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Original Article

Artemisia annua L. Extracts Improved Insulin Resistance via Changing Adiponectin, Leptin and Resistin Production in HFD/STZ Diabetic Mice

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Objectives: Insulin resistance (IR) is major cause of type 2 diabetes (T2D), and adipokines (e.g., adiponectin, leptin, and resistin) play an important role in insulin sensitivity. Medicinal plants are frequently used for T2D treatment. This study investigates the effect of *Artemisia annua* L. (AA) extracts on adipokines in mice with high-fat-diet (HFD)/streptozotocin (STZ)-induced T2D.

Methods: We divided 60 mice into 12 groups (n = 5 per group): control, untreated T2D, treated T2D, and 9 other groups. T2D was induced in all groups, except controls, by 8 weeks of HFD and STZ injection. The treated T2D group was administered 250 mg/kg of metformin (MTF), while the nine other groups were treated with 100, 200, and 400 mg/kg of hot-water extract (HWE), cold-water extract (CWE), and alcoholic extract (ALE) of AA (daily oral gavage) along with 250 mg/kg of MTF for 4 weeks. The intraperitoneal glucose tolerance test (IPGTT) was performed, and the homeostasis model assessment of adiponectin (HOMA-AD) index and blood glucose and serum insulin, leptin, adiponectin, and resistin levels were measured.

Results: Similar to MTF, all three types of AA extracts (HWEs, CWEs, and ALEs) significantly ($p < 0.0001$) decreased the area under the curve (AUC) of glucose during the IPGTT, the HOMA-AD index, blood glucose levels, and serum insulin, leptin, and resistin levels and increased serum adiponectin levels in the MTF group compared to the T2D group ($p < 0.0001$). The HWEs affected adipokine release, while the CWEs and ALEs decreased leptin and resistin production.

Conclusion: Water and alcoholic AA extracts have an antihyperglycemic and antihyperinsulinemic effect on HFD/STZ diabetic mice. In addition, they decrease IR by reducing leptin and resistin production and increasing adiponectin secretion from adipocytes.

Keywords: adiponectin, leptin, resistin, artemisia anuua l., diabetes

INTRODUCTION

Phototherapy has long been used as folk medicine to treat many diseases, such as diabetes mellitus. A few traditional plants used to treat diabetes mellitus have been scientifically and medically assessed [1]. Type 2 diabetes (T2D), which is characterized by obesity and insulin resistance (IR), results in elevated blood glucose levels [2, 3]. Adipocytes release various adipokines, such as leptin, resistin, and adiponectin, which might change the insulin sensitivity and glucose usage in peripheral tissues. Severe IR was observed in leptin-deficient ob/ob mice that was reversed by leptin administration [4, 5]. However, leptin levels are elevated in most obesity-related T2D models, suggesting a role of leptin resistance in T2D. Elevated

resistin levels in an obesity-induced IR model impaired insulin sensitivity and caused glucose intolerance [4]. Adiponectin secreted from adipocytes improves insulin sensitivity, increases the glucose usage in peripheral tissues, and stimulates insulin release [6].

Numerous studies have shown the antidiabetic effects of *Artemisia* spp. For instance, tea produced from *Artemisia herba-alba* is used in Iraq to treat diabetes mellitus [7, 8]. *A. santonicum* is used as an antidiabetic remedy in Turkish folk medicine [9]. *A. annua*, also known as sweet sagewort, sweet annie, sweet wormwood, and annual wormwood (*Chinese Qinghao*), is used as a medicinal plant in East Asia (China and Korea) [10-13]. Studies have reported the antidiabetic effects of *A. annua* extracts on T1D and T2D animal models [14-16]. However, the physiological effects of many *Artemisia* spp., such as *A. annua* L. (AA), for T2D treatment have not been thoroughly assessed.

This study investigated the effects of water and alcoholic extracts of AA on the homeostasis model assessment of adiponectin (HOMA-AD) index, blood glucose levels, and serum insulin, leptin, adiponectin, and resistin levels in mice with high-fat-diet (HFD)/streptozotocin (STZ)-induced T2D.

MATERIALS AND METHODS

1. Animals and treatment

Adult male albino mice (N = 60, 8–10 weeks old, body weight 30–35 g) were used in this study. The mice were randomly divided into 12 groups (n = 5 per group): control, untreated T2D, treated T2D, and 9 other groups. After induction of T2D (see the next section), the control and untreated T2D groups were administered intraperitoneal (IP) saline daily for 4 weeks. The treated T2D group was administered 250 mg/kg of IP metformin (MTF; mahbanchemi, Iran) as a standard drug to treat T2D. The remaining nine groups were treated with hot-water extract (HWE), cold-water extract (CWE), and alcoholic extract (ALE) by daily oral gavage for 4 weeks [17] at doses of 100, 200, and 400 mg/kg [18, 19].

All procedures were performed in accordance with the local ethics committee of the Mazandaran University of Medical Sciences, Sari, Iran (IR.MAZUMS.IMAMHOSPITAL.REC.1399.3274).

2. Induction of type 2 diabetes

To induce T2D, all groups, except controls, were fed with an HFD for 8 weeks to induce hyperglycemia [20]. To induce sustained hyperglycemia, the mice were administered IP STZ (Sigma-Aldrich, St. Louis, MO, USA) a single low dose of 65 mg/kg body weight. After 1 week, blood glucose levels were measured using a glucometer (On-Call EZ, San Diego, CA, USA), and mice with blood glucose levels of ≥ 200 mg/dL were considered diabetic and included in the study [20].

The mice had free access to water and commercial food. A standard pellet (Behparvar Industrial Expansion and Development Co, Iran) consisted of carbohydrates (72.1%), proteins (22.1%), and lipids (7.5%), with a total caloric value of ~2,900 kcal/kg. The HFD consisted of carbohydrates (27.5%), proteins (14.5%), and lipids (58.5%), with a total caloric value of ~4,700 kcal/kg.

3. Plant material

Aerial parts of AA were collected from the north of Iran in May and June 2018. The genus and scientific species of the plant were biotechnically identified by Dr. Masod Azadbakht, professor at the Sana University of Mazandaran, Sari, Iran, and preserved in the School of Pharmaceutics, Mazandaran University of Medical Sciences, Sari, Iran (record no. E1-39-2191).

4. Preparation of *Artemisia annua* L. extracts

The aerial parts of AA were dried at room temperature and then were powdered using a grinder. Next, 500 g of the powder was mixed with a given volume of distilled water. To prepare the CWE, extraction was performed for 3 consecutive days at room temperature away from sunlight. To prepare the HWE, a powder-and-water mixture was boiled for 6 h and then filtered; this process was repeated twice. To prepare the ALE, 500 g of the powder was mixed with a given volume of methanol instead of distilled water and extraction was performed after 48 h. After extraction, all obtained extracts were concentrated using a rotary evaporator, frozen, dried, and stored in a glass container at –20℃.

5. Toxicity test of *Artemisia annua* L. extracts

The median lethal doses (LD_{50}) of the extracts were deter-

mined using a new three-stage method. In stage 1, separate groups of four mice each were injected with different IP doses of the three extracts. Since there was no mortality, stage 2 was started. In all stages, behavioral changes as well as mortality were monitored. LD_{50} was calculated as follows:

$$LD_{50} = [M_0 + M_1]/2,$$

where M_0 is the highest dose of the tested extract without mortality and M_1 is the lowest dose of the tested extract that caused mortality.

6. Intraperitoneal glucose tolerance test

The intraperitoneal glucose tolerance test (IPGTT) was performed to determine the impact of AA extracts on glucose tolerance at the end of the experimental period. To perform the IPGTT, 1 day after the end of the experimental period, all groups were fasted for 16 h and then injected with 2 g/kg IP glucose. Blood glucose levels were measured using a glucometer just before and at 15, 30, 60, and 90 min after glucose loading.

7. HOMA-AD index

The HOMA-AD index was determined as follows [21]:

HOMA-AD = Fasting blood glucose (mmol/L) × Fasting blood insulin (mU/L)/[22.5 × adiponectin (μg/mL)]

8. Serum insulin, leptin, resistin, and adiponectin levels

After 4 weeks of treatment, the fasted mice were anesthetized using 10 mg/kg of xylazine or 110 mg/kg of ketamine and then euthanized via blood drainage from the heart [22]. The blood samples were centrifuged at 664 ×*g*, and the serum was removed and kept at −70℃. The blood parameters were determined using a mice insulin enzyme-linked immunosorbent assay (ELISA) kit (ZelBio, Germany) with a sensitivity of 0.2 MIU/L, a mice leptin ELISA kit (ZelBio) with a sensitivity of 5 ng/L, a mice resistin ELISA kit (ZelBio) with a sensitivity of 0.35 ng/mL, and a mice adiponectin ELISA kit (ZelBio) with a sensitivity of 0.12 mg/L. The intra-assay coefficient of variation (CV) was < 10% and the inter-assay CV was < 12% for all parameters. The blood glucose levels were measured using a glucometer.

9. Statistical analysis

The data for serum glucose, insulin, leptin, resistin, adiponectin, HOMA-AD index, and total area under the curve (AUC) of glucose during the IPGTT were expressed as the mean ± standard error of the mean (SEM) and compared using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test between groups (n = 5). All data were analyzed using Graph-Pad Prism 8.1. $p < 0.05$ was considered statistically significant.

RESULTS

1. Effect of AA extracts on blood glucose and serum insulin levels

Fasting blood glucose and serum insulin levels significantly increased in the T2D group compared to the control group ($p < 0.0001$), which were then reversed in the MTF group ($p < 0.0001$) in the positive control group. Moreover, hyperglycemia and hyperinsulinemia (IR) induced by T2D significantly decreased in all HWE, CWE, and ALE groups ($p < 0.0001$). The efficacy of glucose reduction in the T2D + CWE200 group (43.67%) was significantly lower compared to the MTF group (90.22%; $p < 0.001$) (Fig. 1A).

Efficacy of doses of 100 in T2D + HWE and T2D + CWE groups (respectively, 62.74%, 58.82%) ($p < 0.0001$) and also dose of 200 in T2D + CWE group (64.7%) significantly were less than that of MTF group ($p < 0.001$). While the efficacy of the other groups were similar to MTF group (Fig. 1B).

2. Effect of AA extracts on serum adiponectin levels and HOMA-AD index

Compared to the T2D group, the serum adiponectin levels significantly decreased in the MTF group ($p < 0.0001$) and all three HWE groups ($p < 0.0001$), while no significant difference was observed between the T2D group and the six CWE and ALE groups, indicating that the efficacy of CWE and ALE in enhancing adiponectin (13.51%–44.86%) was significantly lower (1.3 times) compared to the MTF group ($p < 0.0001$); see Table 1. The HOMA-AD index significantly increased in the T2D group ($p < 0.0001$) and significantly decreased in the MTF and all HWE, CWE, and ALE groups ($p < 0.0001$). The efficacy of HOMA-AD index reduction in the T2D + CWE100 and

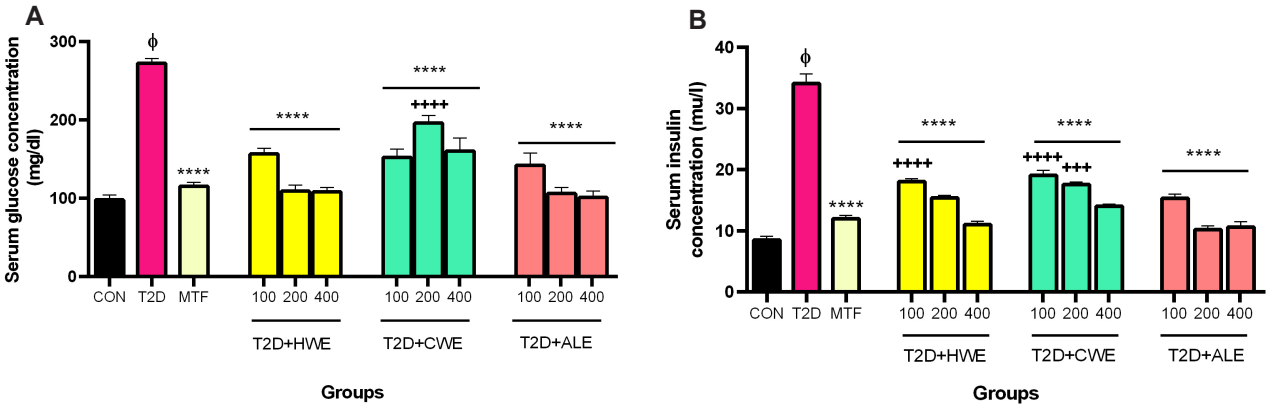

Figure 1. The effects of Metformin (A) or various doses of hot-water, cold-water, and alcoholic Artemisia annua extracts on the serum levels of glucose (A) insulin (B) in HFD/STZ-induced diabetic mice. Data are expressed as the mean ± SEM. (n = 5). $^{\phi}p < 0.0001$ versus the CON group. ****$p < 0.0001$ versus the T2D group. $^{\dagger\dagger\dagger}p < 0.001$, $^{\dagger\dagger\dagger\dagger}p < 0.0001$ versus the T2D + MTF group.

Table 1. The effects of Metformin or various doses of hot-water, cold-water, and alcoholic *Artemisia annua* extracts on the serum levels of adiponectin (A) HOMA-AD (B) in HFD/STZ-induced diabetic mice. Data are expressed as the mean ± SEM (n = 5)

Groups	Adiponectin (μg/mL)	HOMA-AD
CON	3.01 ± 0.05	0.69 ± 0.07
T2D	1.15 ± 0.07$^{\phi}$	20.18 ± 1.35$^{\phi}$
T2D + MTF	3.7 ± 0.19****	0.93 ± 0.06****
T2D + HWE100	2.98 ± 0.15****	2.35 ± 0.11****
T2D + HWE200	3.3 ± 0. 15****	1.26 ± 0.08****
T2D + HWE400	3.9 ± 0.29****	0.77 ± 0.07****
T2D + CWE100	1.4 ± 0.17††††	5.32 ± 0.54****††††
T2D + CWE200	1.56 ± 0.19††††	5.65 ± 0.47****††††
T2D + CWE400	1.76 ± 0.25††††	3.14 ± 0.44****
T2D + ALE100	1.59 ± 0.2††††	3.57 ± 0.5****†
T2D + ALE200	1.98 ± 0.34††††	1.5 ± 0.27****
T2D + ALE400	1.89 ± 0.22††††	1.53 ± 0.22****

$^{\phi}p < 0.0001$ versus the CON group. ****$p < 0.0001$ versus the T2D group. $^{\dagger}p < 0.05$, $^{\dagger\dagger\dagger\dagger}p < 0.0001$ versus the T2D + MTF group.

T2D + CWE200 groups (74.43% and 77%, respectively) and in the T2D + ALE100 group (84.7%) was significantly lower compared to the MTF group (97.89%; $p < 0.001$ and $p < 0.05$, respectively); see Table 1.

3. Effect of AA extracts on serum leptin and resistin levels

Serum leptin levels increased in the T2D group ($p < 0.0001$) but significantly decreased in the MTF, HWE, and ALE groups ($p < 0.0001$) compared to the T2D group. Moreover, serum leptin levels decreased in all the T2D + CWE100, T2D + CWE200, and T2D + CWE400 groups compared to the T2D group ($p < 0.01$, $p < 0.001$, and $p < 0.0001$, respectively). The efficacy of serum leptin reduction was significantly higher ($p < 0.0001$) in the T2D + HWE400 group (80.3%) compared to the MTF group (58.16%), while it was significantly lower ($p < 0.0001$, $p < 0.01$, respectively) in the T2D + ALE100, T2D + ALE200, and T2D + ALE400 groups (30.06%, 43.76%, and 43.13%, respectively) compared to the MTF group (Fig. 2A).

A significant elevation was observed in the levels of serum resistin in diabetic mice ($p < 0.0001$). However, treatment of diabetic mice with Metformin, hot water extract ($p < 0.0001$) and dose of 400 of cold water extract ($p < 0.01$) resulted in a notable reduction in the level of resistin in comparison with T2D group. Except efficacy of doses of 100 and 200 of in T2D + CWE group (respectively, 15.09%, 23.07%) in reduction of resistin that considerably (respectively, $p < 0.0001$, $p < 0.001$) was less than that of MTF group (79.24%) (Fig. 2B).

4. Effect of AA extracts on the IPGTT

The AUC of glucose significantly increased in the T2D group compared with the control CON group ($p < 0.0001$) but was reversed in the MTF and all HWE, CWE, and ALE groups ($p < 0.0001$) except at a dose of 100 mg/kg ($p < 0.05$). The efficacy of the AA extracts in glucose tolerance improvement was not similar to that of MTF except the dose of 200 mg/kg of HWE. The efficacy of the AA extracts in glucose tolerance improvement was lower in the T2D + HWE100 (92.91%, $p < 0.01$),

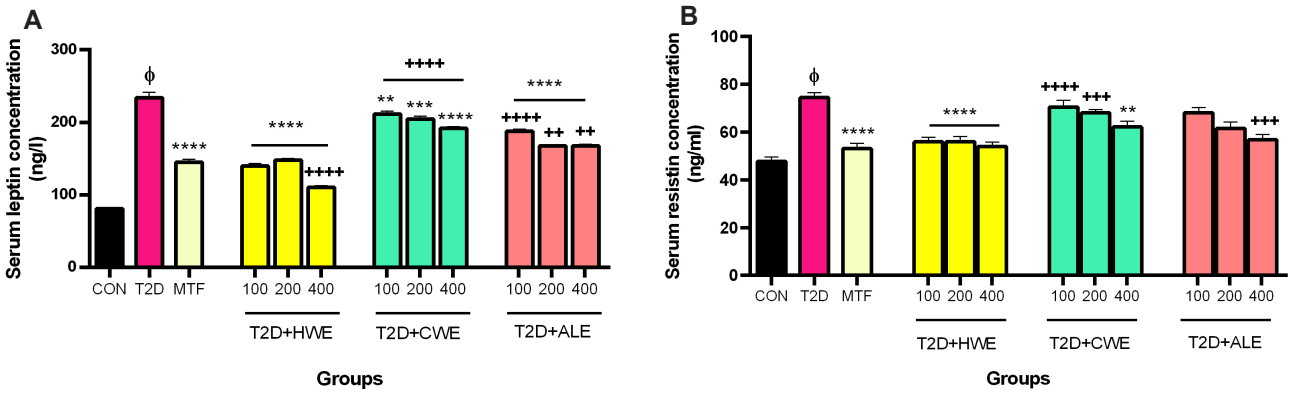

Figure 2. The effects of Metformin or various doses of hot-water, cold-water, and alcoholic Artemisia annua extracts on the serum levels of leptin (A) resistin (B) in HFD/STZ-induced diabetic mice. Data are expressed as the mean ± SEM. (n = 5). $^{\phi}p < 0.0001$ versus the CON group. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ versus the T2D group. $^{\dagger\dagger}p < 0.01$, $^{\dagger\dagger\dagger}p < 0.001$, $^{\dagger\dagger\dagger\dagger}p < 0.0001$ versus the T2D + MTF group.

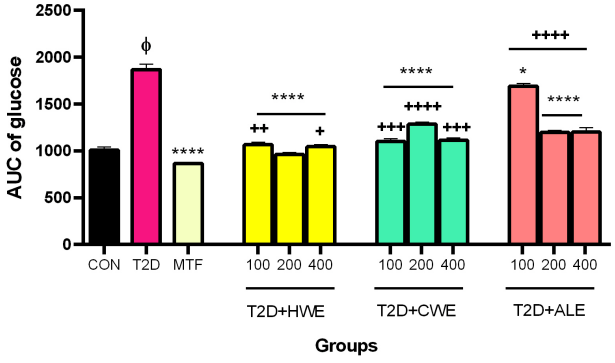

Figure 3. The effects of Metformin or various doses of hot-water, cold-water, and alcoholic Artemisia annua extracts on the serum levels of glucose during IPGTT, AUC of glucose in HFD/STZ-induced diabetic mice. Data are expressed as the mean ± SEM. (n = 5). $^{\phi}p < 0.0001$ versus the CON group. $^{*}p < 0.05$, $^{****}p < 0.0001$ versus the T2D group. $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$, $^{\dagger\dagger\dagger}p < 0.001$, $^{\dagger\dagger\dagger\dagger}p < 0.0001$ versus the T2D + MTF group.

T2D + HWE400 (96.58%, $p < 0.05$), T2D + CWE100 and T2D + CWE400 (89% and 87.68%, respectively, $p < 0.001$), T2D + CWE200 (67.59%, $p < 0.0001$), and all three T2D + ALE groups (20.57%–77.58%, $p < 0.0001$) compared to the MTF group (1.16 times); see Fig. 3.

DISCUSSION

This study shows that indicators of IR, such as hyperglycemia, hyperinsulinemia, HOMA-AD index, and glucose intolerance, in HFD/STZ-induced T2D mice improve after treatment with HWEs, CWEs, and ALEs of AA by increasing circulating serum adiponectin levels and decreasing circulating serum leptin and resistin levels.

All three doses (100, 200, and 400 mg/kg) of AA extracts acted similar to MTF in modulating hyperglycemia induced in mice. Although all AA extracts considerably restored hyperinsulinemia to normal levels, the ALEs (all three doses) were more effective than the HWEs and CWEs in alleviating hyperinsulinemia induced in T2D mice and their effects were similar to those of MTF, although high doses (400 mg/kg) of HWEs and CWEs showed an efficacy like that of MTF. Treatment with all doses of HWEs, CWEs, and ALEs significantly improved glucose intolerance induced in T2D mice, but the efficacy of the 200 mg/kg dose of the HWE was higher compared to 100 and 400 mg/kg.

Studies have shown that 2-week gavage with artemeter, a derivative from artemisin, improves hyperglycemia and hyperinsulinemia (IR) and glucose intolerance in T2D mice [23]. In addition, supplementation of ethanol extracts of *A. princeps* improves glucose intolerance, hyperglycemia, and hyperinsulinemia in T2D mice, although it did not change plasma leptin levels [24]. IR is a principal cause of glucose intolerance in T2D [25], and our findings indicate that AA extracts improved IR in T2D mice. Although all AA extracts significantly lowered the HOMA-AD index in T2D mice and the effect of high doses of CWEs and ALEs was like that of MTF, HWEs at all doses were more effective than CWEs and ALEs and acted similar to MTF in lowering the HOMA-AD index. The HOMA-AD index is a useful novel, simple, and adequate surrogate marker, modified from the HOMA-IR index, for detecting IR [25, 26]. These findings indicate that AA extracts decreased the IR index HOMA-AD in T2D mice. Following these results, we analyzed

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the mechanism of action of AA extracts in the improvement of IR by measuring the serum adiponectin, leptin, and resistin levels.

Treatment with only HWEs of AA significantly increased the low serum adiponectin levels induced in T2D mice, acting like MTF. An increase in circulating serum adiponectin levels by natural compounds selectively ameliorates IR, glucose intolerance, and hyperglycemia in obese and diabetic mice [27]. In the same context, gavage of HFD mice for 2 weeks daily with ethanolic extracts of *A. scoparia* and *A. santolinifolia* increased insulin sensitivity by increasing plasma adiponectin levels [28]. In addition, 6-week treatment with eupatilin isolated from *A. princepes* enhanced plasma adiponectin levels and glucose tolerance and decreased fasting blood glucose levels in progressive T2D mice, proving its antidiabetic function, which is similar to the findings of this study [29]. Adiponectin is an adipokine that improves insulin action and increases glucose uptake in peripheral tissues [6]. Low serum adiponectin levels are a marker of IR and a risk factor for incidence of T2D [25, 30]. Moreover, plasma adiponectin levels are low in patients with T2D [1].

All doses of HWEs, CWEs, and ALEs significantly decreased the increased circulating serum leptin levels in T2D mice. However, only HWEs had an effect similar to MTF, while the efficacy of CWEs and ALEs was lower compared to MTF. These findings are consistent with those of another study, which confirmed that mice with HFD-induced obesity that were treated with mugwort extract of *A. princeps* Pampanini for 6 weeks showed enhanced serum adiponectin and decreased serum leptin levels [31]. Leptin is an insulin sensitizer secreted from adipose tissues that improves insulin sensitivity by direct action on peripheral tissues [30]. High circulating serum leptin levels in obese patients probably indicate leptin resistance and are a high-risk factor for T2D development [5, 32, 33]. Moreover, hyperinsulinemia induced in T2D also acts as an exacerbating factor for releasing leptin from adipocytes [33]. Leptin resistance induced by an increase in serum leptin levels in turn leads to IR [30].

After detecting serum resistin levels, related findings demonstrated that all doses of HWEs and high doses (400 mg/kg) of CWEs and ALEs significantly decreased the increased circulating serum resistin levels in T2D mice, and their effects were similar to MTF. Several antidiabetic medicinal plants affect insulin sensitivity by inhibiting serum leptin and resistin levels and increasing serum adiponectin levels [34, 35]. Resistin is an adipokine that attenuates insulin action in adipocytes, the muscle, and the liver and leads to IR [30, 36]. Therefore, our findings indicate that HWEs of AA act by increasing serum adiponectin secretion and decreasing serum leptin and resistin production, while CWEs and ALEs exerted their IR reduction effects by decreasing leptin and resistin release from adipocytes.

The mechanisms underlying the improvement of IR (ameliorating hyperglycemia, hyperinsulinemia, glucose intolerance, and the HOMA-AD index) in mice with HFD/STZ-induced T2D after 4 weeks of oral treatment with water and alcoholic extracts of AA might be associated with an increase in adiponectin secretion and a decrease in resistin and leptin production from adipocytes. Secondary metabolites, such as flavonoids, alkaloids, and phenols, derived from plants have been used in folk medicine for diabetes mellitus [6]. AA extracts are abundant in flavonoids [16], and flavonoids are effective in adiponectin secretion from adipocytes [37].

CONCLUSION

The improvement of IR in mice with HFD/STZ-induced T2D after treatment with water and alcoholic extracts of AA could be due to an increase in adiponectin secretion and a decrease in leptin and resistin production from adipocytes. Compared to MTF, the only AA extract with a higher efficacy in reducing leptin is 400 mg/kg of HWE. This effect might be attributed to the presence of bioactive compounds, such as flavonoids, in AA. However, bioactive compounds were not isolated in this study. Further studies are necessary to investigate the effect of compounds isolated from AA on IR and the factors involved.

CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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AVAILABILITY OF DATA AND MATERIALS

The datasets used and/or analyzed during the current study

are available from the corresponding author upon reasonable.

AUTHORS' CONTRIBUTION

FS and MSL designed the experiments. MG performed the animal experiments and EH was involved in preparing the extracts. FS and MSL analyzed the data, wrote and edited the manuscript.

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