RESEARCH ARTICLE
10.5152/TrendsPharm.2024.23010

Effects of Ethanolic Extract of *Anethum graveolens* on T3, T4, and TSH Levels in Rat Model of L-Thyroxine Induced Hyperthyroidism

ABSTRACT

Background: Although *Anethum graveolens* (AG) is known for some biological activities such as antibacterial and antifungal effects, its ethnic use in Türkiye for the successful treatment of thyroid dysfunction diseases, including hyperthyroidism, has recently attracted the attention of scientists in this country. To evaluate the scientific basis of this approach, our group conducted a two-step study.

Methods: Firstly, the effective dose of AG ethanolic extract (AGEE) was determined as 300 mg/kg/day in healthy animals which caused a decrease in TSH levels after 30 days. In the current report as the second step, we studied the effects of a higher dose of AGEE in a hyperthyroidism animal model in a shorter time. AG was purchased from a local market and identified by the Pharmaceutical Botany Department. AGEE was prepared by maceration of dried leaves in ethanol and its composition was determined using LC-HRMS and GC-MS where fumaric acid and 2,4-Di-tert-buthylphenol were found to be the major compounds respectively. The hyperthyroidism model was conducted by adding 200 µg/kg/day L-thyroxine (L-thr) to the drinking water of female Wistar rats for 30 days. Afterward, 500mg/kg/day of vacuum-dried AGEE re-suspended in drinking water was applied for seven days without quitting L-thr addition (LAG group). Serum TSH, T3, and T4 levels were compared with the control group (C) and the groups which received only AGEE (AG) and L-thr (L).

Results: T3 level increased in L was decreased in LAG, the opposite was true for TSH. No difference in T4 levels was observed.

Conclusion: AGEE could be used as a potential selective inhibitor of T3.

Key Words: *Anethum graveolens*, Dill, T3, T4, TSH, Hyperthyroidism.

Ahmet Davut Aksu[1]
Fatemeh Bahadori[1,2]
Leyla Elmas[3,4]
Ahmet Ceyhan Gören[5]
Erhan Ayşan[6]

[1] *Department of Pharmaceutical Biotechnology, Bezmialem VakıfUniversity, Faculty of Pharmacy, İstanbul, Türkiye*
[2] *Department of Analytical Chemistry, İstanbul University-Cerrahpaşa, Faculty of Pharmacy, İstanbul, Türkiye*
[3] *Bezmialem Vakıf University, Faculty of Medicine, İstanbul, Türkiye*
[4] *Department of Dermatology, Hacettepe University, Faculty of Medicine, Ankara, Türkiye*
[5] *Department of Organic Chemistry, Gebze Technical University, Faculty of Science, İstanbul, Türkiye*
[6] *Department of General Surgery, Yeditepe University, Faculty of Medicine, İstanbul, Türkiye*

Corresponding author:
Fatemeh Bahadori
Address: Department of Pharmaceutical Biotechnology, Bezmialem Vakıf University, Faculty of Pharmacy, İstanbul, Türkiye; Department of Analytical Chemistry, İstanbul University-Cerrahpaşa, Faculty of Pharmacy, İstanbul, Türkiye
Email address: fatemeh.bahadori@iuc.edu.tr

Received: May 28, 2024
Revision Requested: June 10, 2024
Last Revision Received: June 10, 2024
Accepted: June 11, 2024
Publication Date: June 14, 2024

Cite this article as: Aksu AD, Bahadori F, Elmas F, et al. Effects of ethanolic extract of *Anethum graveolens* on T3, T4, and TSH levels in rat model of L-thyroxine induced hyperthyroidism. *Trends Pharm.* 2024, 1, 10, doi: 10.5152/TrendsPharm.2024.23010

INTRODUCTION

Anethum graveolens L. (AG) known as Dill *(Apiaceae)* is one of the most popular culinary herbs in the world. It is used by "Attars", the men or women who work as herbal healers in Iran, in the treatment of diabetes[1] and is used in the treatment of inflammatory gout diseases in the Najd region of Saudi Arabia.[2,3] A topical formulation prepared from the aerial parts of AG has been used in traditional medicine in Delhi, India in the management of uterus cancer.[4,5] According to many previous studies, AG possesses some pharmacological effects such as antibacterial, antifungal, anti-inflammatory, analgesic, and antioxidant.[3] Recently the successful treatment profile of patients with thyroid gland disorders who have consumed AG on a regular basis along with prescribed medications has attracted the attention of some clinicians in Türkiye.[6,7] Altay et. al. in a letter to the editor of the American Journal of Therapeutics[7] have reported a significant decrease in free triiodothyronine (T3) and free thyroxine (T4) levels in patients with any kind of thyroid disease who used one bunch of dill (100 g). However, in this study, the fresh plant material was used by patients, which could not be counted as a standardized pharmaceutical preparation. Previously also, a scientific study was conducted to determine the effects of AG ethanolic extract (AGEE) on patients with different metabolic syndromes including hypothyroidism. This study failed to show any meaningful results.[8] The contradiction between these two publications is partially resolved by another study made by Panda S.[9] This study focuses on the increase in serum concentra-

tion of insulin and glucose in corticosteroid-induced type 2 diabetes mellitus in female rats which is normalized by consumption of AGEE. This study shows that Dexamethasone-induced alterations in the levels of thyroid hormones were also reversed by the plant extract.

According to the best of our knowledge, no study reveals the effects of AGEE directly on thyroid hormones in hyperthyroidism animal models, thus, our group decided to study this subject in two steps. In the first step, our group reported the effective dose of AGEE on healthy female Wistar albino rats as 300 mg/kg/day which caused a meaningful decrease in the values of thyroid-stimulating hormone (TSH).[10] This was where no statistically meaningful change in the levels of T3 and T4 was observed. In the second step which is being reported in our current study, we report the effects of 500mg/kg/day AGEE on the T3, T4, and TSH levels of L-Thyroxine (L-thr) induced hyperthyroidism rats.[11,12] We aimed to understand the effects of doses above previously determined effective doses of AGEE on a certain disease of the thyroid gland.

MATERIAL AND METHODS

Chemicals

96% Ethanol (#159010) and Tween 80 (#1754) purchased from Merck. All standard materials used in GC-MS and LC-HRMS assays were purchased from Sigma-Aldrich with purities >99%.

Plant Material

AG was purchased from a local market in İstanbul-Türkiye and was identified by Dr. Cagla Kizilarslan Hancer in the Department of Pharmaceutical Botany, Bezmialem Vakıf University, İstanbul-Türkiye.

Preparation of AGEE and Administration to Animals

5 kilograms of plant material was dried in dry and shadow conditions, powdered, and extracted with ethanol at room temperature for 3 × 24 hours. The extract was filtrated at the end of each 24 hours and dried under vacuum at 45oC. The total w/w yield of obtained waxy dry and dark green extract was 6.5%. Since the water solubility of obtained AGEE was slightly low a 0.01% (w/w) solution of Tween 80 was prepared to emulsify the waxy extract in water. The prepared AGEE emulsion was administered orally in a cage bottle in the amount of 500 mg/kg/day prepared for each animal in 40 ml. After complete consumption of the extract by each animal the cage buttle was refreshed with drinking water.

Animals

28 four weeks old Wistar albino female rats, with weights around 190g were maintained in standard light (14 h light: 10 h dark cycle) and temperature (27 ± 2 °C) controlled room with the provision of laboratory feed and water ad libitum. Animals were maintained in accordance with the guidelines of the Central Ethics Committee on Animal Experiments (HADMEK) Republic of Türkiye Ministry of Agriculture and Forestry. Ethics committee approval was obtained from the Bezmialem Vakıf University Experimental, Animal Research Center (Approval no: 1297, Date: 2019).

Wistar rats were randomly divided into four groups. The first group (C) was fed with regular laboratory feed and evaluated as the control group. Two groups namely "L" (for L-thyroxine) and "LAG" (for (L-thr) and AGEE) were determined as hyperthyroidism model groups which were achieved by oral consumption of L-thyroxin for 30 days in 200 µg/kg/day dose in distilled water from the cage bottle. Group L did not receive any treatment. AGEE administration in the amount of 500mg/kg/day was started after 30 days and continued for seven days as a potential treatment in the group LAG. Meantime, L-thr consumption was continued. The last group, namely AG was fed with AGEE for seven days.

Before starting the animal work the average water consumed by each animal was measured as 30-40 ml. The AGEE emulsion was prepared in 30 ml to ensure its complete consumption by the animal. Distilled water was provided for animals after the complete consumption of AGEE. The fourth group signed as "AG" received only AGEE without application of L-thyroxin.

Determination of T3, T4, and TSH Levels and Water Consumption Volumes

Serum T3, T4, and TSH levels were determined using the enzyme immunoassay technique EliKine™ Thyroxine ELISA Kit (Abbkine Optics Valley International Biomedicine Park, Wuhan, China)[10]. The scarification was carried out by intra-cardiac puncture under deep terminal anesthesia by which, whole-body blood volume was obtained (≈7 ml). Plasma was obtained by centrifugation of blood samples.

The water consumption volume of each animal per day was measured for one week prior to starting the experiments to determine the volume of which, AGEE had to be dissolved in, to make sure about the complete consumption of the extract by each animal. Furthermore, the volume of water consumed by each animal was accepted as an indication of the development of the hyperthyroidism animal model. The increase in water consumption was in accordance with the progress of hyperthyroidism in animals.

Determination of the Chemical Composition of AGEE

The chemical composition of AGEE was determined using two different methods, LC-HRMS and GC-MS. The internal standard in the concentration of 100 mg/l was added to AGEE to obtain the final concentration of 3 ppm. The methyl ester derivatives were prepared before conducting the GC-MS method. In LCHRMS analysis the HPLC mobile phase consisted of A: 1% Formik Asit in H2O and Mobil Faz B: 1% Formik Asit in MeOH and Troyasil C18 HS (150 ×3 mm, 5 µm) column. The ratio of mobile phase B was increased from 50% at the zero point to 100% at the 3rd minute, kept

constant for 3 minutes, and reduced back to 50% within 1 minute and, kept constant for eight minutes. The flow rate was 0.35 ml/min. The MS analyses of LC-HRMS were carried out on a Thermo Orbitrap Q-Exactive model ESI gas chromatograph-mass spectrometer. The mass scan range was 100-900 m/z, with a Sheath gas flow rate of 45, Aux gas flow rate of 10, Sprey voltage (kV) of 3.80, Capillary temp. (°C) 320, Aux gas heater temp (°C) 320, and S-lens RF level of 50.0. The results were compared with the compound library data of Bezmialem Vakıf University, Center for Drug Research (ILMER).

GC-MS analysis was carried out in a Thermo Scientific GC-MS instrument equipped with TG-5MS 0.25µm column with the max. temperature of 330/350 °C. The mass scan area was 30-950 m/z with a Sheath gas flow rate of 45, Aux gas flow rate of 10, Sprey voltage (kV) of 3.80, and Capillary temp. (°C) 320, Aux gas heater 4 temp (°C) 320, and S-lens RF level of 50.0. The results were compared with the compound library data of Bezmialem Vakıf University, Center for Drug Research (ILMER)".

Statistical Analysis

The statistical analyses were conducted using the Statistical Package for Social Sciences version 21.0 software (IBM Corp.; Armonk, NY, USA). Group values of fT3, fT4, and TSH were evaluated by the one-way ANOVA test. The results were evaluated at the $P<.05$ significance level.

RESULTS

Determination of T3, T4, and TSH Levels and Water Consumption Volumes

Prior to starting the experiment, the amount of water consumed by each animal from the cage was measured and this observation was continued throughout the experiments. According to these observations, each animal consumed 30-40ml of water each day and the amount of consumed water was increased in accordance with the development of hyperthyroidism caused by L-thyroxine. Maximum water consumption was reached to 80 ml by each animal which was completely normalized after receiving AGEE in the LAG group. The water consumption rate of animals in different groups is given in Figure 1.

Serum TSH, T3, and T4 levels of the group consumed L-thr and AGEE (LAG) group were compared with the control group (C) and the groups which received only AGEE (AG) and L-thr (L) in the equal periods. T3, T4, and TSH levels were determined using ELISA Kit. As it is shown in Figure 2. As it can be seen in Figure 1 the levels of T3 and T4 were significantly increased following L-thr application. The increase in the T3 level was successfully ameliorated by AGEE induction, however, the change in the T4 level

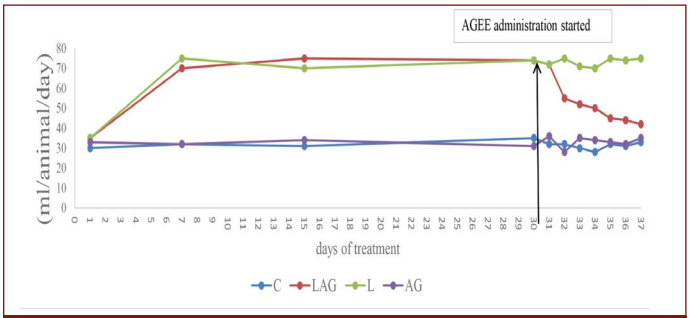

Figure 1. The Water Consumption Rate of Animals in Different Groups

was not statistically meaningful. TSH level which was decreased by L-thr application (probably as a feedback of T3 and T4 increase) was meaningfully increased by AGEE consumption. Administration of AGEE alone, however, was only able to decrease the TSH level which is in accordance with our previous study.[10]

Volatile oils are the most known bio-active constituents of AG. Some pharmacological effects of AG such as antimicrobial, antihyperlipidemic, and anti-hypocholesterolemic activities have been attributed to the volatile oil content of this traditional plant.[13] It has been reported that volatile oils bearing a hydrophobic aromatic core with hydrophilic side chains are able to make hydrogen bonds with bacterial enzymes.[14] AG-containing constituents such as dillapiole and athenol are prone to make hydrogen bonds with thyroid enzymes or probably their receptors.

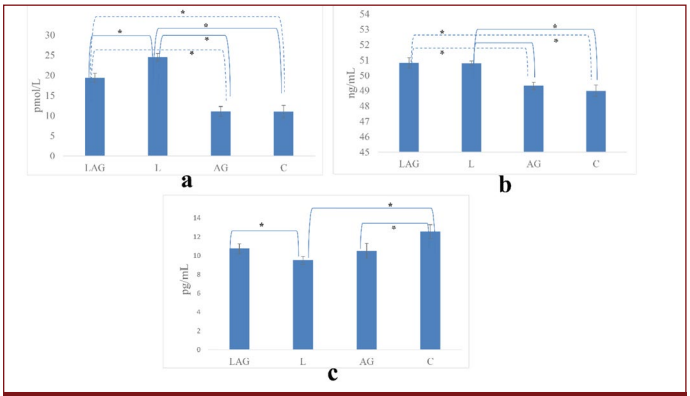

Figure 2. The Levels of Plasma a): T3, b): T4, and c): TSH in LAG, L, AG, and C Groups.

Determination of the Chemical Composition of AGEE

Table 1 shows the results obtained from the LC-HRMS analysis of AGEE. The most abundant compound was found to be fumaric acid with 3064.19 mg per kg of extract followed by Nepetin, Ascorbic acid, and Hispidulin with ratios of 9.61, 8.27, and 1.76 mg per kg of extract. Figure 3 shows

The results show a meaningful decrease in T3 level and an increase in TSH level in animals who received AGEE after L-thr application (LAG) compared to those who received L-thr alone (L). No meaningful difference was observed in the T4 level between these two groups. *:$P<.05$

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the LC-HRMS chromatogram of AGEE, and Table 2 summarizes the experimental conditions.

GC-MS analysis showed that 2,4-Di-tert-buthylphenol is the most abundant volatile component of AGEE. Table 3 shows the results of the GC-MS analysis of this extract and Figure 4 shows the related chromatogram. Figure 4 shows the GC-MS chromatogram of AGEE.

DISCUSSION

With an emphasis on its possible application as a selective inhibitor of T3 in an animal model of hyperthyroidism, the study sought to determine how Anethum graveolens ethanolic extract (AGEE) affected thyroid hormone levels. The results showed promising results, suggesting that AGEE could control levels of thyroid hormones, specifically by raising TSH and lowering T3, while having no effect on T4 levels.

Table 1. Amounts of Compounds Determined in Dill Extract (mg/kg extract) Determined by LC-HRMS

Compound	mg/kg AGEE	U %
ascorbic acid	8.27	3.94
fumaric acid	3064.19	2.88
luteolin-7-rutinoside	0.07	3.06
apigenin 7-glucoside	0.12	3.59
nepetin-7-glucoside	0.11	3.07
naringenin	0.04	4.20
nepetin	9.61	2.19
kaempferol	0.61	3.56
apigenin	0.78	2.87
hispidulin	1.76	3.41
acacetin	0.04	3.98

Table 2. The Experimental Conditions of LC-HRMS Assay

Compound	m/z	Ionization mode	Linear range	LOD/ LOQ	R^2	Recovery
Ascorbic acid	175.0248	Negatif	0.5-10	0.39/1.29	0.9988	96.2
Fumaric acid	115.0037	Negatif	0.1-10	0.05/0.17	0.9991	97.13
Luteolin 7-rutinoside	447.0933	Negatif	0.1-7	0.01/0.03	0.9961	96.31
Nepetin-7-glucoside	479.1184	Pozitif	0.05-10	0.01/0.03	0.9997	102.18
Apigenin 7-glucoside	431.0984	Negatif	0.3-7	0.01/0.03	0.9962	96.07
Naringenin	271.0612	Negatif	0.1-10	0.01/0.03	0.9995	86.65
Nepetin	315.0510	Negatif	0.05-10	0.01/0.03	0.9992	97.76
Kaempferol	285.0405	Negatif	0.5-7	0.01/0.03	0.9958	90.25
Apigenin	269.0456	Negatif	0.3-10	0.01/0.03	0.9998	81.55
Hispidulin	301.0707	Pozitif	0.05-10	0.01/0.03	0.9993	98.36
Acacetin	283.0612	Negatif	0.05-7	0.01/0.03	0.9995	87.52

Table 3. The Fatty Acid Methyl Ester Compounds Determined in GCMS and Their % Ratios in AGEE

RT	Compound	Molecular formula	% area
4.59	Butanedioic acid	$C_6H_{10}O_4$	3.27
7.10	1-Dodecene	$C_{12}H_{24}$	3.43
8.02	2-isopropyl-1-methoxy-4-methylbenzene	$C_{11}H_{16}O$	0.28
10.37	1-tridecanol	$C_{13}H_{28}O$	5.97
10.49	Undecane,2.3-dimethyl	$C_{13}H_{28}$	1.62
12.27	2,4-Di-tert-buthylphenol	$C_{14}H_{22}O$	16.78
13.43	Hexadecen-1-ol, trans-9	$C_{16}H_{32}O$	8.53
16.21	1-Octadecene	$C_{18}H_{36}$	9.90

Table 3. The Fatty Acid Methyl Ester Compounds Determined in GCMS and Their % Ratios in AGEE

RT	Compound	Molecular formula	% area
17.60	7,10,13-Hexadecaatrienoic acid	$C_{17}H_{28}O_2$	1.49
17.83	Methyl palmitoleate	$C_{17}H_{32}O_2$	1.04
17.91	Hexadecanoic acid	$C_{17}H_{34}O_2$	5.25
18.73	1-Eicosanol	$C_{20}H_{42}O$	10.25
19.28	Phytol isomer	$C_{20}H_{40}O$	0.64
19.62	Phytol	$C_{26}H_{40}O_5$	1.81
19.95	9,12-Octadecadienoic acid	$C_{19}H_{34}O_2$	2.96
20.03	9,12,15-Octadecatrienoic acid	$C_{19}H_{32}O_2$	5.07
20.30	Octadecanoic acid	$C_{19}H_{32}O_2$	0.54
20.78	9-Octadecenoic acid	$C_{20}H_{38}O_2$	0.54
21.04	Lignoceric alcohol	$C_{24}H_{50}O$	8.02
	Unknown		12.60
	Total		99.99

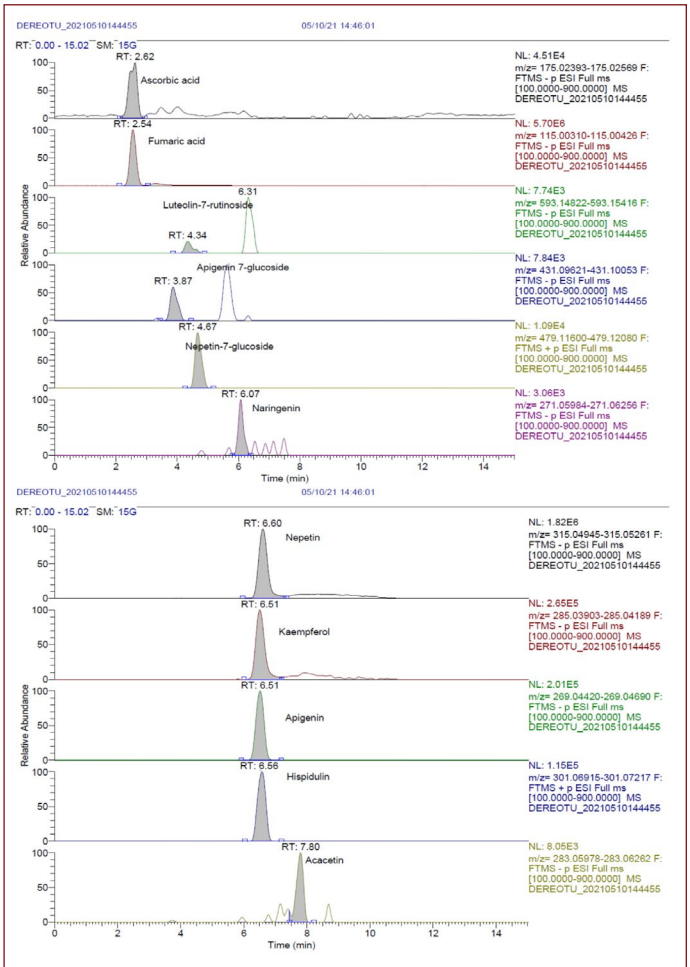

Figure 3. LC-HRMS Chromatogram of AGEE

The data showed that in the L-thyroxine (L-thr) induced hyperthyroidism model (LAG group), AGEE treatment significantly decreased T3 levels and increased TSH levels. This implies that AGEE selectively inhibits T3, an important feature for hyperthyroidism management since high T3 levels are the main cause for worry. Targeting certain thyroid dysfunctions may benefit from AGEE's more noticeable effect on T3, rather than T4, as seen by the LAG group's unchanged T4 levels as compared to the L group.

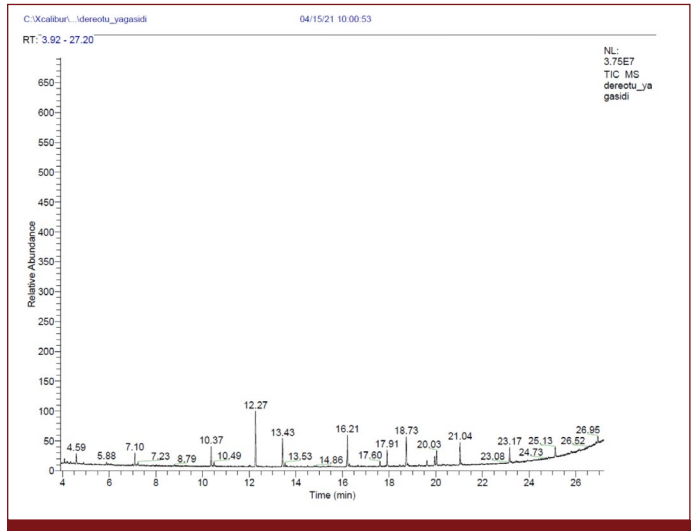

Figure 4. GC-MS Chromatogram of AGEE

Two of the main chemicals found in the AGEE's chemical composition analysis were fumaric acid and 24-Di-tert-butylphenol. These components might contribute to the pharmacological effects that have been noted. Due to its anti-inflammatory and antioxidant characteristics, fumaric acid may play a role in regulating the production or release of thyroid hormones. Furthermore, AGEE's volatile oils, like dillapiole and athenol, may interact with thyroid enzymes or their receptors to change hormone levels.[13]

The results are consistent with the effects of AG that have been documented in conventional medicine, where thyroid problems have been treated with it. Nonetheless, prior research yielded inconsistent findings concerning the effectiveness of AGEE on thyroid function. As an illustration, Altay et al.[6,7] reported significant reductions in T3 and T4 levels in patients consuming fresh dill, while a study on

metabolic syndrome patients did not show meaningful results with AGEE. The present study helps reconcile these discrepancies by demonstrating the effectiveness of AGEE in an animal model of hyperthyroidism, particularly at a higher dose (500 mg/kg/day) than previously tested.[10]

The capacity of AGEE to specifically lower T3 levels raises the possibility of therapeutic uses in the treatment of hyperthyroidism. The observation of elevated TSH levels may serve as a compensatory mechanism to offset the decrease in T3, underscoring the function of AGEE in re-establishing hormonal equilibrium. These findings call for more research to be done in clinical settings to determine the safety and effectiveness of AGEE in hyperthyroid individuals.

CONCLUSION

Although the study offers insightful information, there are several issues that need to be resolved. Seven days was a relatively short time for AGEE treatment; longer term trials are required to assess long-term effectiveness and potential negative effects. Furthermore, it will be essential to investigate the precise molecular pathways behind AGEE's influence on thyroid hormones in order to create tailored treatments.

Future studies ought to look into how AGEE affects further thyroid function factors and associated metabolic processes. Human subjects must participate in clinical trials in order to confirm preclinical results and determine dosage schedules and safety profiles.

In conclusion, using an animal model of hyperthyroidism, this study shows that AGEE significantly affects the regulation of thyroid hormones. The promise of this compound as a treatment agent for hyperthyroidism is shown by the compensatory increase in TSH levels and the selective reduction of T3 levels. These results open the door for more clinical uses of Anethum graveolens and add to the increasing body of data in favor of its medicinal usage.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of the Bezmialem Vakıf University Experimental, Animal Research Center (Approval no: 1297, Date: 2019)

Peer-review: Externally peer-reviewed.

Author Contributions: Concept - E.A.; Design - F.B., E.A.; Supervision - F.B.; Data Collection - A.D.A., L.E., A.C.G.; Analysis and/or Interpretation - L.E., A.C.G, A.D.A., F.B.; Literature Search - A.D.A., F.B., E.A., L.E.

Declaration of Interests: The authors have no conflict of interest to declare.

Funding: This work was fully supported by the grant of Bezmialem Vakıf University, Scientific Research and Development Department (Grant No: 12.2014/21).

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