Toxic Effect of Carbendazim on Thyroid of Male albino Rats with Possible Protective Role of Linalool

Eman Fawzi Aboualkhair [1], Zakaria. A .Elzawahry [1], Khaled Hamed Elmosalamy [2], Eman El-Sayed Khayal [1],Mohammad Zayed Mohammad Hassan [1]

1 Forensic Medicine and Clinical Toxicology Department, Faculty of Medicine, Zagazig University

2 Histology and Cell Biology Department, Faculty of Medicine, Zagazig University

Email: emanaboualkhair@gmail.com

Article History: Received 10th June, Accepted 5th July, published online 10th July 2023

Abstract

Background: The increasing use of toxic pesticides is a major environmental concern. Carbendazim (CBZ) is a systemic fungicide that is considered a persistent environmental contaminant. Linalool is known to reveal various pharmacological activities such as antimicrobial, anti-inflammatory, antioxidant, and anticancer properties.

Objectives: The present study investigated the protective mechanism of linalool in CBZ induced thyroid gland damage in rats.

Subjects and methods: This experimental study consisted of negative control, positive control, linalool (50 mg/kg), CBZ (500 mg/kg), and combined CBZ and linalool groups for 8 consecutive weeks. 35 rats were randomly divided into five equal groups, G (I): Negative control group: Regular diet and tap water. G (II): Positive control group: 1 ml corn oil (as a vehicle) once daily by oral gavage for 8 weeks. G (III): Linalool treated group: linalool 50 mg/kg B.W. once daily by oral gavage for 8 weeks. G (IV): Carbendazim treated group: 500 mg/kg B.W. of carbendazim (1/10 LD50) dissolved in 1ml corn oil once daily by oral gavage for 8 weeks (Oral LD50 of carbendazim in rats equal 5000 mg/kg). G (V): Carbendazim and linalool-treated group linalool 50 mg/kg then after 1 hour received carbendazim 500 mg/kg once daily by oral gavage for 8 weeks. Serum smples used for evaluating thyroid hormones (TSH, FT3, FT4). Thyroid tissues was used to prepare tissue homogenates, which were used for estimating oxidative stress biomarkers [malondialdehyde (MDA), and superoxide dismutase (SOD)], and pro-inflammatory cytokines [interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α)]. Thyroid tissues blocks were prepared for histopathological examination and immunohistochemistry (IHC) staining using anti-NF-κB and anti-TLR4 antibodies.

Results: linalool alleviated CBZ induced a decrease in SOD levels and an increase in MDA levels in thyroid tissues. These biochemical results were supported by histopathological changes which were improved by co-treatment with linalool. Immunohistochemical examination revealed that linalool significantly suppressed CBZ-mediated increase in NF-κB and TLR-4 in the thyroid tissues.

Conclusion: linalool-mediated thyroid protection in CBZ-treated rats involves antioxidant and anti-inflammatory.

Keywords: carbendazim, fungicide, linalool, thyroid, acetylcholinesterase.

DOI: 10.53555/ecb/2023.12.Si12.253

Introduction

Environmental pollution is a significant contributor to health risks worldwide. According to the World Health Organization (WHO), around 25% of the diseases that affect humans are caused by long-term exposure to environmental pollution, such as pesticides, including insecticides, fungicides, and herbicides [1].

Carbendazim (CBZ), also known as methyl N-(1H-benzimidazol-2-yl) carbamate, is a fungicide that is widely used in agriculture worldwide to control fungal infections. It is a broad-spectrum benzimidazole fungicide and is applied to various crops such as bananas, cereals, cotton, fruits, grapes, mushrooms, ornamentals, peanuts, sugar beets, soybeans, and vegetables [2]. In addition to its use in agriculture, CBZ is also used as a biocide to protect a wide range of products such as film, leather, rubber, fiber, polymerized materials, and building facades [3]**.**

Carbendazim is a major global concern due to its frequent transfer via rain to areas where it harms the environment, and animal and human health, and leaves a residual concentration in soil, fruits, and vegetables. Additionally, CBZ is a persistent environmental contaminant because of its benzimidazole ring, which is difficult to break down, leading to slow degradation. Several countries have reported that CBZ is often found in soil and water for periods ranging from 3 days to 12 months [4].

The World Health Organization (WHO) has classified CBZ as a hazardous chemical. Additionally, the European Commission has placed CBZ on its priority list of endocrine disruptors due to its toxicity on different cells, tissues, and organisms under in vitro and in vivo conditions [5,6].

Toll-like receptors (TLRs) are a group of transmembrane proteins that function as signal transduction molecules. Among them, toll-like receptor 4 (TLR4) is believed to be the primary sensor for recognizing pathogen-associated molecular patterns. The stimulation of TLR4 can activate the nuclear factor kappa beta protein (NF-κB) through either the myeloid differentiation factor 88 (MyD88)-dependent or independent pathway. NF-κB activation induces the expression of genes that encode proinflammatory cytokines, including interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α), which can lead to cell apoptosis [7].

Natural products have a long history of medicinal importance and are currently used to treat a variety of human ailments. Linalool (LINL) is a non-cyclic monoterpenoid commonly extracted from lavender (*Lavandula* spp.), rose (Rosa spp.), basil (*Ocimum basilicum*), and neroli oil (*Citrus aurantium*). Several in vivo studies have confirmed various effects of linalool on the central nervous system. It has been shown to have antimicrobial, anti-inflammatory, anticancer, and anti-oxidant properties [8].

The study aimed to investigate the potential protective effect of linalool administration on the toxic impact of carbendazim on the thyroid in adult male albino rats.

Material and methods:

Materials:

a) **Carbendazim:** A 50% pure commercial product called OCCIDOR, including inactive ingredients, was purchased from a local market in the form of white powder.
b) **Linalool:** A colorless liquid with over 97% purity of linalool was purchased from Sigma Aldrich Co in Cairo, Egypt.
c) **Corn oil:** The item was bought from a nearby market.

Animals & Experimental Design:

We conducted an experimental study using 35 adult male albino rats weighing around 150-200 gm. These rats were obtained from the animal house of the Faculty of Medicine at Zagazig University. The study was conducted in collaboration with the departments of Forensic Medicine, and Clinical Toxicology and Histology and Cell Biology department at the Faculty of Medicine, Zagazig University.

All animal procedures were reviewed by the Institutional Animal Care and Use Committee (IACUC) for appropriateness and gentleness. Approval number: **ZU-IACUC/3/F/59/2023.**

All animals were cared for in compliance with the Animal Care Guidelines and Ethical Regulations, as per "The Guide for the Care and Use of Laboratory Animals" (ILAR, 2011). Prior to the experiment, the animals were healthy and underwent 7 days of passive preliminaries to adjust to their new environment, ensure their physical well-being, and exclude any diseased animals.

The animals were kept in plastic cages, free from chemical pollution, under controlled conditions. The ambient temperature ranged from 22 ± 2 ºC, relative humidity was 50 ± 5%, and there was a 12-hour light cycle. Softwood shavings were used as bedding for the animals, and they were changed during cage cleaning to ensure that the animals remained clean. Overcrowding and isolation were avoided. The rats were fed a well-balanced diet that provided all the necessary nutrients to maintain their health before and during drug administration. The diet included bread and barley. Water was provided in clean and separate containers.

Grouping of animals:

After acclimating to their new housing, 35 rats were randomly divided into five equal groups,

G (I): Negative control group Regular diet and tap water. G (II): Positive control group: 1 ml corn oil (as a vehicle) once daily by oral gavage for 8 weeks. G (III): Linalool treated group: linalool 50 mg/kg B.W. according to Nagappana and Jayaram [9] once daily by oral gavage for 8 weeks. G (IV): Carbendazim treated group: 500 mg/kg B.W. of carbendazim (1/10 LD50) dissolved in 1ml corn oil once daily by oral gavage for 8 weeks (Oral LD50 of carbendazim in rats equal 5000 mg/kg). G (V): Carbendazim and linalool-treated group linalool 50 mg/kg then after 1 hour received carbendazim 500 mg/kg once daily by oral gavage for 8 weeks.

At the end of 8 weeks, which is 24 hours after the last dose, the rats were anesthetized with pentobarbital at the dose of 60 mg per kg of body weight. Then, 2 ml venous blood samples were collected from the animals using microcapillary glass tubes from the retro-orbital plexuses. The blood samples were left without anti-coagulant to clot at room temperature. After that, centrifugation was used to separate the serum for 10 minutes at 3000 rotation per minute (rpm) and then stored at -20°C for estimating thyroid hormones (TSH, FT3, FT4). The rats were then euthanized, and their thyroid tissues were dissected from each rat and divided into two equal parts. One part from thyroid tissues was used to prepare tissue homogenates, which were used for estimating oxidative stress biomarkers [malondialdehyde (MDA), and superoxide dismutase (SOD)], and pro-inflammatory cytokines [interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α)].

The other part of thyroid tissues were put in 10% neutral buffered formalin, which was prepared for histopathological examination.

II- Methods:

a) Thyroid hormones assay:

Serum levels of TSH, FT3, and FT4 were assayed by ELISA according to the method of Lalli and Sassone [10], Baumgartner-Parzer et al. [11], and Elsyade et al. [12]respectively. FT3 and FT4 levels were expressed as pmol/l and TSH levels were expressed as ng/ml.

b) Biochemical analysis of thyroid and brain homogenates:

The thyroid gland and cerebral hemispheres were isolated from all the groups to prepare a homogenate. An electrical homogenizer was used to prepare the homogenate by mixing 0.5 g of tissue with 5 ml of phosphate-buffered saline (PBS) at 4◦C. The homogenates were then centrifuged at 3000 rpm for 15 minutes. The resulting supernatants were collected and stored at −20◦C until further use.

Oxidative stress parameters in thyroid tissue homogenates:

- **Malondialdehyde (MDA):**

Tissue MDA was assayed using Biodiagnostic kits (Egypt), according to the method proposed by **Ohkawa et al.** [13]**.** MDA activity was expressed in umol/gm tissue.

- **Superoxide Dismutase (SOD):**

Tissue SOD activity was measured using Biodiagnostic kits (Egypt), according to the method of **Misra and Fridovich** [14]**.** SOD activity was expressed in U/mg protein.

c) Pro-inflammatory cytokines in thyroid tissue homogenates:

Tissue TNF-α and IL-6 were measured using commercial ELISA kits (catalog number: RAB0480 for TNF-α and RAB0312 for IL-6**,** Sigma Aldrich) following manufacturer instructions. The data are expressed as pg/mg protein.

Histopathological studies:

Several groups of rats were used to collect thyroid specimens, which were then fixed in neutral buffered formalin at a concentration of 10% for 24 hours. The specimens were dehydrated using ascending grades of

ethanol, followed by clearing agents like xylene I and xylene II. They were then impregnated in wax, and 5-micron thicknesses of paraffin sections were obtained using a microtome (Leica RM 2155, London, UK). The sections were stained using hematoxylin and eosin (H&E) for histopathological examination [15].

Immunohistochemical staining:

Paraffin sections of thyroid tissues from all experimental groups were dewaxed and hydrated. Immunohistochemistry (IHC) staining was performed using an Anti-NF-κB p65 antibody (ab16502) and an Anti-TLR4 antibody (ab22048), obtained from Abcam, Cambridge, UK. The staining was carried out following the manufacturer's protocols and using the DAB chromogenic agent (Expose mouse and rabbit specific specific horseradish peroxidase/ 3,3'Diaminobenzidine (HRP/DAB) detection kit, Abcam; Ready–to–use; catalog number: ab80436). Counterstaining was done using hematoxylin. For each antigen, three immuno-labeled sections were analyzed per animal, with a total of 7 animals per group.

Statistical Analysis:

The data collected were expressed as mean + SD. The statistical analysis was performed using the Epi-info statistical package program version 6.04d, January 2001, and Graph Prism version 9. One-way analysis of variance (ANOVA or F-test) was used to compare the means of more than two groups, while the Least Significant Difference (LSD) was used for comparison between two groups. Pearson correlation was used to compare between different groups.

In all the above-mentioned statistical tests, the threshold of significance was set at a 5% level (P-value). A P value of > 0.05 was considered non-significant, a p value of < 0.05 was considered significant (*), and a p value of < 0.001 was considered highly significant (**).

Results

Biochemical examination:

After conducting biochemical tests on thyroid hormones (TSH, FT3, FT4), oxidative stress biomarkers (MDA, SOD), and pro-inflammatory cytokines (TNF-α, IL-6) in thyroid tissues of the negative control, positive control, and linalool-treated groups, no significant changes ($p> 0.05$) were observed among these groups (Table 1). Therefore, we used the negative control group (I) as a point of reference for comparison with the other treated groups.

- Thyroid hormones assay:

A one-way ANOVA test revealed significant differences between the negative control group, the group treated with carbendazim, and the group treated with both carbendazim and linalool in terms of the mean values of FT3, FT4, and TSH ($p<0.001$) (Table 1).

Rats that received carbendazim treatment for 8 weeks had a significant decrease in the mean values of FT3 and FT4, and a significant increase in the mean value of TSH compared to the negative control group ($p<0.001$). Administering linalool along with carbendazim for 8 weeks significantly protected against the reduction in the mean values of FT3 and FT4, as well as the increase in TSH observed in the carbendazim group ($p<0.001$). However, the group treated with both carbendazim and linalool displayed a significant reduction in the mean values of FT3 and FT4 and significant increases in the mean value of TSH, when compared to the negative control group ($p<0.05$) (Table 1).

Oxidative stress parameters in thyroid and brain tissue homogenates

The study found significant differences in SOD and MDA levels in thyroid homogenates among the negative control group, carbendazim-treated group, and carbendazim and linalool-treated group ($p<0.001$). (Figures 3,4).

Table 1 indicated that the carbendazim-treated group had significantly lower mean values of thyroid SOD levels compared to the other groups ($p<0.001$). On the other hand, the carbendazim and linalool-treated group showed a significant increase in the mean value of thyroid SOD when compared to the carbendazim-treated group ($p<0.001$). However, the carbendazim and linalool-treated group still exhibited significantly lower SOD levels in thyroid compared to the negative control group ($p<0.05$) (Table 1).

The results also showed that the mean values of MDA in thyroid tissues were significantly higher in the carbendazim group than in both the negative control and carbendazim and linalool-treated group ($p<0.001$).

Comparing the carbendazim and linalool-treated group with the negative control group displayed significantly higher MDA levels ($p<0.001$) (Table 1).

- Pro-inflammatory cytokines in thyroid and brain tissue homogenates

The results of a one-way ANOVA analysis showed a significant difference between the negative control group, the carbendazim-treated group, and the carbendazim and linalool-treated group in terms of TNF-α and IL-6 levels in thyroid homogenates ($p<0.001$). Rats treated with carbendazim displayed a significant increase in TNF-α and IL-6 levels compared to the negative control group and the combined carbendazim and linalool-treated group ($p<0.001$). On the other hand, the carbendazim and linalool-treated group showed significantly higher levels of both TNF-α and IL-6 compared to the negative control group ($p<0.001$ for TNF-α and $p<0.05$ for IL-6) (Table 1).

The study on correlation coefficient revealed some important findings. The thyroid tissues showed a positive correlation between thyroid hormones (FT3 & FT4) and SOD, and a negative correlation between thyroid hormones (FT3 & FT4) and MDA, TNF-α, and IL-6. However, there was a negative correlation between TSH and SOD, and a positive correlation between TSH and MDA, TNF-α, and IL-6 (Table 2).

Histopathological changes of the thyroid:

Sections of thyroid glands of both control groups and linalool treated group showed normal histological configuration of variable-sized thyroid follicles lined by cuboidal epithelium with centrally rounded nuclei or flat follicular epithelium with flat nuclei. The follicles were filled with intraluminal homogenous acidophilic colloid materials. There is interfollicular tissue that is formed mainly from several parafollicular cells and a fine network of reticular fibers with numerous capillaries.

After 8 weeks of treating rats with carbendazim, the thyroid gland of these rats (Figure 9) exhibited a denuded large number of follicular epithelia within eosinophilic secretions with pyknotic some parafollicular cells. Some follicles were distended with colloid secretions with flattened epithelium. Moreover, the destruction of basement membranes of some follicles was seen devoid of their colloid secretions and invaded by inflammatory cells primarily lymphocytes and plasma cells. Hyperemic interstitial blood vessels between the follicles were also observed. Microscopic examination of the thyroid specimens of the rats of the carbendazim and linalool-treated group revealed ameliorations of thyroid glandular tissue with colloid eosinophilic secretions in their lumina beside a normal quantity of parafollicular cells. Few follicles contain vacuolated eosinophilic colloids with the presence of dilated blood vessels (Fig. 1).

Immunohistochemistry:

Immunohistochemistry staining of the thyroid gland against TLR4 and NF-κB revealed no immuno-expression in the control group and linalool-treated group. The carbendazim-treated group showed moderate establishment of immunostained cells against (TLR4) and an increased number of immuno-expressions for NF-κB. Reduction of immunoreactive levels was in carbendazim and linalool-treated groups compared with that in carbendazim treated group. The expression of TLR4 proteins was seen in follicular epithelium and within stromal cells. The immunoreactivity of NF-κB was observed mostly within nuclei of thyroid follicular epithelium (**Fig. 2)**.

Table 1: Comparison of mean values of serum levels of FT3, FT4, TSH, and levels of SOD, MDA, TNF-α, and IL-6 in thyroid tissues among negative control, positive control, and linalool-treated groups was conducted using one-way ANOVA**.**

Parameter	Negative Control group	Positive Control group	Linalool treated group	F	p-value
	Mean± SD				
FT3 (pmol/l)	8.83±0.53	8.52±0.40	8.37±0.50	1.631	0.223 NS
FT4 (pmol/l)	24.07±1.00	23.43±0.95	22.81±0.65	3.515	0.051 NS
TSH (ng/ml)	0.97±0.07	0.92±0.06	0.95±0.05	0.834	0.450 NS
T.SOD (U/mg)	225.14±3.57	224.00±3.21	224.31±7.60	0.090	0.914 NS
T.MDA (umol/gm)	1.02±.05	0.98±0.06	0.97±0.05	1.088	0.358 NS
T.TNF-α (pg/mg)	51.92±2.25	52.10±1.58	52.33±1.05	0.099	0.906 NS
T.IL-6 (pg/mg)	43.84±2.96	43.59±2.82	42.06±2.56	0.8430	0.4467 NS

All values are expressed as mean± SD, Number of rats in each group =7 rats. All values are expressed as mean± SD, Number of rats in each group=7 rats. NS: non-significant (p >0.05) FT3: free tri-iodothyronine, FT4: free tetra-iodothyronine, TSH: thyroid stimulating hormone, T.SOD: Thyroid superoxide dismutase, T.MDA: Thyroid malondialdehyde
pmol/l: Pico mol per liter, ng/ml: nanogram per milliliter, umol/gm: micro mol per gram
Pg/mg: Picogram per milligram, TNF-α: Tumor necrotic factor alpha, IL-6: Interlukin 6

Table (2): Correlation Coefficient between thyroid hormones (FT3, FT4, TSH), SOD, and MDA levels in thyroid gland tissues.

	T. SOD (U/mg)		T. MDA (umol/gm)		T. TNF-α (pg/mg)		T. IL-6 (pg/mg)	
	r	p	r	P	r	p	r	p
FT3 (pmol/l)	0.966	<0.001	-0.933	<0.001	-0.9247	<0.001	-0.8196	<0.001
FT4 (pmol/l)	0.989	<0.001	-0.958	<0.001	-0.7495	<0.001	-0.6235	<0.001
TSH (ng/ml)	-0.972	<0.001	0.953	<0.001	0.9384	<0.001	0.8240	<0.001

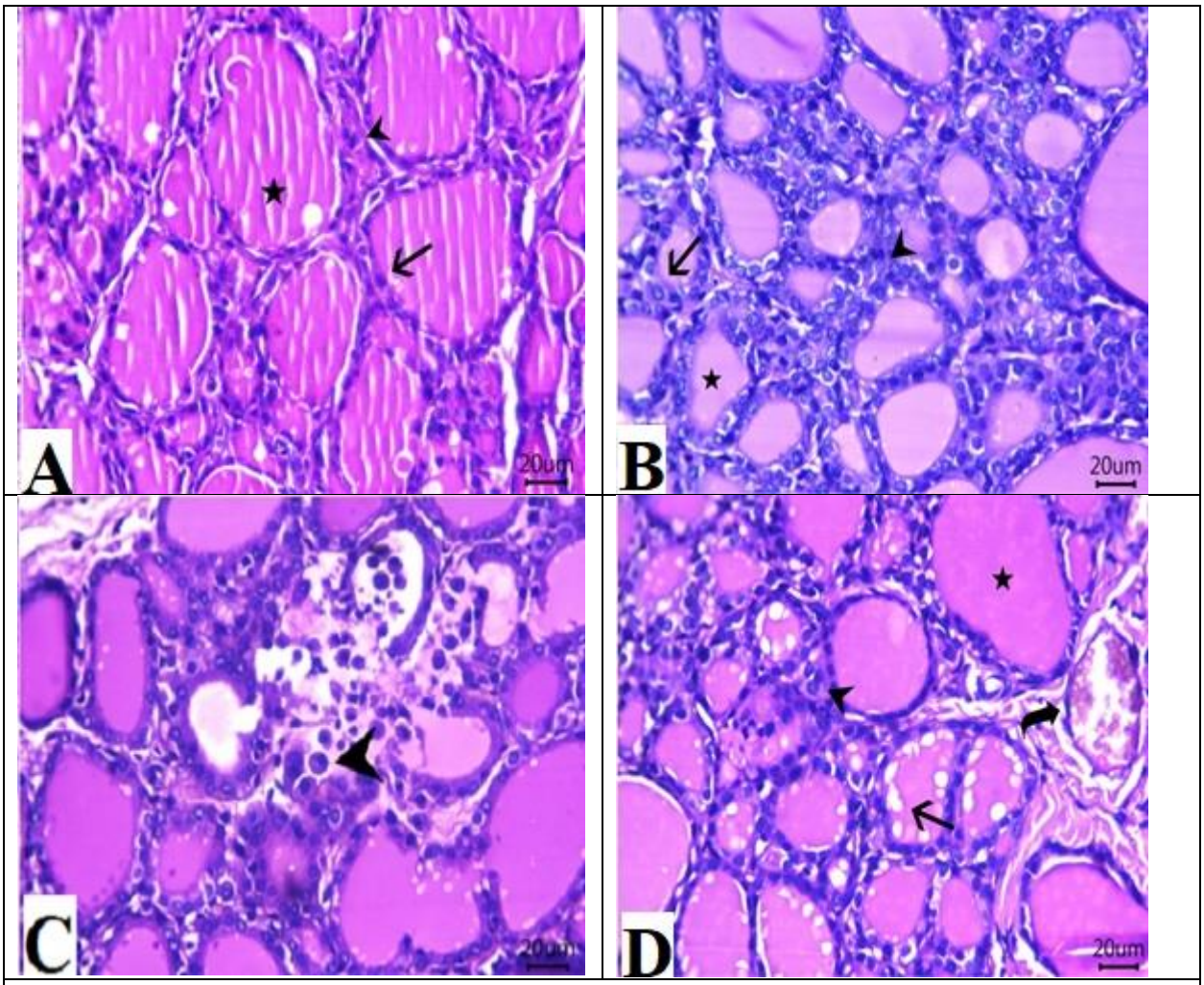

Fig.1. (A) Photomicrograph of H&E-stained sections of the control group from thyroid gland showing: A, B: Normal histomorphology of thyroid follicles with cuboidal epithelium (arrow), intraluminal homogenous acidophilic colloid (star) and Parafollicular cells (arrowhead). (B) Photomicrograph of H&E-stained sections of linalool treated group from thyroid gland showing: A, B: normal histological configuration of variable sized thyroid follicles lined by cuboidal epithelium with centrally rounded nuclei (arrow) and filled with acidophilic colloid materials (star) beside a normal number of parafollicular cells (arrowhead). (C) Photomicrograph of H&E-stained sections of carbendazim treated group from thyroid gland showing dilated interfollicular blood vessels (arrow), destruction of basement membranes of some follicles, and invasion of inflammatory cells primarily lymphocytes and plasma cells (arrowhead). (E) Photomicrograph of H&E-stained sections of carbendazim and linalool treated group from thyroid gland showing thyroid glandular tissue with colloid eosinophilic secretions (star), normal quantity of parafollicular cells (arrowhead), vacuolated eosinophilic colloid in few follicles (arrow) and dilated blood vessels (curved arrow).

Discussion

Environmental contaminants, especially pesticides, are now responsible for developing various diseases in wildlife and humans [1]. Carbendazim (CBZ) is a systemic broad-spectrum fungicide in the benzimidazole class. It is a widely used industrial fungicide that led to an overuse of CBZ in fields, resulting in adverse effects on humans and wildlife [6].

Linalool is a well-known essential oil component produced by several aromatic plants. It has been used in the fragrance industry and traditional medicine due to its ability to scavenge reactive oxygen species (ROS) and to stimulate other antioxidants from their inactive state [6,16].

The aim of the work was to study the effects of CBZ on the thyroid gland of adult albino rats and to evaluate the possible protective effect of linalool.

Thyroid hormones, including triiodothyronine (T3) and thyroxine (T4), play critical roles in growth, differentiation, development, and metabolic homeostasis, as well as behavior [12]. Tetra-iodothyronine (T4) is produced in the follicular cells of the thyroid gland, and its production is regulated by thyroid stimulating hormone (TSH), which is secreted by the pituitary gland. TSH secretion, in turn, is regulated by the thyroid-releasing hormone (TRH) produced by the hypothalamus. TSH stimulates the thyroid gland to produce T4 and T3, which are responsible for regulating the metabolism of virtually all tissues in the body. When there is a low level of thyroid hormones in the blood, the hypothalamus releases high levels of TRH, which stimulates the pituitary gland to secrete more TSH, thereby increasing the production of thyroid hormones. It is worth noting that all the serum T4 originates from the thyroid gland, while more than 80% of T3 is produced by the deiodination of T4 in other tissues [17].

The results of the present study demonstrated a significant increase in the mean values of serum TSH and a significant decrease in the mean values of serum FT3 and FT4 after administration of CBZ for 8 weeks.

The observations of the present work were in line with the results of **Salihu et al.** [18]**, Alghamdi** [1]**, and Salem et al** [19] who observed a significant increase in TSH levels with a decrease in levels of FT3 and FT4 among rats who received CBZ. Alteration of endocrine function is tightly associated with an increase of reactive oxygen species (ROS) and free radicals. However, CBZ-induced decreases in the serum T4 are probably caused by direct damage to the thyroid gland structure and function.

The results of the present study showed an increase in oxidative stress markers in the carbendazim-treated group as reflected by a significant decrease in the mean values of SOD levels and a significant increase in the mean values of MDA, IL-6, and TNFα levels in the thyroid gland tissues when compared with control groups.

Many studies observed a significant reduction in the level of SOD and a significant elevation in MDA levels in different tissues such as the testes, liver, kidney, brain, and thyroid gland after exposure to carbendazim [1,19]**.**

A possible reason for the decrease in SOD activity is the elimination of free radicals inside the cells. CBZ's inhibitory effect on SOD can also be explained by an overproduction of ROS. The lower SOD activity may also be due to an increase in the amount of reactive oxygen species and hydroxyl radicals that can deactivate SOD's chemical structure, ultimately leading to enzyme activity loss [2].

Superoxide dismutase accelerates the transformation of endogenous cytotoxic superoxide radicals to H2O2, and the increase of SOD expression levels may contribute to improving the enzyme activities in order to eliminate the superoxide radicals induced by carbendazim and to prevent the occurrence of cellular dysfunction during exposure to carbendazim [20].

Carbendazim increases oxidative stress, as the levels of ROS are significantly elevated due to the reduction in the cellular activities of antioxidant enzymes such as SOD, catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione-S- transferase (GST) and decreased concentrations of nonenzymatic antioxidant glutathione (GSH), which results in the production of free radicals, ROS and reactive nitrogen species(RNS) leading to changes in enzymatic and nonenzymatic antioxidant defenses. CBZ also increases the level of malondialdehyde, H2O2, nitric oxide (NO), and myeloperoxidase (MPO) activity by inhibiting antioxidant enzymes and lowering the nonenzymatic antioxidants including vitamins E, C, and A [5,21].

These results are consistent with elevated inflammatory markers NF-κB, IL-6, and TNFα reported in other CBZ exposure studies [19,22].

The biochemical changes observed after exposure to CBZ in this study were associated with histopathological changes with predominant inflammatory cells in thyroid tissues.

The observations of the present work were in line with the results of **Barlas et al** [23] who observed histopathological changes in the thyroid tissue of rats treated with carbendazim in the form of enlargement of the interstitial tissue, congestion, and lymphoid cell infiltration between the follicles.

These results were in accordance with **Omonona and Jarikre** [24]**, and Patil et al.** [25] who found histopathological changes in the brain tissue after carbendazim administration. They attributed this tissue damage to oxidative stress as a result of an imbalance between antioxidants and pro-oxidants in the living system. These results were in line with oxidative stress caused by CBZ in this study.

Inflammation is a common defense mechanism against various stressors and pathogenic diseases, including microbial and viral infections, radiation exposure, toxic chemicals, autoimmune and chronic diseases, and unhealthy eating habits [26]**.** There is a documented relationship between oxidative stress and inflammation, with scientific evidence suggesting that oxidative stress contributes to the development of chronic inflammatory diseases [27]**.** When the body is exposed to stress, it produces an excessive amount of ROS, which in turn triggers an inflammatory response.

Nuclear factor kappa beta is a transcription factor that regulates the expression of numerous genes, such as cytokine genes, that elaborated in immune and inflammatory responses. The disruption of cellular homeostasis leads to the activation of NF-κB via proteolytically degraded inhibitory kappa beta (IκB). As a result of NF-κB's activation, it will translocate into the nucleus for the induction of its proinflammatory cytokines [28].

The nuclear factor kappa B can also modulate oxidative stress by having anti- and prooxidant roles depending on the stress levels**.** NF-κB is involved in a variety of cellular processes like cell proliferation and apoptosis, neural development, response to infection, and inflammation. Malfunctioning of the NF-κB results in the onset of chronic inflammatory diseases like cancers and neurodegenerative disorders. The regulation of the pathway is very essential for treating these diseases [29]**.**

The nuclear factor kappa B pathway is triggered by several stimuli, the most important of which is the activation of Toll-like Receptors (TLRs), including TLR4**.** Toll-like receptors are part of the innate immune system which responds to factors derived from pathogens or cellular damage to elicit an effective defense. Endogenous molecules termed damage-associated molecular patterns are believed to activate TLRs to initiate an inflammatory response. Studies have demonstrated TLR4 activation in conditions associated with oxidative stress. TLR4 has also been implicated in the development and progression of diseases by inducing oxidative stress and endothelial dysfunction. TLR4 activation has also been reported to cause oxidative stress [30]**.**

The oxidative stress and inflammation observed in the carbendazim treated group were consistent with the immunohistochemical results of the present study that showed increased areas of immunostaining for NF-κB and TLR4 in thyroid tissues.

This study was in agreement with the findings of **Madboli and Seif** [31]**, Salem et al** [19]**, and Ebedy et al** [21] who reported positive immunoreaction for NF-κB with an increase in inflammatory markers.

All the biochemical and histopathological changes induced by carbendazim were relieved by linalool co-administration.

Linalool is a reducing agent due to its ability to scavenge ROS and to stimulate other antioxidants from their inactive state [32]**.** Several studies demonstrated the antioxidant effect of linalool in different tissues such as brain tissues. **Xu et al.** [33] found that linalool has a neuroprotective effect on cognitive deficits induced by Amyloid-beta (Aβ) by decreased levels of MDA and increased levels of SOD. **Zheng et al.** [34] described the protective effect of linalool against isoproterenol (ISO)-induced myocardial infarction in adult male rats. The treatment with linalool significantly inhibited the MDA activities as well as restoring the decreased SOD activities. Also, **Oner et al** [35] observed that linalool could remove doxorubicin (DOX)-induced cardiotoxicity by restoring MDA and SOD levels at different doses.

Furthermore, **Mohamed et al.** [32] found that linalool could protect against cisplatin-induced kidney function and tissue damage as they found that pretreatment with linalool showed significant increases in SOD and declines in MDA. These results were also consistent with the findings of **Ola and Sofolahan** [36] who found administration of linalool improved antioxidant activity evidenced by decreased levels of MDA and increased levels of SOD in hepatic tissues of rats exposed to benzene.

These results are parallel to the results of **Zheng et al.** [34] **and Mohamed et al.** [32] who found significant inhibition of NF-κB expression by linalool in myocardial tissues and renal tissues respectively. **Periyasamy et al.** [37] **and Zhang et al.** [8] reported that linalool can suppress the expression of NF-κB and TLR4, which alleviates hepatic and spinal cord injury through its anti-inflammatory and antioxidant activities.

Our results suggest that improvement in thyroid hormone levels, ACHE activity, and histopathological changes were due to reduced oxidative stress and inflammation induced by CBZ. This was achieved by increased anti-oxidant SOD and decreased inflammation caused by linalool.

Conclusion:

Carbendazim administration in a dose of 500 mg/kg/day for 8 weeks has resulted in thyroid hormones with evidence of oxidative stresses in the form of increased MDA and decreased SOD in thyroid tissues. These biochemical changes were associated with marked histopathological changes and an increase in immunostaining for NF-κB and TLR4 in thyroid tissues. Linalool administration in a dose of 50 mg/kg/day for 8 weeks caused amelioration of all these changes through its anti-oxidant and anti-inflammatory.

References:

1. Alghamdi SA. Effect of Nigella sativa and Foeniculum vulgare seeds extracts on male mice exposed to carbendazim. Saudi journal of biological sciences. 2020;27:2521–30.
2. Ezeoyili IC, Mgbenka BO, Atama CI, Ngwu GI, Madu JC, Nwani CD. Changes in brain acetylcholinesterase and oxidative stress biomarkers in African catfish exposed to carbendazim. Journal of Aquatic Animal Health. 2019;31:371–9.
3. Owumi SE, Nwozo SO, Najophe ES. Quercetin abates induction of hepatic and renal oxidative damage, inflammation, and apoptosis in carbendazim-treated rats. Toxicology Research and Application. 2019;3:2397847319849521.
4. Mo E, Ebedy YA, Ibrahim MA, Farroh KY, Hassanen EI. Newly synthesized chitosan-nanoparticles attenuate carbendazim hepatorenal toxicity in rats via activation of Nrf2/HO1 signalling pathway. Sci Rep. 2022;12:9986.
5. Sharma M, Maheshwari N, Khan FH, Mahmood R. Carbendazim toxicity in different cell lines and mammalian tissues. J Biochem Mol Toxicol. 2022;36:e23194.
6. Yossef H, Badr M, Elsdody H. Protective Effect of Ethanolic Kumquat Fruit (Fortunella margarita) Extract Against Carbendazim Induced Hepatotoxicity in Rats. Journal of Home Economics - Menofia University. 2022;32:118–30.
7. Wu Y, Liu Y, Huang H, Zhu Y, Zhang Y, Lu F, et al. Dexmedetomidine inhibits inflammatory reaction in lung tissues of septic rats by suppressing TLR4/NF-κB pathway. Mediators Inflamm. 2013;2013:562154.
8. Zhang L, Sun W, Li L, Zhang X, Tong J. Neuro-protective effect of Linalool against spinal cord injury in rats and the mechanism involved. Boletín Latinoamericano y del Caribe de Plantas Medicinales y Aromáticas; Vol 22 No 2: March 2023; 214-223 [Internet]. 2022 [cited 2024 Jan 24]; Available from: http://revistaschilenas.uchile.cl/handle/2250/215795
9. Nagappan A, Jayaram PB, Namasivayam N. Protective Effects of Linalool on Diethynitrosamine and AAF-induced Hepatocellular Carcinoma in Wistar Rats. International Journal of Engineering Research & Technology [Internet]. 2019 [cited 2024 Jan 24];8. Available from: https://www.ijert.org/research/protective-effects-of-linalool-on-diethynitrosamine-and-aaf-induced-hepatocellular-carcinoma-in-wistar-rats-IJERTV8IS110130.pdf, https://www.ijert.org/protective-effects-of-linalool-on-diethynitrosamine-and-aaf-induced-hepatocellular-carcinoma-in-wistar-rats
10. Lalli E, Sassone-Corsi P. Signal transduction and gene regulation: the nuclear response to cAMP. Journal of Biological Chemistry. 1994;269:17359–62.
11. Baumgartner-Parzer SM, Wagner L, Reining G, Sexl V, Nowotny P, Müller M, et al. Increase by tri-iodothyronine of endothelin-1, fibronectin and von Willebrand factor in cultured endothelial cells. J Endocrinol. 1997;154:231–9.
12. Elsyade RH, Zaher SM, Waly NE, Kamel MM, Wahb AMSE-D. Protective Role of Selenium versus Melatonin on Mercury Induced Toxicity on Thyroid Follicular Cells in Adult Male Albino Rat: Histological and Biochemical Study. Ain Shams Medical Journal. 2023;74:281–301.
13. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem. 1979;95:351–8.
14. Misra HP, Fridovich I. The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. J Biol Chem. 1972;247:3170–5.

15. Suvarna SK, Layton C, Bancroft JD, editors. Bancroft's theory and practice of histological techniques. Eighth edition. Amsterdam: Elsevier; 2018.
16. Khattab M. Theoretical study of the geometric and electronic characterization of carbendazim-based drug (Nocodazole). Heliyon. 2020;6.
17. Pirahanchi Y, Tariq MA, Jialal I. Physiology, Thyroid. StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2024 [cited 2024 Jan 24]. Available from: http://www.ncbi.nlm.nih.gov/books/NBK519566/
18. Salihu M, Ajayi BO, Adedara IA, de Souza D, Rocha JBT, Farombi EO. 6-Gingerol-rich fraction from Zingiber officinale ameliorates carbendazim-induced endocrine disruption and toxicity in testes and epididymis of rats. Andrologia. 2017;49.
19. Salem MA, Ismail RS, Zaki HF, Arafa HMM, El-Khatib ASN. L-carnitine extenuates endocrine disruption, inflammatory burst and oxidative stress in carbendazim-challenged male rats via upregulation of testicular StAR and FABP9, and downregulation of P38-MAPK pathways. Toxicology. 2021;457:152808.
20. Li J, Zhou X, Zhang C, Zhao Y, Zhu Y, Zhang J, et al. The effects of carbendazim on acute toxicity, development, and reproduction in Caenorhabditis elegans. Journal of Food Quality. 2020;2020:1–6.
21. Ebedy YA, Elshazly MO, Hassan NH, Ibrahim MA, Hassanen EI. Novel insights into the potential mechanisms underlying carbendazim-induced hepatorenal toxicity in rats. Journal of Biochemical and Molecular Toxicology. 2022;36:e23079.
22. Seif M, Aati H, Amer M, Ragauskas AJ, Seif A, El-Sappah AH, et al. Mitigation of Hepatotoxicity via Boosting Antioxidants and Reducing Oxidative Stress and Inflammation in Carbendazim-Treated Rats Using Adiantum Capillus-Veneris L. Extract. Molecules. 2023;28:4720.
23. Barlas N, Selmanoglu G, Kockaya A, Songür S. Effects of carbendazim on rat thyroid, parathyroid, pituitary and adrenal glands and their hormones. Human & experimental toxicology. 2002;21:217–21.
24. Omonona A, Jarikre T. Effect of Carbendazim Exposure and Vitamin E Supplementation in African Giant Rats. JAERI. 2015;4:1–9.
25. Patil NV, Lonare MK, Sharma M, Deshmukh S, Gupta K, Sharma SK. Ameliorative Effect of Quercetin on Neurotoxicogical Alterations Induced by Carbendazim: Oxidative Stress, Biochemicals, and Histopathology. Proc Natl Acad Sci, India, Sect B Biol Sci. 2023;93:351–64.
26. Vithoulkas G. An integrated perspective on transmutation of acute inflammation into chronic and the role of the microbiome. J Med Life. 2021;14:740–7.
27. Burgos-Morón E, Abad-Jiménez Z, Martinez de Maranon A, Iannantuoni F, Escribano-López I, López-Domènech S, et al. Relationship between oxidative stress, ER stress, and inflammation in type 2 diabetes: the battle continues. Journal of clinical medicine. 2019;8:1385.
28. Scalavino V, Liso M, Cavalcanti E, Gigante I, Lippolis A, Mastronardi M, et al. miR-369-3p modulates inducible nitric oxide synthase and is involved in regulation of chronic inflammatory response. Sci Rep. 2020;10:15942.
29. Biswas R, Bagchi A. NFkB pathway and inhibition: an overview. Computational Molecular Biology. 2016;6.
30. Pushpakumar S, Ren L, Kundu S, Gamon A, Tyagi SC, Sen U. Toll-like Receptor 4 Deficiency Reduces Oxidative Stress and Macrophage Mediated Inflammation in Hypertensive Kidney. Sci Rep. 2017;7:6349.
31. Madboli AE-NA, Seif MM. Adiantum capillus-veneris Linn protects female reproductive system against carbendazim toxicity in rats: immunohistochemical, histopathological, and pathophysiological studies. Environ Sci Pollut Res Int. 2021;28:19768–82.
32. Mohamed ME, Abduldaium YS, Younis NS. Ameliorative Effect of Linalool in Cisplatin-Induced Nephrotoxicity: The Role of HMGB1/TLR4/NF-κB and Nrf2/HO1 Pathways. Biomolecules. 2020;10:1488.
33. Xu P, Wang K, Lu C, Dong L, Gao L, Yan M, et al. The Protective Effect of Lavender Essential Oil and Its Main Component Linalool against the Cognitive Deficits Induced by D-Galactose and Aluminum Trichloride in Mice. Evid Based Complement Alternat Med. 2017;2017:7426538.
34. Zheng X-H, Liu C-P, Hao Z-G, Wang Y-F, Li X-L. Protective effect and mechanistic evaluation of linalool against acute myocardial ischemia and reperfusion injury in rats. RSC Adv. 2017;7:34473–81.

35. Oner Z, Altınoz E, Elbe H, Ekinci N. The protective and therapeutic effects of linalool against doxorubicin-induced cardiotoxicity in Wistar albino rats. Hum Exp Toxicol. 2019;38:803–13.
36. Ola OS, Sofolahan TA. A monoterpene antioxidant, linalool, mitigates benzene-induced oxidative toxicities on hematology and liver of male rats. Egyptian Journal of Basic and Applied Sciences. 2021;8:39–53.
37. Periyasamy T, Sathibabu Uddandrao VV, Ponnusamy C, Ganapathy S, Pudhupalayam SP, Singaravel S, et al. Linalool Mitigated High-Fat Diet–Induced Non-alcoholic Fatty Liver Disease by Regulating the Intestinal-Hepatic Axis via TGF-β/NF-kB/TLR4/ZO-1 Pathway. Rev Bras Farmacogn. 2023;33:617–28.