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Protective effects of *Artemisia herba-alba* in diabetic peripheral artery disease mediated via inhibition of advanced glycation end products

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Abstract

The rationale for undertaking this study is lethality of diabetes mellitus as predicted by 6.7 million deaths in 2021 and immense pharmacological potential of *Artemisa herba-alba*. The current research examined how *Artemisia herba-alba* extract (AHE) affects the peripheral artery disease in diabetic rats through lowering of advanced glycation end products (AGEs). The *in-vitro* AGE inhibiting potential of AHE was determined by spectrofluorimetric method. The blood glucose levels and HbA1c (A1C) of the rats from each group were determined by automatic analyser. The levels of AGEs in vascular smooth muscle cells (VSMCs) of different rat groups were observed through western blotting. Expression of COX-1 and COX2 were determined by qRT-PCR. The AHE inhibition of AGEs formation was reported *in vitro* and exhibited an IC_{50} of 45 µg/mL which was significantly lower than that of standard AGEs inhibitor aminoguanidine (IC_{50}: 60 µg/mL). Analysis of metabolic profiles revealed that AHE normalised the blood glucose, cholesterol, and triglycerides with no apparent changes on Hb1Ac levels. Western blot analysis showed that AHE exhibited protective effects in VSMCs of diabetic rats by inhibiting fabrication of AGEs. Moreover, the manifestation of COX-2 was inhibited by AHE in diabetic rats. However, the expression of COX-1 remained unaltered. Collectively, the results revealed AHE inhibits AGEs formation *in vitro* and in VSMCs of diabetic rats. These findings point towards the prospective of AHE applications towards the management of diabetic peripheral artery disease.

Keywords: *Artemisia herba-alba*; glycation; diabetic peripheral artery disease; blood glucose.

1. Introduction

Diabetes mellitus (DM) has been linked to the advancement of peripheral artery disease (PAD) (Goldin *et al.,* 2006). Diabetic patients are at a greater risk for having or developing PAD in a due course of time. Although the ethology of PAD is poorly understood, the involvement of a wide array of inflammatory mediators including advanced glycation end products (AGEs) are main players in PAD advancement (Lutgers *et al.,* 2006). Currently employed treatment strategies generally involve change in lifestyle and certain drug targeting peculiar PAD/DM. PAD is also accompanied by severe oxidative stress which ultimately triggers inflammation (Kralev *et al.,* 2009).

Natural products have served as a repository of chemical scaffolds which have the potential to cure human diseases (Rates, 2001). Several lifesaving drugs owe their origin from plants and microbes (Shakya, 2016). The benefit of using natural products as drugs lies in the fact that they are much safer and with negligible toxicity as compared to their synthetic drugs (Zhao *et al.,* 2020). Moreover, around 80% of the of the world's population also uses natural products as their primary source of healthcare. Therefore, natural sources such as plants find enormous use in pharmacology (Veeresham, 2012).

Artemisia herba-alba is an important medicinal herb commonly known as desert wormwood (Moufid *et al.,* 2012). It has been used in the management of several ailments such as hypertension and diabetes (Abou El Hamd *et al.,* 2010). Studies have also reported its analgesic and haemostatic properties. *Artemisia herba-alba* is a rich source of secondary metabolites which mainly include sesquiterpene lactones such as eudesmanolides and germacranolides which are reason behind its medicinal properties (Ayad *et al.,* 2022). Given the pharmacological importance and the anti-diabetic efficacies associated with *Artemisia herba-alba,* the purpose of the current study was to assess the effects of ethanolic extract of *Artemisia herba-alba* (AHE) in murine model of diabetic peripheral artery disease. Herein we for the first-time report that AHE exerts its effects via inhibition of advanced glycation end products. Moreover, we suggest that AHE may prove beneficial to diabetic patients in overcoming the advancement of peripheral artery disease.

2. Materials and Methods

2.2 Plant material and extraction

The aerial parts of *Artemisia herba-alba* were harvested from natural habitat. The desiccated aerial parts were crushed to powder in a grinder and subsequently extracted with denatured ethanol (50%) at room temperature for 48 h. Filtration of the crude extract was performed followed by concentrating under reduced pressure by evaporation of excess solvent. Finally, the mixture was lyophilized to acquire a pure ethanolic extract from AHE above ground parts. Different AHE were made by dissolving it within dimethyl sulphoxide (DMSO).

2.2 *In vitro* AGE assay

For AGE assay, a reaction mixture consisting of 10 mg/L bovine serum albumin in phosphate buffer (50 mM; pH 7.4 with sodium azide), fructose and glucose (100 mL; 0.2 M) was placed in 1.5 mL screw cap tubes and different concentrations of AEH and aminoguanidine (AGE inhibitor) were added at 37 °C for 2 weeks. Thereafter, 200 mL of each reaction product were assayed by spectrofluorimetric detector IC50 values were calculated as described previously (Miwa *et al.,* 1996).

2.3 Animals and diabetes

Animals were procured from Central Hospital Affiliated to Shandong First Medical University and the study was approved by animal research ethics committee of the institute. Induction of diabetes was carried by injection of streptozotocin (STZ, 60 mg/kg, ip) in 6 week-old rats. Control rats of same age received each amount of citrate buffer (0.01 M, pH 4.5). After 2 days, levels of blood glucose in tail vein were determined. Diabetic rats were defined as those with blood sugar levels over 300 mg/dl. To examine the effects of AHE, treatment was started 1-week post-onset of diabetes. AHE extract was given orally to rats once daily for twelve weeks. Four groups of rats were tested: (i) normal rats (N) receiving vehicle only (ii) normal rats receiving vehicle plus 100 mg/kg of AEH (N + AEH) (iii) diabetic rates (DM) (iv) diabetic rats receiving 100 mg/kg of AEH (DM + AEH) and (v) positive control group of diabetic rats receiving 100 mg/kg of aminoguanidine, AGEs inhibitor (DM + AG) with n = 9 for each group. Isolation of vascular smooth muscle cells (VSMCs) was executed as described in our previous study (14).

2.4 Metabolic analysis

After fasting for 16 hours, blood samples were taken from the rats' tails and analysed for glucose and HbA1c (A1C) levels using an automated analyzer.

2.5 Expression analysis

Extraction of the total RNA from the rat VSMCs was carried with the help of RNAiso Plus (TaKaRa Biotechnology). Subsequently, the RNA reverse transcribed to cDNA using PrimeScript™ RT Reagent kit (TaKaRa Biotechnology) using protocol of manufacturer. SYBR™ Premix Ex Taq kit (TaKaRa Biotechnology) was used to carry out the RT-PCR following the cycling conditions; 95°C for 30 s, 95°C for 10 s and 57°C for 35 s, for 38 cycles, and 95°C for 20 s, 60°C for 1 min, and 95°C for 20 s. The $2^{-\Delta\Delta CT}$ technique was utilized to assess relative expression and GADPH was considered to be reference.

2.6 Western blot analysis

Rat proteins were isolated from PBMCs using RIPA buffer 1% protease inhibitor cocktail and subsequently BCA assay was used to determine the protein concentration. Proteins of same mass (40 g) were separated by electrophoresis on a 12% SDS-polyacrylamide gel and then loaded over nitrocellulose membranes. Next the membranes were subjected to incubation in rabbit anti-AGE antibody followed by visualization in an enhanced chemiluminescence detection system.

2.7 Statistical analysis

Data is presented as mean ± standard deviation (SD). The data was analyzed by one-way ANOVA followed by turkey's test or by student's t-test and $P < 0.05$ was used to indicate the statistically significant difference. GraphPad prim 7 was used to perform the tests.

3. Results

3.1 AHE inhibits AGE *in vitro*

The effects of different concentrations of AHE (0 to 640 µg/mL) were examined on AGE. It was revealed that AHE inhibited AGE generation following concentration-reliant manner. The IC_{50} of AHE was found to 45 µg/mL which was comparatively lower than that of AGEs inhibitor aminoguanidine which exhibited an IC_{50} of 60 µg/mL (**Table 1**).

Table 1. Effect of AHE on AGE formation expressed as IC_{50}

	AGEs formation (IC_{50})
AHE	45 ± 2.5 µg/mL
Aminoguanidine	60 ± 2.8 µg/mL

3.2 AHE normalizes blood glucose, cholesterol, and triglyceride levels in blood

The analysis of all fasting blood glucose levels revealed that in DM group the glucose levels in blood were significantly elevated as compared to the normal rats. After 12-week treatment with AHE the levels were considerably decreased suggestive of the protective effects of AHE (**Figure 1**). The effects of AHE were also evaluated on the levels of total Hb1Ac, cholesterol and triglycerides. Whereas diabetic rats showed considerably higher levels of cholesterol and triglycerides. However, no apparent changes were observed on the levels of Hb1Ac (**Table 2**).

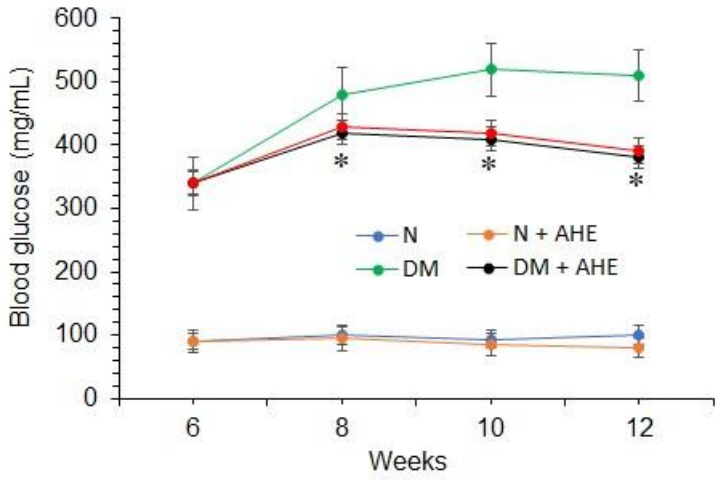

Fig. 1. Blood glucose levels in different rat groups post AHE treatment, (N, DM, N + AHE, and DM + AHE); the results how AHE normalize blood glucose levels in diabetic rats (data represents ± SD (*P < 0.05))

Table 2. AHE effectivities on total cholesterol, HbA1c, and triglycerides in different groups of rats (*P < 0.05 for DM Vs DM + AHE and DM Vs DM + AG)

	N (n=9)	N+AHE (n=9)	DM (n=9)	DM+AHE (n=9)	DM+AG (n=9)
HbA1c (%)	3.72 ± 0.06	3.63 ± 0.04	6.64 ± 0.18	6.62 ± 0.22	6.66 ± 0.16
Total cholesterol (md/dl)	63.2 ± 2.8	59.1 ± 3.1	90.4 ± 5.3	48.14 ± 1.8*	46.3 ± 1.2*
Triglycerides (md/dl)	117.1 ± 10.6	68.4 ± 2.2	470.8 ± 14.3	131.6 ± 16.5*	97.7 ± 18.8*

3.3 AHE inhibits AGEs in VSMCs of rats

The effects of AHE on the AGEs production was examined by SDS-PAGE. The results showed that levels of AGES were considerably elevated in VMCs of diabetic rats than in normal rats. However, the AGES levels of rats were considerably lower in case of diabetic rats administrated with AHE and were compared to those of diabetic rates treated with aminoguanidine (**Figure 2**).

3.4 Effect of AHE on the expression of COX (-1 and -2) expression

As AGEs induce inflammation, we looked at how AHE affected the levels of two cyclooxygenases, COX-1 and -2. The COX-2 expressions were observed to be elevated in diabetic rats. In contrast, AHE exposure significantly reduced COX-2 expression (**Figure 3A**). Nonetheless, no changes significant changes were observed on the expression of COX-1 (**Figure 3B**).

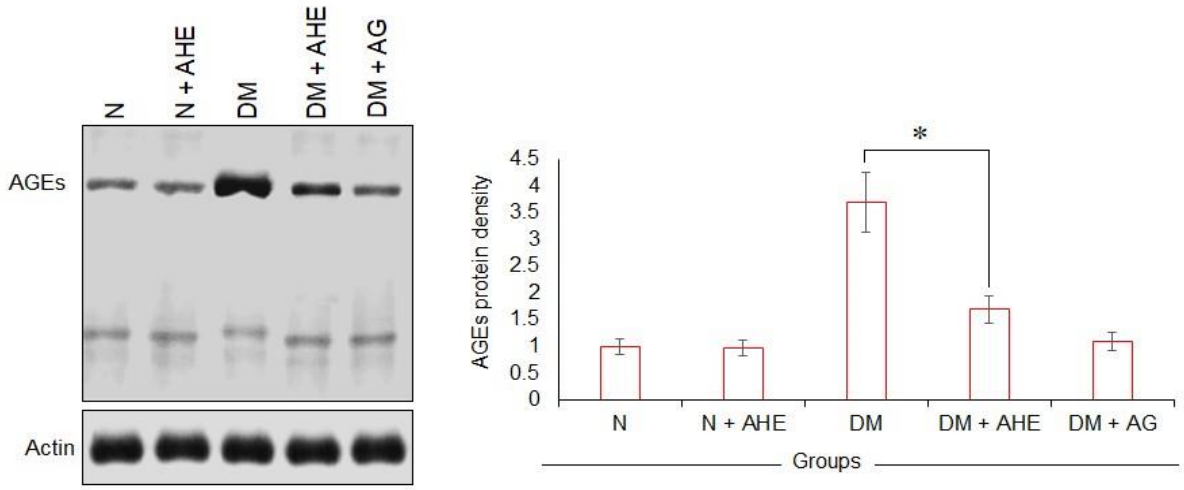

Fig. 2. AHE effects on the AGEs formation in different rat groups, (N, DM, N + AHE, and DM + AHE); the results show AHE inhibits the formation of AGEs in VSMCs of diabetic rats. (triplicate experiments were executed (*P < 0.05))

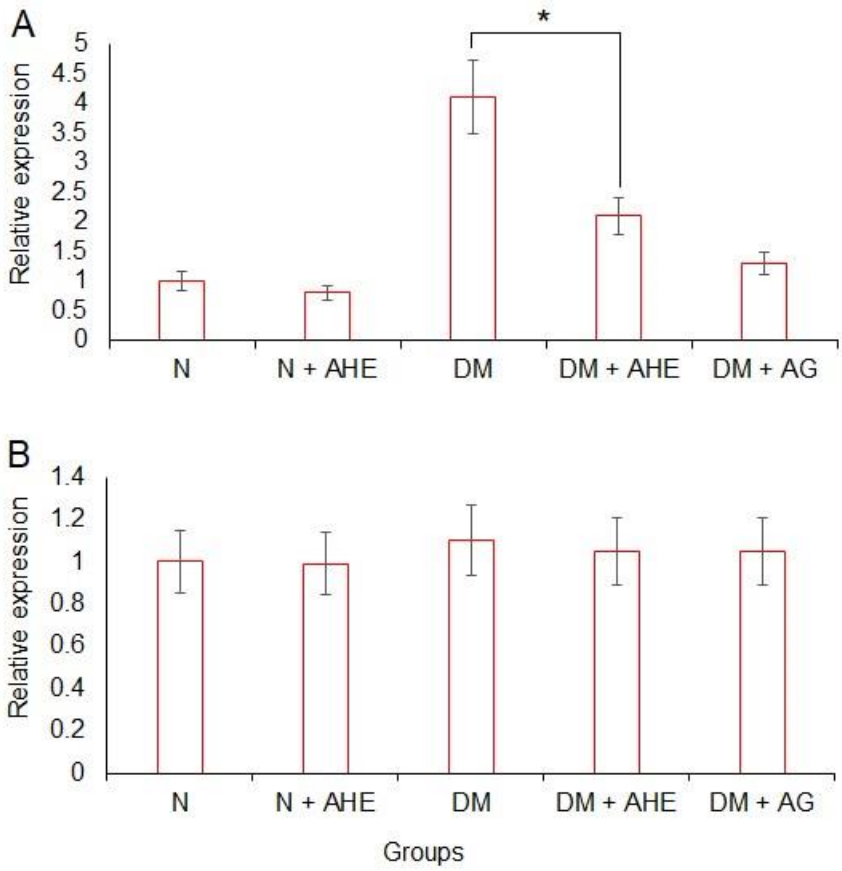

Fig. 3. Effect of AHE on the expression of cyclooxygenases in VSMCs of different rat groups (N, DM, N + AHE, and DM + AHE) (A) effect of AHE on the expression of COX-2 and (B) (A) effect of AHE on the expression of COX-1 (experiments were performed in triplicate and shown as mean ± SD (*P < 0.05))

4. Discussion

Peripheral artery disease (PAD) remains one of the common health issues across the globe. With increased awareness about the cardiovascular diseases, there has been more than 20% increase in the incidence of PAD (Klarin *et al.*, 2019). Stroke, myocardial infarction, and mortality have all been linked to PAD, and patients have been shown to have a much increased risk of these complications. Therefore, PAD patients are always in need of secondary cardiovascular prevention (Wan *et al.*, 2022).

Diabetic patients are associated with the development other complications which include but are not limited to nephropathy, neuropathy, and PAD (Wukich *et al.*, 2022). AGEs are promising markers of PAD in diabetic patients (Chen *et al.*, 2022). The treatment strategies for diabetic peripheral neuropathy are limited. As such there is pressing need to identify leads for the development of drugs that can specifically target PAD.

Plants herbal products find enormous use in pharmaceutical industry due to their negligible side effects (Khursheed *et al.,* 2022). *Artemisia herbal-alba* has been previously reported to exhibit antidiabetic effects (Réggami *et al.,* 2021). However, its effects on AGEs production in PAD have been evaluated. With this background this study was designed to evaluate its effects on PAD in diabetic rats. Results showed that AHE not restored blood sugar, triglyceride and cholesterol levels could remarkably inhibit AGEs production *in vitro* and in VSMCs of diabetic rats revealing the significant ability of AHE in overcoming PAD. These results are in confirmation with previous studies wherein several plant extracts or plantderived compounds have been shown to exhibit potential to inhibit AHE. For instance, *Litsea japonica,* a medicinal herb, has been previously shown to inhibit the production of AGEs in diabetic rats (Xu *et al.,* 2020). Similarly, Cassia semen which are basically the mature seed of *Cassia obtusifolia* have been shown to inhibit the production of AEs in diabetic rats (Kim *et al.,* 2014).

Results have shown that COX-2 expression is considerably enhanced under conditions such as diabetic peripheral artery disease while as COX-1 expression remains unchanged (Isworo, 2020). Therefore, we examined the effects of AHE on the expression of COX-1 and COX-2. Our observations revealed that AHE suppressed the expression levels of COX-2 in VMCs of diabetic rats preventing inflammation. Although, the present study preliminarily showed that AHE exhibits protective effects in diabetic peripheral artery disease via inhibition of AGEs, more in-depth studies are required to further confirm the findings. Moreover, identification of the active constituents of AHE is also need of the hour.

Conclusion

Taken together, the results of the present study revealed AHE exhibits protective effects in VSMCs of diabetic rats via inhibition of AGEs and expression of COX-2. The findings suggest that AHE may be utilized in the management of diabetic PAD. However, the effect of AHE on other biomarkers of PAD needs to be evaluated and the active components of AHE need to be identified. Our study could be a platform to identify novel bioactive ingredients in AHE and to identify targets by which AHE induce inhibition of AGEs and expression of COX-2.

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