, β-sitosterol, and piperine was 104, 48, 38, and 22, respectively (Supplementary data Table 1). Results of GO analysis performed by the ShinyGO web server for all these compounds are presented in terms of molecular function, biological activity, and cellular component of different target genes. It also gives the enrichment analysis in the form of KEGG pathways in which given genes participate (Fig. 10). Supplementary data Tables 2, 3, Table 4, and Table 5 show the results of enrichment analysis for target genes of four different compounds, i.e. salicylic acid, apigenin, β-sitosterol and piperine respectively. These results give an idea about the potential role these compounds can play in different pathological conditions, and especially for hypertension, inflammation, and related conditions. Similarly, other data provide detailed insight into potential cellular, molecular, and biological targets of salicylic acid, apigenin, β-sitosterol, and piperine compounds are given in Figs. 10, 11, 12, and 13, respectively. These results demonstrate and support the data obtained by different assays that have been performed for this study. Although these compounds can actively participate in other activities, the anti-hypertensive and anti-inflammatory response is non-negligible.

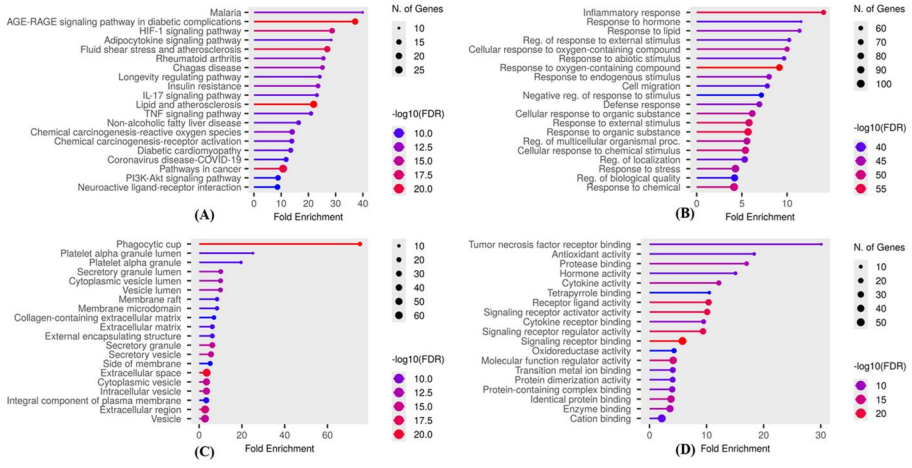

Fig. 10 Gene ontology analysis of Salicylic acid target genes by ShinyGO. Data were represented **A** Summary of top 20 KEGG pathways related to target genes, **B** Involvement of target genes in different biological activities, **C** Involvement of target genes in different cellular components, and **D** Molecular functions

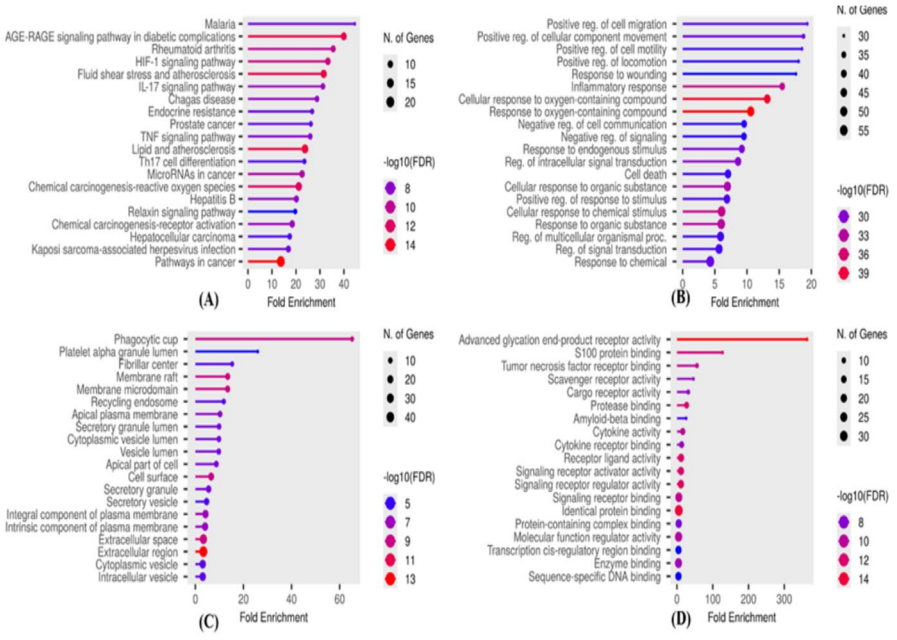

Fig. 11 Gene ontology analysis of Apigenin target genes by ShinyGO. Data were represented **A** Summary of top 20 KEGG pathways related to target genes, **B** Involvement of target genes in different biological activities, **C** Involvement of target genes in different cellular components, and **D** Molecular functions

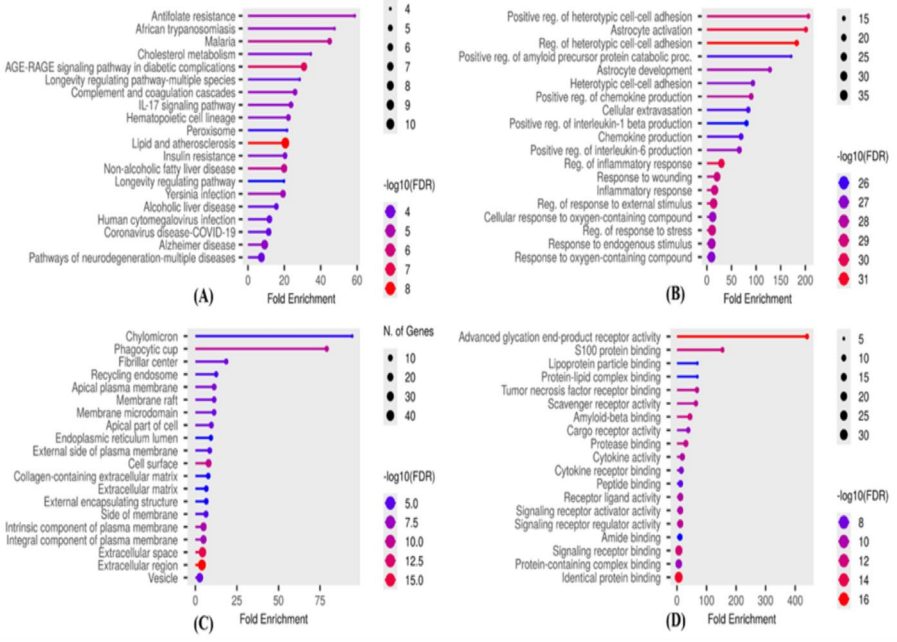

Fig. 12 Gene ontology analysis of β-sitosterol target genes by ShinyGO. Data were represented (A) Summary of top 20 KEGG pathways related to target genes, **B** Involvement of target genes in different biological activities, **C** Involvement of target genes in different cellular components, and **D** Molecular functions

3 Discussion

The present study aimed to investigate the antihypertensive property of a folklore herbal formulation (Stress-WIN) in a deoxycorticosterone acetate (DOCA) salt-induced hypertensive animal model. The treatment of DOCA salt was administered at a dose

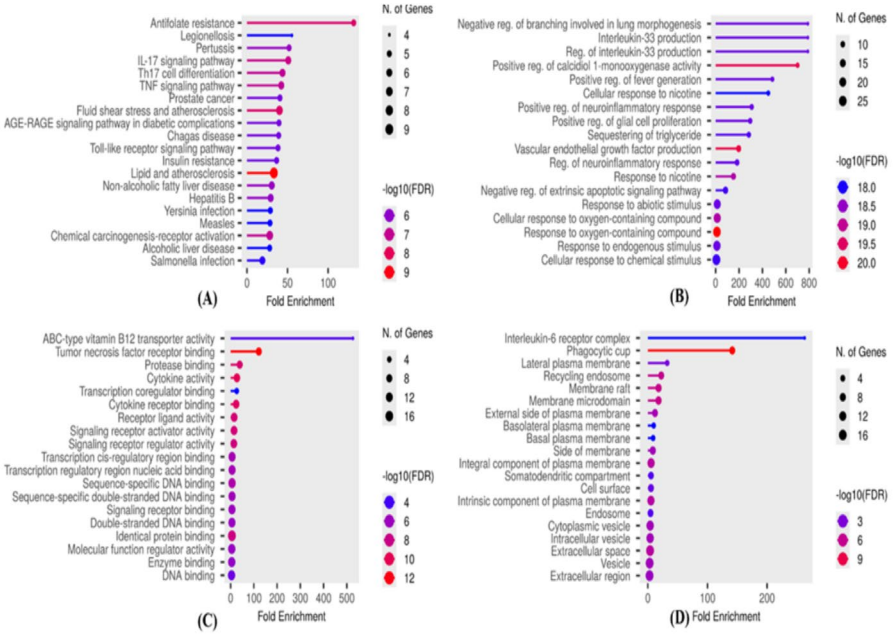

Fig. 13 Gene ontology analysis of piperine target genes by ShinyGO. Data were represented (A) Summary of top 20 KEGG pathways related to target genes, **B** Involvement of target genes in different biological activities, **C** Involvement of target genes in different cellular components, and **D** Molecular functions

of 20 mg/kg body weight dissolved with olive oil twice a week for five weeks to induce hypertension. DOCA salt treatment was able to induce an inflammatory response as well as increased oxidative and nitrosative stress. It was observed that MDA (μM/mg) and nitrite (μM/mg) levels were significantly elevated in the DOCA salt-treated groups. DOCA-salt treatment in the Wister rats exhibited morphological changes in the heart and kidney tissues. It was seen that the infiltration of immune cells in the heart and kidney tissues was reversed by the oral supplementation of Stress-WIN. Moreover, the treatment of Stress-WIN reversed the pathological changes in renal tissue, such as tubule atrophy, necrosis, and glomerular congestion, which were thought to be the effects of elevated levels of reactive oxygen species as well as increased expression of proinflammatory cytokines.

DOCA salt treatment results in activation of the brain Renin Angiotensin System (RAS) and induces the angiotensin II type I receptor (AT1R) [29]. Angiotensin II is a potent vasoconstrictor and plays a pivotal role in regulating blood pressure [30]. Further, in DOCA salt-mediated hypertension, the expressions of proinflammatory cytokines are elevated. In the present study, we observed that Stress-WIN supplementation reduced the expression of proinflammatory cytokines in the heart and kidney tissues, where we found the reduced expression of IL-1β and TNF-α translationally as well as transcriptionally. This may suggest that the administration of Stress-WIN might suppress the expression of angiotensin I receptor (ATIR) of immune cells (T-lymphocytes, dendritic cells, and macrophages). Therefore, angiotensin II may not bind sufficiently to the ATIR of Immune cells. Thus, immune cells may not overproduce the pro-inflammatory cytokines. Further, the reduced level of SGOT and SGPT indicated that angiotensin II did not contribute to hypertension-mediated organ damage.

Immune cells (T-lymphocytes, dendritic cells, macrophages, etc.) collaborate to produce an inflammatory response, with increased production of pro-inflammatory

cytokines, ROS, and adhesion molecules [31–34]. It induces inflammatory-mediated hypertension through different signalling pathways. In the present study, it was found that pro-inflammatory cytokines and ROS production were reduced by the treatment with Stress-WIN. A recent study indicated that the interleukin pathway appears to play a significant role in atherosclerosis, with IL-1α and/or β boosting the production of VCAM-I, ICAM-I, and E-selectin [35], with increased endothelial cell permeability and adhesion molecule expression. High doses of IL-1 and TNF-α can cause endothelial-mediated vasoconstriction. IL-1β increases hypertensive inflammation by releasing IL-6 through STAT signalling via the gp130 subunit and a particular isoform. This enhances NADPH oxidase and eNOS, resulting in lower nitric oxide levels and increased vascular superoxide, leading to endothelial dysfunction [36, 37]. This study shows that Stress-WIN significantly decreases the nitric oxide level, which helps to reduce the inflammation via the STAT signal pathway. Angiotensin II, IL-1β, TNF-α, and tissue hypoxia/ischemia promote IL-6 secretion and ROS generation, leading to hypertension, inflammation, stiffness, and endothelial dysfunction. Furthermore, it promotes artery wall collagen synthesis, inhibits its breakdown, and stimulates fibrinogen production, resulting in elevated blood pressure. The polyherbal medication (Stress-WIN) similarly decreased the level of reactive oxygen species in the heart and kidney tissues. These data demonstrated a considerable decrease in pro-inflammatory cytokines (IL-1β and TNF-α), which indirectly reduces the production of IL-6 and helps to maintain normal blood pressure and ameliorates DOCA-salt mediated hypertension.

Further, we performed gene enrichment analysis and metabolite profiling of our polyherbal formulation. We found four anti-hypertensive molecules that might play a key role in regulating blood pressure by promoting vasodilation, reducing levels of proinflammatory cytokines, and decreasing oxidative stress. Apigenin, a flavonoid, is reported to lower the expression of TGF-β1/Smad 2/3 signalling pathway. Apigenin exerts its protective role against hypertension via the transient receptor potential vanilloid 4 (TRPV4) mediated activation of AMPK/SIRT1 pathway, ultimately inhibiting the TGF-β1/Smad 2/3 signalling pathway [38]. Similarly, β-sitosterol ameliorates hypertension-induced renal collagen deposition, glomerular matrix expansion, and infiltration of inflammatory immune cells [39]. Additionally, salicylic acid and piperine are also reported to reduce hypertension. It was seen that salicylic acid helps in lowering blood pressure by increasing the production of r-cortexin in the kidneys [40]. Also, oral administration of piperine showed a partial prevention of increased blood pressure induced by chronic L-NAME administration [41]. All these molecules showed anti-hypertensive effects via different pathways. Therefore, it can be suggested that the anti-hypertensive effects observed by the supplementation of Stress-WIN in our experiment might be the synergistic result of these molecules.

4 Conclusion

The results of the above experiments provide consistent evidence that Stress WIN can reverse the state of hypertension by lowering serum inflammatory cytokines and nitric oxide levels. Apart from their lowering effect on serum cholesterol, it also reduces the SGOT and SGPT, which play a major role in making a proper balance between ROS production and degradation that is involved in the hypertension mechanism. The effects of polyherbal drug (Stress-WIN) in hypertension are not limited to lowering blood

pressure but also protect the heart and kidney by reducing the level of malondialdehyde (MDA) and nitric oxide. Future prospective clinical trials are needed to determine the effect of Stress WIN targeting particular IL on blood pressure, end-organ damage, and systemic inflammation in hypertension patients.

5 Methods

5.1 Plant material and preparation of Stress-WIN formulation (SOP)

All the root parts of plants were collected, powdered, and dried for the preparation of polyherbal formulation as described in Table 1 of our previous work [23]. The poly herbal formulation was prepared by taking specific doses from the roots of all plants, and the preparation was divided into the following two phases:

- Phase I: Formation of fine root powder (20 mesh size) of *Withania somnifera* L. Dunal and *Inula racemosa* Hook. F.
- Phase II: Mixing and trituration with the hot infusion of coarse root powder of *Nardostachys jatamansi* (D. Don) DC. Into fine root powder (20 mesh size) of *Ashwagandha* and *Pushkarmoola*.

The polyherbal Ayurvedic formulation (Stress WIN) was prepared from an equal part of Ashwagandha (Withania somnifera L. Dunal), Pushkarmoola (Inula racemosa Hook.F.), root powder, and hot infusion of Jatamansi (Nardostachys jatamansi (D. Don) DC.) root. The formulation was made by trituration of the powder of Ashwagandha and Pushkarmoola with hot infusion of Jatamansi and then dried. Here, for isolation of bioactive compounds fractionation method has been used with four different solvents (n-hexane, acetone, chloroform, and ethyl acetate) represented by SwH, SwA, SwC, and SwE, respectively. In this method, four different solvents (n-hexane, acetone, chloroform, and ethyl acetate) were used for the isolation of bioactive compounds from a polyherbal formulation. The fractionation of bioactive compounds was initiated by moistening or complete dissolution of the polyherbal formulation with 200 mL of water. Now it was followed by transfer into a separating funnel, shaken, and allowed to settle. Furthermore, 250 ml of n-hexane, the most polar solvent among them, was added and shaken allowed to settle. A tap of the separating funnel is opened to remove the aqueous layer, and the remaining content is pooled to get the n-hexane extract. An equal volume of n-hexane was added again, shaken, allowed to settle, and separated. This process continued three times. A similar cycle was performed for acetone, chloroform, and ethyl acetate to get acetone, chloroform, and ethyl acetate extracts. The ethyl acetate, acetone, chloroform, and n-hexane extracts were dried under a rotatory evaporator and reloaded on a Sephadex LH-20 column (3 cm diameter) with dissolve in 80% methanol and 60% n-hexane separately. A total of 22 fractions were obtained from the extract of ethyl acetate by elution with 80:20 (chloroform: methanol) as mobile phase, in which fractions 17 to 19 showed a positive result for glycoside, and fractions 21 to 24 showed a positive result for salicylic acid by ferric chloride test. A total of 21 fractions were obtained from the extract of chloroform, in which fractions 10 to 14 showed positive results for alkaloids. Here, 18 fractions were obtained from the extract of n-hexane by elution with 60:40 (chloroform: methanol) as mobile phase, in which fractions 9 to 11 showed a positive result for steroid test (Table 1).

5.2 Experimental animals

Adult male Wistar rats weighing between 200 and 250 g were obtained from the Central Animal House of the Institute of Medical Science at Banaras Hindu University in Varanasi. All the rats were housed under controlled humidity and temperature (21 ± 2 °C) with alternate light and dark cycles for 12 h. Food and water were provided ad libitum, and the rats were randomly divided into different experimental groups (n = 5–6/group). The Committee for Control and Supervision of Experiments on Animals (CPCSEA), Government of India, New Delhi, carried out all animal-based experiments. The Institute Animal Ethics Committee at the Institute of Medical Sciences, Banaras Hindu University, Varanasi, Uttar Pradesh, India, authorized all of the experimental procedures (Dean/202I/IAEC/3040). In this research work Clinical trial number is not applicable.

5.3 Experimental design

Hypertension was induced by oral administration of deoxycorticosterone acetate (DOCA) salt at a dose of 20 mg/kg diluted with olive oil twice a week for five weeks. A solution containing 1% sodium chloride (NaCl) was used instead of drinking water. The animals were divided into six groups at random, with n = 6 Wistar rats in each group. Group 1 is a healthy control group, group 2 received DOCA-salt via oral route, and groups 3, 4, and 5 received polyherbal formulation A (Stress-WIN A) by oral gavage at doses of 250, 500, and 1000 mg/kg. Whereas group 6 animals were administered Stress-WIN A at the dose of 2000 mg/kg body weight. At the end of the treatment, blood samples were obtained, animals were euthanized, and the heart and kidney tissues were collected and preserved at − 80 °C for subsequent biochemical and molecular biology analysis, and fixed in 4% paraformaldehyde (PFA) for histological analysis.

5.4 Biochemical analysis

All biochemical analyses were carried out on rat heart and kidney tissues. After carefully isolating the tissue samples, they were washed with 0.9% normal saline and placed in a -80 °C deep freezer. To prevent protein breakdown, the samples were homogenized on ice using RIPA buffer (pH 7.4). Centrifuged tissue lysate and supernatant were separated at 12,000 rpm for 20 min at 4 °C. Finally, colorimetric tests were used to assess oxidative stress markers such as MDA, nitric oxide, and GSH.

5.5 Estimations of lipid peroxidation (LPO)

Malondialdehyde (MDA) is a byproduct of lipid peroxidation, and its levels were determined using the thiobarbituric acid reactive substance (TBARS) assay. Lipid peroxidation was evaluated by measuring the production of thiobarbituric acid reactive substance (TBARS), as described in Halliwell and Chirico et al., 1993, with minor adjustments [42]. This test is based on the formation of a pink colour when MDA forms a complex with TBA. Acetic acid and sodium dodecyl sulphate (SDS) were added to the supernatant together with thiobarbituric acid and heated at 100 °C for 1 h. Samples were allowed to cool before being placed in a 96-well plate, and absorbance was measured at 532 nm with a multimode microplate reader. MDA concentration is measured in micrograms per milligram of tissue protein.

5.6 Determination of reduced glutathione (GSH)

A large number of reactive oxygen species are produced inside a cell, causing extensive cell damage. GSH is an antioxidant enzyme that defends the cell against such harm. Determining decreased glutathione levels is an indirect indication of the cell's pathogenic status. The assay of reduced glutathione was estimated by the method described by Caito et al., 2015, with minor modifications [43]. The tissue lysate is combined with 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) and incubated at 48 °C until the colour turns yellow. Later, using a multimode microplate reader, the absorbance at 412 nm is measured. The concentration of GSH is measured in μM/mg protein in the tissue.

5.7 Determination of nitric oxide

Nitric oxide levels were assessed using the Griess reagent to indicate the degree of nitrosative stress present in a cell. This assay was done by the method described by Sun et al. [44]. Griess reagent is mixed with equal quantities of each sample and incubated at room temperature for 30 min. Finally, the microplate reader was used to detect absorbance at 540 nm and nitric oxide content in μM/mg of tissue.

5.8 Western blotting

Following isolation, the tissue samples were homogenized in RIPA buffer with an automated hand homogenizer and incubated on ice before being centrifuged at 16,000*g* at 4 °C to create a tissue lysate for protein quantification. Western blotting was done by the method described by Lv et al. 2020, with slight modification [45]. Now, 20 micrograms of protein were injected per lane onto 10% resolving and 5% stacking polyacrylamide gels. The protein-loaded gel was transferred to a 0.22-μm nitrocellulose membrane and blocked with 3% bovine serum albumin (BSA) for 2 h. The membranes were probed and treated with primary antibodies to TNFα (1:1000), IL-1β (1:1000), and Gapdh (1:80,000) at 4 °C overnight. They were then washed three times with TBST and incubated for 2 h at room temperature with the appropriate secondary antibodies. The target proteins were identified with an improved chemiluminescence substrate (Biorad, USA). The blots were visualized using ChemiDocTM (BioRad, Hercules, California, USA). Finally, the Image Lab 6.1 software was used to quantify the blots, and the data were normalized to the corresponding Gapdh blots.

5.8.1 Histopathological studies

Histopathological studies of Kidney and heart tissue were done by the method described by Regner et al. 2010 and Gobinath et al. 2022, with slight modifications of Kidney and heart tissue, respectively [46, 47]. Kidney and heart tissue from all experimental groups were immediately washed in 0.9% saline and fixed in 4% paraformaldehyde for 24 h at 4 °C. The tissues (kidney and heart) were fixed and then embedded in paraffin wax. The blocks were sectioned at 5 μm with a Leica RM2245 microtome machine. The pieces were then deparaffinized in xylene and hydrated with a graduated series of alcohols. The sections were then immersed in hematoxylin for 10 min, followed by 30 min of blueing in flowing tap water. Sections were then immersed in eosin for 2 min before being dehydrated with graded alcohol. Finally, the sections were cleaned with xylene and mounted in DPX. The histological slices were then inspected under a brightfield microscope (Nikon Eclipse E200), and photomicrographs were created with Nixon software.

5.8.2 GC–MS analysis

Clarus 680 PerkinElmer (Auto system XL) Gas chromatograph equipped and coupled to a mass detector SQ8T-PerkinElmer, 30 m × 0.25 mm × 0.25 mm of capillary column. A GC–MS analysis of Stress-WIN's hexane extract was carried out using this instrument. We required 70 eV ionization energy for GC–MS detection, which means an electron ionization system operated in electron impact mode with this much energy. The initial temperature necessary for this instrument is about 5000 °C and is maintained at this temperature for at least 6 min. Then the oven temperature was raised to 18,000 °C from 5000 °C, its simple meaning is that we raised the temperature at the rate of 10,000 °C/min and maintained it for 6 min. Lastly, the temperature was raised to 28,000 °C from 18,000 °C, and this was maintained for 8 min 1 ml/min rate of helium gas was used as a carrier gas, and 3 µl of Stress-WIN was injected using a split mode injection system. The injector temperature was maintained at 25,000 °C. Mass spectra were taken at 70 eV. The solvent delay was 0 to 3 min, and the total GC–MS running time was 40 min. Stress-WIN was injected in split mode. The mass spectral scan range was from thirty to five hundred twelve (30–512) m/z. Finally obtained chromatograms were compared with the NIST (National Institute of Standards and Technology) and Wiley, which provided fatty acids present in Stress-WIN.

5.9 NMR

NMR spectrum was acquired on a Bruker (500 MHz) NMR (Bruker, Billerica, MA, USA) at a constant temperature controlled and adjusted to room temperature, and chemical shifts are shown in δ values (ppm) with tetramethylsilane as an internal standard. 1H and 13C NMR spectra were recorded using methanol-d4 as the solvent.

5.10 Gene enrichment analysis

Four major compounds were isolated from the formulation, which are salicylic acid, β-sitosterol, apigenin, and piperine. All these compounds have been reported to have ameliorative effects on hypertension, inflammation, and other related conditions [48–54]. In this study, the antihypertensive and anti-inflammatory effect of the formulation can be mainly because to all these compounds. To support this hypothesis, gene enrichment analysis was performed. Gene enrichment analysis gives an idea about the interaction of a compound with gene targets [55]. Initially, gene targets for all the compounds and genes involved in hypertension were retrieved from the GeneCards database (https://www.genecards.org/, accessed on 16th November 2024) [56, 57]. Co mmon genes between both of these were identified using the Multiple List Comparator programs of the Molbiotools web server (https://molbiotools.com/listcompare.php, Accessed on 16 303 th November 2024). Then, using the Shiny GO 0.8 web server (http://bioinformatics.sdstate.edu/go/, accessed on 16th November 2024), these genes were analysed for gene ontology (GO) and KEGG pathways [58]. Gene ontology is a method of displaying comprehensive details on genes and their products in terms of molecular function, biological activity, and cellular component [59].

5.11 Statistical analysis

Statistical analysis was carried out using GraphPad Prism 8.0. All results were shown as Mean ± S.E.M. All data were compared using analysis of variance, followed by Tukey's multiple comparison test. Values were judged statistically significant at $p < 0.01$.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1007/s42452-025-07420-4.

Supplementary file 1

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Author contributions
SK and AR were responsible for designing the study and drafting the manuscript. SKJ, PP, and AY carried out the isolation and characterization of compounds. The animal experiments and cytokine assessments were conducted by VT and SA. AKS and AR performed the histological evaluations and statistical analyses. HHC contributed to the gene enrichment analysis.

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Availability of data and materials
Data from this study will be available upon reasonable request to the corresponding author.

Declarations

Ethics approval and consent to publication
The study protocol involving animals was approved by the Institutional Animal Ethics Committee (IAEC) of Institute of Medical Sciences, Banaras Hindu University, Varanasi, Uttar Pradesh, India, following the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India (Approval Reference No: (Dean/202I/IAEC/3040 on dated 22-11-21), authorized all of the experimental procedures. In vitro experiments were conducted in accordance with institutional laboratory biosafety guidelines.

Consent to participate
Not applicable. This study did not involve human participants.

Consent to publish
Not applicable. This study did not involve human participants or personal data requiring consent for publication.

Competing interests
The authors declare that they have no competing interests.

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