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Levels of Expression of Hsp70 and iNOS and Effect of *Artemisia Sieberi* (*A. herba-alba*) on Activity of Hypothalamic-Pituitary-Thyroid (HPT) Axis in Diabetic Rats

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This work was carried out to evaluate levels of expression of the Heat Shock Protein 70 (Hsp70) and inducible nitric oxide synthase (iNOS) biomarkers in extracts of Artemisia sieberi (A. herba-alba) and their impacts on the activity of hypothalamic-pituitary-thyroid (HPT) axis in diabetic rats. 50 rats were separated into five experimental groups: a normal control group, a positive control group treated with dilute A. herba alba (AHE) oil extract, a diabetic non-treated group, a diabetic group treated with AHA extract, and a diabetic group treated with Metformin. Results: Orally administered 8.1 mg/kg body weight (BW) of dilute AHA oil and 14.2 mg/kg BW of Metformin were administered for 6 weeks. Serum triiodothyronine (T3) levels decreased significantly in diabetic rats and increased significantly in the rats treated with the dilute AHA oil. Furthermore, no significant differences were observed in thyroid gland Hsp70 expression between the diabetic and non-diabetic rats. Metformin and dilute AHA oil treatments significantly increased the expression of Hsp70 in the thyroid gland. The results also demonstrated that diabetes significantly increased the rate of iNOS expression in the thyroid gland. Treatment with Metformin and dilute AHA oil significantly reduced the expression of iNOS in the thyroid gland. These results suggest that dilute AHA oil plays a role in the peripheral regulation of thyroid function and provide empirical evidence that it contributes to the stimulation or improvement of thyroid function.

Keywords: Artemisia Sieberi (A. herba-alba); Hypothalamic-Pituitary-Thyroid; Hsp70; iNOS; Diabetic Rats.

Artemisia sieberi (*A. herba-alba*) belongs to the Asteraceae (Compositae) family and includes annual, perennial, and biennial plants[1]. *Artemisia herba-alba* is a short shrub usually found in Northern Africa and the Middle East. The parts that grow above the ground are used as medicine[2]. The herb is used traditionally to treat wounds, parasites (especially helminths), diabetes, respiratory and intestinal disorders, and injuries[2]. Moreover, several *Artemisia* herbs have

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historically been used to treat seizures and their efficacy has been proven by *in vivo* animal trials[3,4,5]. The *Artemisia* species have been studied *in vitro* and *in vivo*, as well as in clinical trials, for their anticancer, antimalarial, antibacterial, antiviral, and antidiabetic properties[6]. Vegetables, culinary herbs, and medicinal plants are commonly used to control diabetes in Jordan, where traditional medicine is an essential component of primary health care[7].

The synthesis of nitric oxide (NO) involves enzymes that convert the amino acid L-arginine into NO[8]. The inducible nitric oxide synthase (iNOS) is a key physiological signaling molecule that regulates insulin production, angiogenesis, nociception, inflammation, and pain[8,9]. Enzymatic synthesis of NO is controlled by isoenzymes, including endothelial, neuronal, and inducible isoforms[8]. The concentration of the resultant NO depends on numerous parameters, which mainly include the isoform involved in NO production and duration of the production process[8,9]. The iNOS is more likely to generate NO than other isoforms[10]. Diabetic rats have high levels of iNOS[8,9,10].

The Heat Shock Protein 70 is one of the central components of the cellular network of molecular chaperones. It is naturally generated in lymphocytes, macrophages, epithelial cells, dendrites, muscles, and hepatocytes. Having a molecular mass of 70 kDA, this protein molecule has a role in regulating homeostasis of other proteins and is dependent in its action on adenosine triphosphate (ATP)[11]. Some Hsp70 chaperones are characterized by their genes and cell sites. Both Hsp70 and the heat shock 70 protein (Hsc70) are found in the nucleus and cytoplasm; the binding immunoglobulin protein (BiP) Grp78 is placed in the endoplasmic reticulum (ER) and mitochondrial Hsp70 (mtHsp70) Grp75 is located in the mitochondria[11, 12]. Changes in Hsp70 expression have been seen in people with obesity and metabolic disorders, including type 2 diabetes as evidenced by results of several studies. According to some studies, increasing insulin sensitivity through Hsp70 modulation might affect blood glucose levels[12,13,14]. The hypothalamus-pituitary-periphery (HPT) feedback loop regulates the production, storing, and secretion of thyroid hormones by the thyroid gland. The presence of two crucial trace elements—selenium and iodin—is necessary for these processes[1]. The thyroid-stimulating hormone (TSH), which is secreted by the anterior pituitary gland, is the primary stimulator of thyroid hormone synthesis. It enhances all the processes required for thyroid hormone biosynthesis, including protein biosynthesis, intra-thyroidal iodide organification, and stimulation of thyroglobulin (Tg) release[15]. Diabetes mellitus (DM) and thyroid problems are associated with endocrine system dysfunction. Research[16] has shown that diabetes and thyroid diseases frequently co-exist in individuals. Considering their roles in cellular metabolism, insulin and thyroid hormone excesses or deficiencies can lead to functional abnormalities in the other hormones[16]. Bearing this in mind, this study was carried out to evaluate the levels of expressions of the Hsp70 and iNOS biomarkers in dilute oil extract of *A. herba-alba* (AHA) and their effects on the action of the hypothalamic-pituitary-thyroid (HPT) axis in rats suffering from diabetes.

Experimental Part

Preparation of oil

Artemisia sieberi (A. herba-alba) aerial parts were used to extract the essential oil using the extraction method described in references[17,18]. Dry parts of this plant were cut into 0.5 mm pieces and allowed to air dry in the dark at room temperature for five days. Afterwards, a mass corresponding to 200g of the dried material was hydrodistilled for 3 h using Clevenger apparatus. The oil yield of the dried tops (leaves, stems, and flowers) was 1.1% (v/w).

Diabetes induction in rats

For this study, adult rats weighing 150–200 g were bought from the Jordan University of Science and Technology (JUST) Faculty of Medicine's central animal house. The animals were maintained on a 12-hour light-dark cycle. Water and food were available *ad libitum*. A stock diet meal consisting of 50% wheat, 21% maize, 20% soybean, 8% concentrated protein, and 1% of a combination of salts, vitamins, and dicalcium phosphate was supplied to the rats, who were kept in normal metal cages with ten rats per cage. Struggle and restraint were minimized to avoid the influence of stress on the test results as highlighted in[18]. The rats were administered intraperitoneal injections of alloxan monohydrate (BOH Chemicals Ltd, England) dissolved in fresh normal saline at a dose of 150 ml per kg body

weight (BW) after fasting for 18 h. Rats in this study were classified as diabetics if their blood glucose concentrations ranged between 200 and 450 mg/100 ml. [17, 18].

Experimental design

50 rats were randomly divided into five experimental groups of 10 each. Normal rats have been split into two groups: 1 and 2. Group 1 rats received dimethyl sulfoxide (DMSO) at a 0.5 mL/kg dosage and were used as the negative control group. *A. sieberi* essential oil extract was administered by gavage to group 2 at 80 mg/kg. Rats with alloxan-induced diabetes were divided into three groups: 3, 4, and 5. Group 3 rats were only given DMSO at a 0.5 mL/kg dosage. Meanwhile, Group 4 rats received treatment with dilute AHA oil extract (80 mg/kg), whereas Group 5 rats received Metformin (14.2 mg/kg). The Metformin was obtained from Bristol-Myers Squibb Company in the United Kingdom. For six weeks, the essential oil extract and Metformin were given daily via an intragastric tube. Rats in each group received the recommended treatment for 6 weeks, with unlimited access to water and food during the trial. Blood samples were obtained from the rats at the end of the study period after the heart puncture procedure, and tissues specimens were collected from the thyroid gland to look at histological alterations. The mice were killed by cervical dislocation after the study procedure while under light ether anesthesia. The experiment was conducted in compliance with the guidelines of the animal ethics committee of JUST, which were expressed in accordance with international standard principles for laboratory animal use and care (Number 27045128/17).

Determination of thyroid hormone

The micro particle enzyme immunoassay (MEIA) is the basis for quantification of the Axsym thyroid hormone (triiodothyronine (T3), thyroxine (T4), and TSH). The following order is followed while pipetting the axsym-free (T3, T4, and TSH) reagents and samples. The sampling probe pipettes the sample and the assay (T3, T4, and TSH) chemicals that are needed for a single test into different reaction vessel (RV) wells. The RV is moved right away into the processing facility [18]. Using the processing probe, further pipetting is performed at the processing center. An antibody-antigen combination is formed when the sample and anti-T3, anti-T4, and anti-TSH-coated micro particles are pipetted into one well of the RV. An aliquot of the reaction mixture comprising T3, T4, and TSH bound to anti-T3, anti-T4, and anti-TSH-coated micro particles was added to the matrix cell. The glass fiber matrix and the micro particles form an irreversible bond. To remove unbound components, solubilizer solution was used to wash the matrix cell. When applied to the matrix cells, T3-alkaline phosphatase interacts with the open antibody binding sites. Unbound materials are eliminated by washing the matrix cell. After adding the substrate, that is 4-methyumbelliferyl phosphate, to the matrix cell, the MEIA optical assembly measures the fluorescence[18].

T3, T4, and TSH levels

AxSYM-free T3 and T4 MEIA measures free T3 quantitatively in human blood or plasma using the AxSYM analyzer[17,18]. The AXSYM 3rd Generation TSH Test is a useful method of concluding thyroid function, identification of thyroid conditions, and management of thyroid-related illnesses[18].

Interpretation of the Results

The resulting number of pixels that represented the immunological reaction was divided by the total number of pixels. Following that, the number of pixels with brown color, which is the color of the marker in the present study, was determined and divided by the whole number of pixels to estimate the expression of HSP70 and iNOS based on the interpretation of output of Adobe Photoshop Version: 22.3.[19].

Statistical analysis

Results are expressed as mean ±SE. One-way analysis of variance (ANOVA) was used to estimate statistical differences by using SPSS version 22. A value of $p < 0.05$ was considered significant[18,20]. Descriptive statistics for HSP70 for every group were calculated then presented as "Mean ± Standard deviation".

RESULTS

Effects of *Artemisia* and Metformin on T3, T4, and TSH levels in diabetic rats

The study found significant drops ($p < 0.05$) in blood T3, T4, and TSH levels in the diabetic rats below the corresponding levels in the control group (Table 1). Rats in the control group

have substantially increased blood T4, T3, and TSH levels ($p < 0.05$) than the comparable values in the diabetic rats. Furthermore, compared to the control rats, the diabetic rats had greater blood TSH levels. These findings imply that AHA oil therapy significantly raised the control group's blood T4 levels ($p < 0.05$).

Immunohistochemistry results

Expression rates of hsp70 in control and diabetic groups

Our results demonstrate that diabetes significantly decreased Hsp70 expression in the kidneys and liver ($p < 0.05$). Diabetes had a molecular impact on the liver and kidneys. In addition, there was no significant difference ($p > 0.05$) in Hsp70 expression in the thyroid gland between diabetes and non-diabetic rats (Table 2).

The effects of Metformin and dilute AHA oil on levels of Hsp70 expression.

The outcomes of the experiment (Table 3) revealed that both Metformin and dilute AHA oil treatments at significant ($p < 0.05$) raised the levels of Hsp70 expression in the livers, kidneys, and thyroid glands of the treated diabetic rats (Group 3 and Group 4) compared with the levels in non-treated diabetic rats (Group 3).

Levels of expression of iNOS in rats of the control and diabetic groups

Diabetes resulted in a statistically significant ($p < 0.05$) increase in the levels of iNOS expression in the livers, kidneys, and thyroid glands of the rats (Table 4). Diabetes affects the thyroid gland, liver, and kidney at the molecular level.

Effects of Metformin and dilute AHA oil on levels of expression of iNOS

Table 5 illustrates that the rats' thyroid glands, livers, and kidneys all had considerably lower levels of iNOS expression ($p < 0.05$) after receiving both the Metformin and diluted AHA oil treatments.

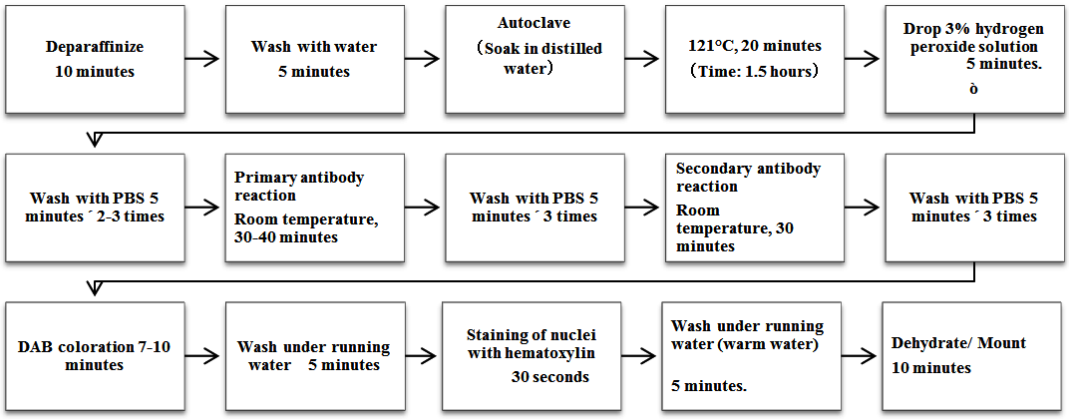

Chart 1. Schematic Overview of Immunohistochemistry

Table 1. Levels of TSH, T3, and T4 in the control and diabetic rats

Group	T_4(nmol/L)	T_3(nmol/L)	TSH(ng/ml)	*N*
1. Untreated Control	48.65 ± 2.45	1.46 ± 0.05	0.93 ± 0.02	10
2. Control treated with dilute AHE oil	54.82± 4.84*	2.21± 0.13*	1.67±0.01*	10
3. Rats with alloxan-induced diabetes	30.58± 2.27	0.82 ± 0.03*	0.48 ± 0.03*	10
4. Diabetic rats treated with dilute AHE oil	40.66 ± 1.28**	1.04 ± 0.06**	0.83 ± 0.04**	10
5. Diabetic rats treated with Metformin	38.54 ± 1.43**	1.08 ± 0.07**	0.76 ± 0.03**	
10				

Values are the mean values ± standard deviation of 10 rats.* Statistically significant when compared to the control and vehicle groups, i.e., Groups 1 and 2, at $\acute{a} = 0.05$. ** Statistically significant when compared to the rats with alloxan-induced diabetes (Group 3) at $\acute{a} = 0.05$.

DISCUSSION

The thyroid is one of the major endocrine glands in the body. It affects how quickly the body utilizes energy, makes proteins, and influences its response to other hormones [21]. With the production of thyroid hormones T4 and T3, it aids in these processes. The growth and operation of several other bodily systems are impacted by these, which also regulate metabolism. Tyrosine and iodine are used to make T3 and T4[22]. The thyroid gland also generates calcitonin, which is a hormone that regulates calcium levels. The hypothalamus and pituitary glands regulate thyroid function[23]. TSH secreted by the anterior pituitary, which is produced by TRH release by hypothalamus, controls the synthesis of T4 and T3. In a negative feedback loop, the thyroid and thyrotropes produce less TSH when T4 levels are high and vice versa[24].

Thyroid disorders are two to three times more common in patients with diabetes than in non-diabetic individuals. It also increases with age and is significantly impacted by autoimmune diabetes mellitus. In addition, they were substantially greater in female patients than in male patients. If a thyroid disease is linked to deterioration

Table 2. Levels of Hsp70 expression in rats of the control and diabetic groups

Hsp70 Expression					
Liver		Kidney		Thyroid gland	
Control Rats	Diabetic Rats	Control Rats	Diabetic Rats	Control Rats	Diabetic Rats
0.40	0.08	0.40	0.15	0.30	0.10
0.39	0.10	0.39	0.13	0.31	0.09
0.38	0.09	0.38	0.14	0.32	0.08
0.42	0.11	0.37	0.16	0.35	0.11
0.39	0.10	0.36	0.12	0.29	0.99

Table 3. Effects of Metformin and dilute AHA oil treatments on Hsp70 expression in diabetic rats

Hsp70 Expression								
Liver			Kidney			Thyroid gland		
Group 3[1]	Group 4[2]	Group 5[3]	Group 3[1]	Group 4[2]	Group 5[3]	Group 3[1]	Group 4[2]	Group 5[3]
0.08*	0.90**	0.70**	0.15*	0.93**	0.60**	0.10*	0.80**	0.5**
0.10*	0.89**	0.69**	0.13*	0.90**	0.70**	0.09*	0.79**	0.49**
0.09*	0.80**	0.71**	0.15*	0.89**	0.59**	0.08*	0.75**	0.52**
0.11*	0.95**	0.76**	0.16*	0.92**	0.58**	0.11*	0.81**	0.55**
0.10*	0.93**	0.75**	0.12*	0.91**	0.62**	0.99*	0.82**	0.49**

[1] Rats with alloxan-induced diabetes, [2] Diabetic rats treated with Metformin. [3] Diabetic rats treated with dilute AHA oil.
* Statistically significant when compared to the control group at $\acute{a} = 0.05$. ** Statistically significant when compared to the untreated diabetic group, i.e., Group 3, at $\acute{a} = 0.05$.

Table 4. Levels of expression of iNOS in rats of the control and diabetic groups

iNOS Expression					
Liver		Kidney		Thyroid gland	
Control rats	Diabetic rats	Control rats	Diabetic rats	Control rats	Diabetic rats
0.01	0.75	0.06	0.62	0.12	0.62
0.02	0.76	0.04	0.74	0.13	0.59
0.02	0.77	0.07	0.83	0.14	0.70
0.03	0.82	0.07	0.56	0.09	0.69
0.04	0.79	0.05	0.70	0.10	0.63

functions, its clinical significance, especially for diabetic individuals, increases dramatically. This is because declining functions consistently lead to several issues related to metabolic compensation of diabetes[25]. The two most dangerous outcomes of thyrotoxicosis are the development of potentially fatal ketoacidosis and an increased frequency of hypoglycemia in hypothyroidism. Research has demonstrated correlations between DM and several endocrine disorders, including gonadal dysfunction[26]. It has additionally been demonstrated that there are physiological and molecular connections between insulin and how iodothyronines and insulin affect the metabolism of lipids, proteins, and carbohydrates[27]. Triiodothyronine and tetraiodothynine are two thyroid hormones that serve as insulin antagonists and indirectly enhance insulin activity. T3 and T4 levels are often reduced in people and experimental animals with diabetes[26,27]. Diabetes causes alterations in hypothalamic thyrotropin-releasing hormone (TRH) excretion and pituitary thyrotropin (thyroid-stimulating hormone (TSH)) release and has direct effect on the thyroid gland[28]. Serum T3 concentrations are known to decline in diabetic patients, possibly due to inhibition of

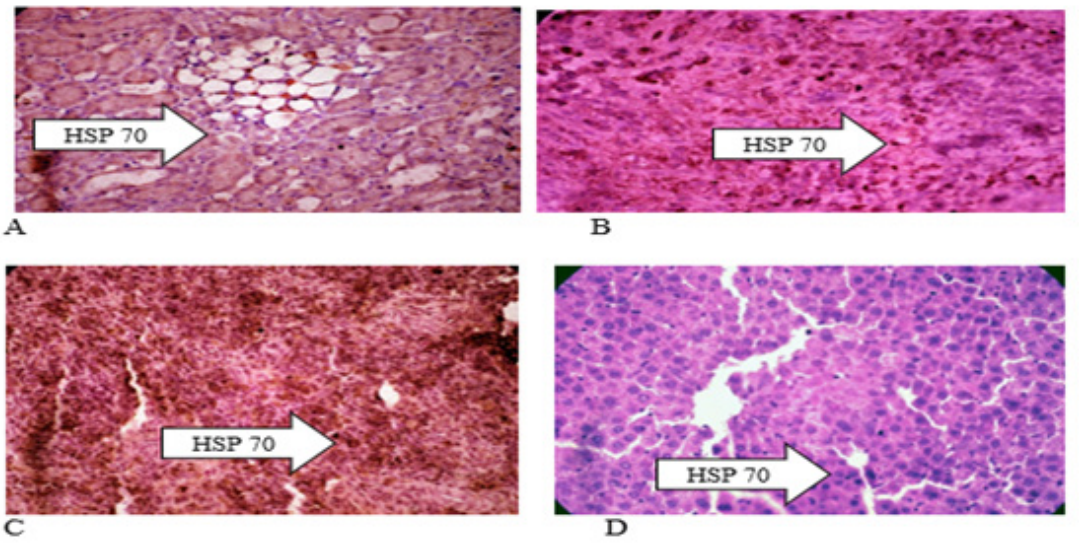

Fig. 1. Levels of HSP70 expression in the thyroid gland from the rate, A: control (HSP70 staining arrow). B: diabetic rats (HSP70 staining arrow). C: diabetic rats treated with diluted oil extracts of AHA (HSP70 staining arrow). D: diabetic rats treated with metaformin (HSP70 staining arrow)

Table 5. Results of analysis of differences in levels of iNOS expression between rats of the control group and those receiving Metformin and dilute AHA oil treatments

iNOS Expression								
Liver			Kidney			Thyroid gland		
Diabetic rats	Metformin	Dilute AHA oil	Diabetic rats	Metformin	Dilute AHA oil	Diabetic rats	Metformin	Dilute AHA oil
0.75*	0.19**	0.23**	0.62*	0.22**	0.28**	0.62*	0.41**	0.50**
0.76*	0.20**	0.23**	0.74*	0.20**	0.25**	0.59*	0.39**	0.49**
0.77*	0.18**	0.22**	0.83*	0.23**	0.26**	0.70*	0.30**	0.51**
0.82*	0.17**	0.24**	0.56*	0.15**	0.24**	0.69*	0.40**	0.48**
0.79*	0.16**	0.25**	0.70*	0.18**	0.23**	0.63*	0.42**	0.52**

* Statistically significant when compared to the control group rats at á = 0.05. ** Statistically significant when compared to untreated diabetic rats at á = 0.05.

peripheral 5´-deiodinase enzyme activity, which catalyzes conversion of T4 to T3.

For thousands of years, conventional medicine has used natural therapies to maintain the robust health of the endocrine system, promoting and regulating optimal hormone production and thyroid function[29]. Several published clinical trials have confirmed the efficacy of various herbs in strengthening the immune system and a healthy thyroid function. Thyroid hormones are required for metabolic homeostasis, and impaired thyroid function can be a serious consequence of diabetes[30]. Thyroid function is mostly determined by serum T3 and T4 levels[28,30]. In diabetic mice, the level of T_3 decreased was lower than that in the control mice. The T3/T4 ratio in the diabetic mice significantly decreased, according to these findings. Abnormal T3 levels are a sign of reduced microsomal ability to convert T4 to T3, most likely due to the emergence of oxidative conditions in many systemic non-thyroid disorders[24,28]. Therefore, we conclude that liver damage may be the mechanism underlying the observed decrease in T_3 expression in diabetic mice. Diabetes suppresses thyroid hormones (T_3 and T_4) and serum insulin levels that regulate the basal metabolic rate[31].

Diabetic rats administered with dilute AHE oil or Metformin had substantially decreased T3, T4, and TSH levels compared to the control group ($p < 0.05$). Current research suggests that the diluted AHE oil has a moderating function during endocrine system disruptions. Related findings were reported in the literature study: oral treatment of *A. herba-alba* significantly increased thyroid blood levels of T3 and TSH in the protective group compared to the control group. On the other hand, it was revealed that AHA extract had a protective impact on serum T4 levels like that of the control. *A. herba-alba* includes several phytochemicals, including phenols and flavonoids, which are known to have a variety of pharmacological properties [37]. A similar role can be argued for Metformin in view of the study findings. While the exact reasons of thyroid dysfunction in diabetes mellitus are yet unclear, several elements of thyroid function can be directly impacted by metabolic changes brought on by diabetes or by insulin deficiency[28,31].

Our study showed that the single dose of alloxan was effective in inducing DM in the early stages of type 2 DM. Serum T_3 and T_4 levels dropped in short-term diabetic rats. Similar results were found in literature[32]. This demonstrates a shift in the variables that determine reduced thyroid hormone production during the early stages of insulinopeinia[33]. Short-term insulinopeinia in diabetic mice cannot be attributed to decreased TSH activation of the thyroid gland, according to researchers [33, 34]. However, dilute AHA oil or Metformin treatment independently returned blood T3 levels to normal levels, which appears to be

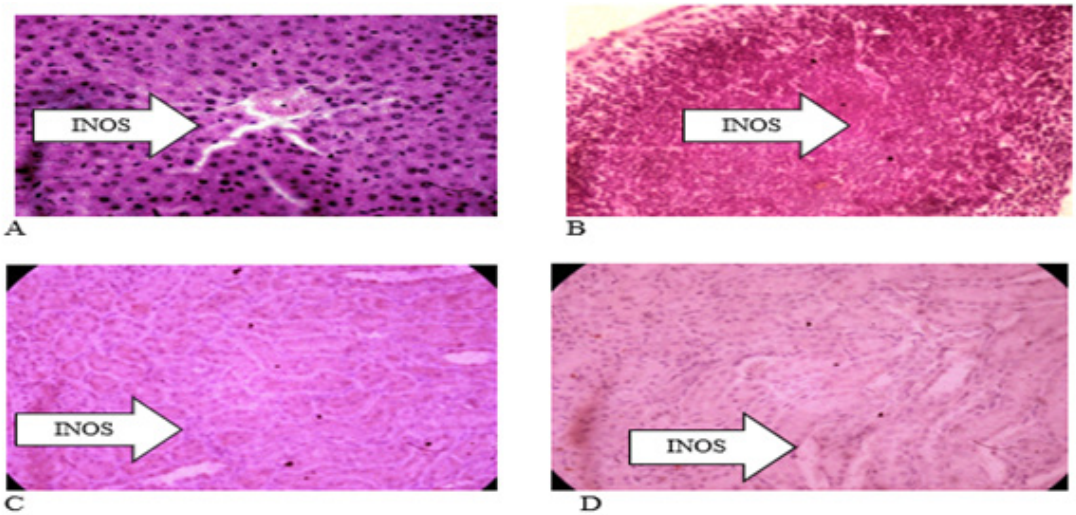

Fig. 2. Levels of INOS expression in the thyroid gland from the rate, A: control (INOS staining arrow). B: diabetic rats (INOS staining arrow). C: diabetic rats treated with diluted oil extracts of AHA (INOS staining arrow). D: diabetic rats treated with metaformin (INOS staining arrow).

related to its antioxidant activity. It is possible that the effect of the extract on raising thyroid hormone concentrations reflects its influence on increasing insulin secretion. Insulin stimulates hepatic T_4/T_3 conversion and improves the synthetic capacity of thyroid cells. Hence, the present results suggest a role for dilute AHE oil or metformin. As a result, the effect of dilute AHE oil on the increase in the level of hormones may indicate the influence on insulin secretion. Our findings indicate that dilute AHE oil participates in the peripheral control of thyroid function and may stimulate or enhance thyroid roles. However, further research is needed into the processes and modes of activity.

The results of this investigation demonstrated that diabetes significantly reduced Hsp70 expression in both the rat liver and kidney ($p < 0.05$). This conclusion is consistent with earlier investigations that reveal a limited impact of HSP70. This conclusion is consistent with earlier investigations that reveal a limited impact of HSP70[35]. We believe that diabetes may mediate its adverse effects by lowering the expression of Hsp70.

Our results indicate that treatment with dilute AHA oil and Metformin substantially ($p < 0.05$) raised the expression of Hsp70 in the livers, kidneys, and thyroid glands of rats. This finding may explain the positive changes embodied in lowered glucose levels and other associated chemicals, such as lipid profile. We believe that both treatments could restore the normal functions associated with Hsp70 and reverse some of the passive effects of diabetes. In addition, iNOS expression was significantly increased ($p < 0.05$) because of induced diabetes in the liver, kidney, and thyroid gland. However, its expression was significantly decreased ($p < 0.05$) by both treatments.

According to a research study, NO is involved in several critical body processes, including blood pressure regulation, brain neurotransmitter function, and immune system defense against viruses and bacteria[34]. But NO may also be produced excessively, or mis regulated, which adds to the etiology of several illnesses, including diabetes[34].

CONCLUSION

The outcomes of the current research indicate that the diluted AHE oil stimulates or enhances thyroid activity, and they also point to a potential role for the oil in peripheral thyroid control. From a molecular point of view, Hsp70 and iNOS seem to act in a counteracting mechanism in both disease and treatment. Therefore, these molecules are good targets for therapeutic and clinical applications[36].

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None.

Data availability

All data generated or analyzed during this study are included in this article.

Conflict of Interest

The authors declare no conflict of interest.

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