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Protective Effects of *Hibiscus sabdariffa* L. Against Chlorpyrifos-Induced Thyroid Dysfunction: A Comparative Study of Antioxidant Efficacy

Abstract

Hibiscus sabdariffa L. calyces (H. sabdariffa), widely consumed as a herbal tea, are recognized for their rich content of antioxidant phytochemicals and associated therapeutic properties. This study aimed to evaluate the potential protective effects of *H. sabdariffa* extract on thyroid function under conditions of oxidative stress induced by chlorpyrifos (CPF) exposure. A total of thirty rats were randomly assigned to five groups. The control group (G1) received no treatment. Group (G2) was administered CPF at a dose of 10 mg/kg/day. Groups (G3) and (G4) received *H. sabdariffa* extract at doses of 250 mg/kg and 750 mg/kg, respectively, in combination with CPF. Group (G5) received vitamin C at a dose of 100 mg/kg along with CPF. All treatments were administered orally for 28 days. At the end of the experimental period, blood samples were collected to assess serum levels of thyroid-stimulating hormone (TSH), triiodothyronine (T3), and thyroxine (T4). Additionally, thyroid tissues were collected for histopathological examination. The results demonstrated that groups G3, G4, and G5 exhibited no significant alterations in thyroid hormone levels compared to the control group, whereas G2 showed a significant reduction in T3, T4, and TSH levels. Histological findings further supported these results, revealing notable improvements in thyroid architecture compared to G2. In conclusion, the findings suggest that *H. sabdariffa* extract exerts a protective effect against CPF-induced oxidative damage to the thyroid gland. Its efficacy was comparable to that of vitamin C. Moreover, no significant difference was observed between the two administered doses of *H. sabdariffa* extract.

Keywords: *Hibiscus sabdariffa* L, Oxidative Stress; Thyroid Hormones.

Article Info.

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Introduction

Herbal medicine has been a respected healing practice for centuries and remains widely used today. Its natural compounds promote health, and due to their proven benefits, safety, affordability, and accessibility, herbal treatments are valuable in both traditional and modern healthcare systems (1). One of the most significant plants in this context is *H. sabdariffa*, a member of the Malvaceae family, which encompasses over 300 species (2). This remarkable plant has been utilized for over 3,000 years, both as a source of nourishment and as a therapeutic remedy (3). It is believed to be native to West Africa, particularly western Sudan, although some scholars argue that its origins may lie in India. Today, this species is extensively cultivated in tropical regions worldwide, including Arab nations such as Saudi Arabia, Sudan, and Egypt, as well as in various other countries such as Mexico, India, the Philippines, China, Thailand, and many others (4). This plant is known by a variety of names across different regions, including Sour Tea, Gujarat, Karkade, Roselle, Bissap, and Red Sorrel (5). Calyces, with their elevated bioactive compound concentration, are the most exploited plant component. Consequently, these calyces serve as the primary raw material in the formulation of the renowned tea (6). They are rich in phytochemicals, especially polyphenolic compounds such as flavonoids and phenolic acids. Additionally, they contain organic acids, vitamins, minerals, and essential nutrients (carbohydrates, proteins, and fats), providing approximately 49 calories per 100 grams (7).

Anthocyanidin, the main flavonoid in *H. sabdariffa* calyces, gives the extract its deep red color (8). This water-soluble pigment is packed with beneficial compounds and is safe even at high doses (9). They are primarily found as glycosides, notably delphinidin-3-O-sambubioside (hibiscin) and cyanidin-3-O-sambubioside (gossypicyanin) (10). Anthocyanins contribute to the potent antioxidant properties observed, effectively neutralizing free radicals, chelating metals, and other pro-oxidant agents. These capacities originate from the unique structural features of polyphenolic compounds, which facilitate the rapid donation of hydrogen atoms (electrons), thereby stabilizing reactive species (11).

Much research has dealt with the therapeutic uses of *H. sabdariffa*, including cardioprotective (12), prevention and treatment of high blood lipids (13), as a vasodilator (14), exhibits diuretic effects (15), hepatoprotective (16), antidiabetic (17), neuroprotective (18), improve hematological parameters (19), decreased risk of overweight and obesity, (20), stimulate insulin secretion and inhibit glucagon secretion (21), anti-inflammatory (22), and antioxidant properties (23).
Chlorpyrifos irreversibly inhibits acetylcholinesterase, leading to accumulation of acetylcholine at nerve endings and neuromuscular junctions, which is associated with neurotoxicity (24). Moreover, CPF inflicts cellular damage through the induction of oxidative stress (OS) (25). This OS may result from either an increased production of Reactive Oxygen Species (ROS) (26) or a diminished Antioxidant System Capacity (27). In either case, the intracellular accumulation of ROS adversely affects cellular function and can ultimately lead to carcinogenesis or cell death (28). The antioxidant system (AOS) removes excess ROS from various sources and plays a crucial role in preserving cellular vitality against potential damage induced by OS (29). Therefore, it has become essential to enhance this system and support its functions. In this context, natural extracts rich in antioxidants play a crucial role (30), with *H. sabdariffa* extract being one of the most significant.

This study's objective was to assess the antioxidant properties of an *H. sabdariffa* extract prepared using traditional methods and examine its potential to shield the thyroid gland from CPF-induced oxidative damage. Moreover, the mitigation effects were compared with those of vitamin C.

Materials and Methods

Materials:

Vitamin C, Chlorpyrifos, and Dried calyces of *H. sabdariffa* were bought from the local Ashar market in Basrah state, Iraq.

The kit that was used to examine the T3, T4, and TSH in the rat serum was purchased from Sunlong Biotech CO., Ltd., China.

Animal handling.

The experiment commenced with 30 adult male rats (weighing 175-200 g) purchased from an animal house farm in Tikrit, Iraq. After the rats arrived at the animal house, they were kept in plastic cages prepared earlier with wooden chips and a 24-hour free source of drinking water and food. The temperature maintained in the animal's house is between 20 and 24 C^{o}. The weights of the rats were measured weekly, beginning one day before the start of the treatments, until the completion of the experiment period, as shown in Figure 1 (31). Following a two-week adaptation period, rats were divided randomly into five groups, each consisting of six rats. All treatments are given orally and continue for 28 days, as shown in Table 1.

The animals are handled according to ethical principles of the International Ethical Guidance for Health-related research involving humans or animals, prepared by the Council of International Organizations of Medical Science (CIOMC) in collaboration with the World Health Organization (WHO), and following the framework of the Office International des Epizootic (OIE) principles. Authorized by the University of Basra/College of Pharmacy in Approval number EC50 on 1/9/2023

Aqueous extract of *H. sabdariffa* preparation*:*

The aqueous extract of *H. sabdariffa* was prepared using the traditional method commonly employed by the general public to make hibiscus tea. Dried calyces were soaked in hot water and left to steep for 1–2 hours (32). Afterward, the infusion was filtered to separate the liquid from the wet calyces. The solution was then concentrated by evaporating the water in a water bath maintained at a temperature not exceeding 70 °C until a dark red residue was obtained (33).

After weighing the resulting precipitate, an appropriate volume of water is added based on the ' 'residue's weight to achieve the desired weight/volume (w/v) concentration. This procedure ensures the preparation of a solution containing the required amount of *H sabdariffa* extract per milliliter for oral administration to the rats in groups G3 and G4.

The doses are freshly prepared each day and carefully transferred to the animal housing facility in black glass dropper bottles, each clearly labeled with its specific concentration. The solutions are then administered to the rats by oral gavage using a 1 mL syringe.

Table 1: Group design dividing the rats (30 rats) into 5 groups (each group has six rats), the dosing is given orally and continues for 28 days.

Group name	Group type	Group treatment	Dose reference
G1	Negative control	Receive no treatment, only food and water	
G2	toxic control	Receive CPF 10 mg/kg/day.	(34)
G3	Experiment group	Receive *H. sabdariffa* extract 250 mg/kg/day + CPF 10 mg/kg/day.	(35)
G4	Experiment group	Receive *H. sabdariffa* extract 750 mg/kg/day + CPF 10 mg/kg/day.	(36)
G5	antioxidant treatment group	Receive Vit C 100 mg/kg/day + CPF 10 mg/kg/day.	(37)

CPF (chlorpyrifos), Vit C (vitamin C).

Serum sampling

After the 28-day trial, rats were sacrificed for blood samples, which were centrifuged at 5000 RPM for 20 minutes. The serum was stored at -20 °C until the testing process was conducted.

Biochemical analysis

Serum levels of thyroid-stimulating hormone (TSH), triiodothyronine (T3), and thyroxine (T4) were analyzed by the Enzyme-linked Immunosorbent Assay (ELISA) method.

Tissue Sampling.

Tissue samples were obtained from the thyroid gland, and all collected samples were washed with normal saline and fixed in a 10% formaldehyde solution. They were dehydrated with ethanol and cleared with xylene to allow paraffin infiltration at 60°C. After cooling to 20°C, the solid block was cut into thin sections (4-6 µm) and placed on glass slides for Hematoxylin and Eosin staining (38).

Statistical analysis

The analysis of the results was performed using IBM's SPSS software, specifically employing One-Way Analysis of Variance (ANOVA) in conjunction with the Tukey Post-Hoc Test to evaluate mean differences among multiple groups. A p-value of less than 0.05 was established to indicate the presence of statistically significant differences between groups. The data are presented as mean ± SE (Standard Error of the Mean) (27).

Results

Rats' Weight Throughout Experiment Periods.

No statistically significant differences in 'rats' weight were observed among the groups at the beginning and the end of the experiment, as shown in Figure 1.

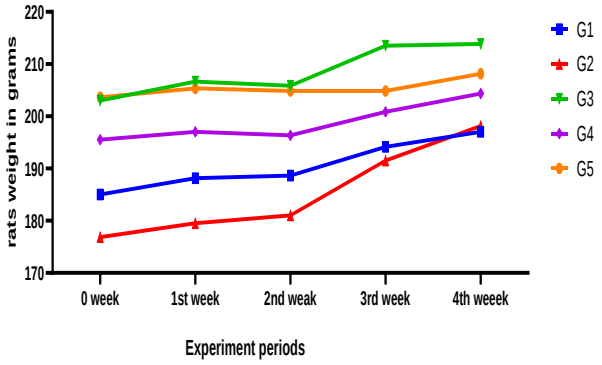

Figure 1: The mean weight of rats increased across all groups during the experiment period.

The Thyroid Protection Effect of HBSC Extract:

There is no statistically significant difference in (T3, T4, and TSH) levels between the G3 and G4 groups when compared to the control group (G1), nor when compared to the G5 group. In contrast, a significant decrease in these hormone levels in the G2 group relative to all other groups (G1, G3, G4, and G5). Furthermore, no significant differences in hormone levels were found between the G3 and G4 groups. The G5 group also did not exhibit any significant difference from the control group (G1). These findings are illustrated in Figures 2, 3, and 4.

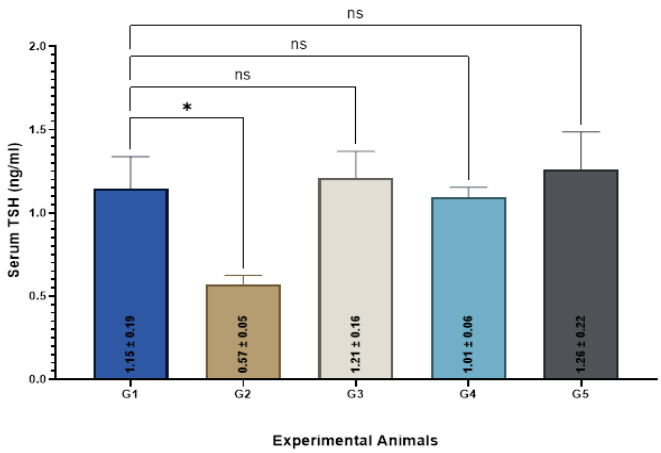

Figure 2: Only group G2 showed a significant reduction in TSH level below other groups, while other groups (G1, G3, G4, and G5) showed no significant differences between them. Expressed values as mean ± SEM, (*) indicating p-value <0.05.

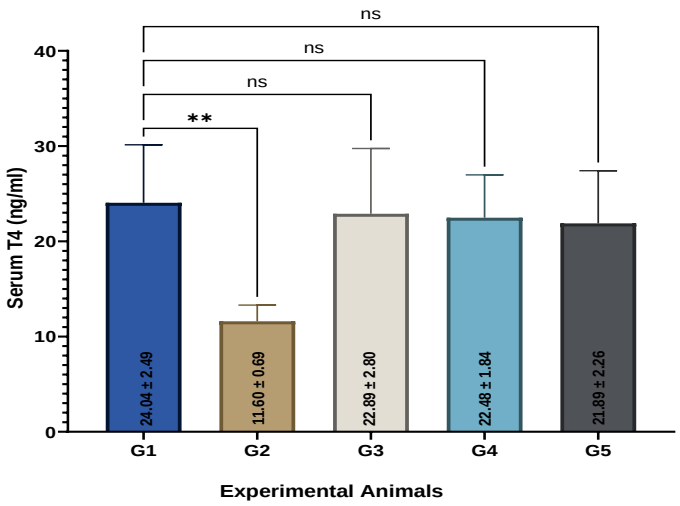

Figure 3: Only group G2 showed a significant reduction in T4 level below other groups, while other groups (G1, G3, G4, and G5) showed no significant differences between them. Expressed values as mean ± SEM, (**) indicating p-value <0.05.

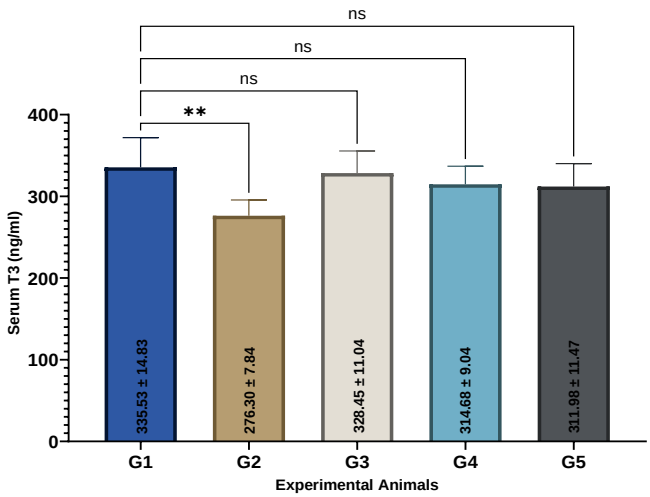

Figure 4: Only group G2 showed a significant reduction in T3 level below other groups, while other groups (G1, G3, G4, and G5) showed no significant differences between them. Expressed values as mean ± SEM, (**) indicating p-value <0.05.

Histopathological study of thyroid section:

Figures 5, 6, 7, 8, and 9 present light micrographs of the thyroid gland tissue samples stained with hematoxylin and eosin (H&E) at a magnification of 40X. Figure 5 illustrates the thyroid architecture of group G1 (control). The thyroid follicles in this group exhibit a normal parenchymal structure, characterized by intact, circular or oval shapes that vary in size, are filled with colloid, and are surrounded by a thin layer of cuboidal epithelial cells.

In contrast, Figure 6 depicts the thyroid sample from group G2, which exhibited significant destructive alterations in the thyroid parenchyma, evidenced by irregularly shaped follicles, a high proportion of

follicle lumens with diminished or diffuse colloid, and some empty lumens encircled by a single layer of flattened cells.

Furthermore, degenerative changes were observed in the connective tissue within the interfollicular spaces. Figure 7 shows the thyroid section from group G3, indicating a restoration of the architectural integrity of the thyroid follicles, which were filled with colloid and lined by a cuboidal epithelial layer. Figure 8 presents the thyroid sample from group G4, revealing some thyroid follicles filled with colloid, alongside evidence of some hemorrhage. Finally, Figure 9 illustrates the thyroid tissue from group G5, demonstrating normal thyroid follicles filled with colloid.

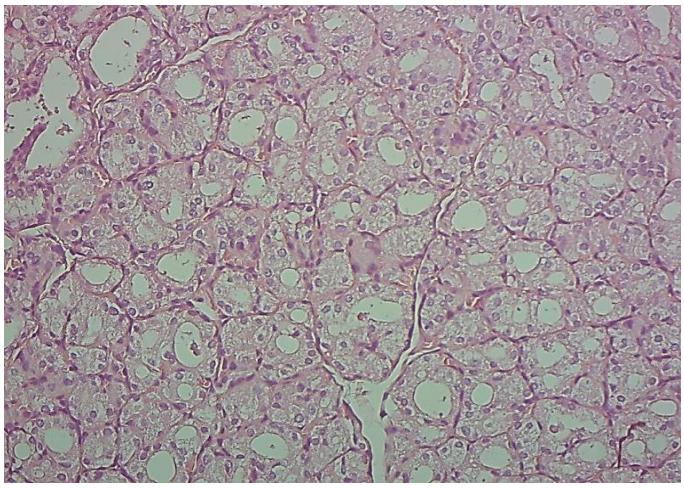

Figure 5: (H&E stain, 40X) for group G1 (control), which did not receive chlorpyrifos (CPF), reveals a normal parenchymal architecture. The thyroid follicles are intact, circular or oval configurations, vary in size, filled with colloid, and are encased by a thin layer of cuboidal epithelial cells, as indicated by the white arrow.

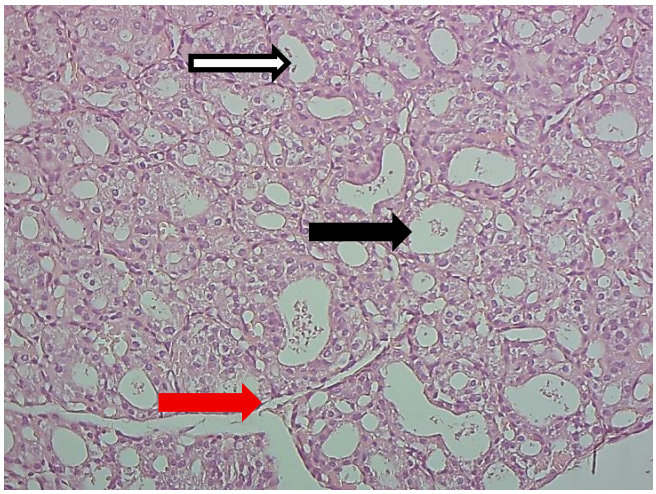

Figure 6: The microscopical examination (H&E stain, 40X) of the thyroid sample from group G2 showed a destructive effect on the thyroid parenchyma, characterized by irregular follicles, a high percentage of follicle lumens appear with low colloid or diffused, and some lumens are empty as indicated with the white arrow surrounded by a single line of flattened cells (black arrow). Additionally, the interfollicular spaces displayed degenerative changes in the connective tissue (red arrow).

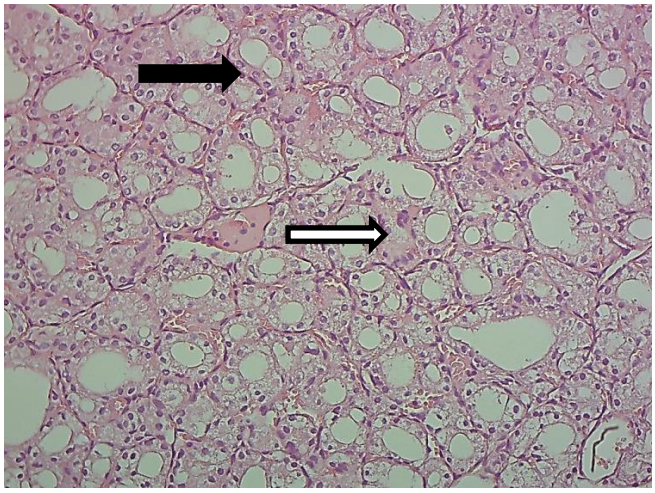

Figure 7:(H&E stain, 40X) of the thyroid section from G3 shows improvement in the architecture of thyroid follicles, filled with colloid and lined by cuboidal thyrocytes. As shown in the (white and black arrows).

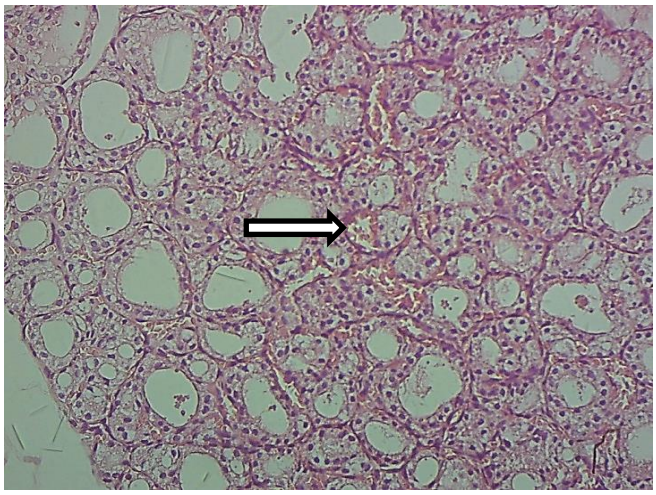

Figure 8: (H&E stain, 40X) of the thyroid sample of the G4 group shows some filled thyroid follicles (white arrow).

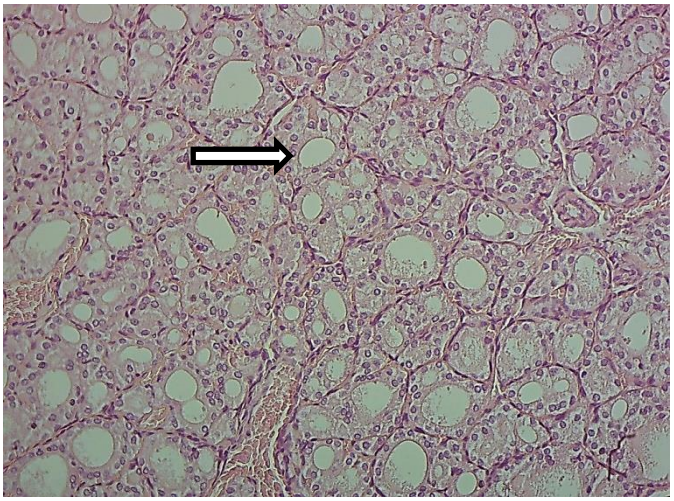

Figure 9: (H&E stain, 40X) of thyroid tissue of G5, shows normal thyroid follicles fully with colloid (white arrow).

Discussion

Hormonal levels analysis obtained from the G2 group, which was administered CPF, showed a significant reduction in T3 and T4 hormone levels relative to group G1 (which serves as the criterion for comparison). This finding suggests that the oxidative stress induced by CPF has led to direct dysfunction of the thyroid cells, resulting in decreased T3 and T4. Furthermore, the results indicate that TSH levels secreted by the pituitary gland are also significantly lower in G2 compared to G1. This is the opposite of what is expected, that TSH would increase in response to the feedback mechanism triggered by the decreased levels of T3 and T4. This observation may indicate that the pituitary gland has sustained damage due to oxidative stress or that the thyrotropin-releasing hormone (TRH) from the hypothalamus has diminished as a result of hypothalamic impairment, ultimately leading to a reduction in TSH secretion by the pituitary gland. Regardless of the underlying mechanism of the defect observed in the G2 group, it is established that oxidative stress is the causative factor. The findings align with a previous study, which indicated that CPF use induces oxidative stress in rats and results in decreased serum (T3, T4, and TSH) hormone levels (39).

Notably, the incorporation of vitamin C as a protective agent for the G5 group has nearly restored the measurements to the standard levels found in the G1 group, resulting in no significant differences in the readings of (TSH, T3, and T4). These results align with what was found, that vitamin C, when used as an antioxidant, can restore thyroid hormone levels to normal and protect the structure of thyroid tissue (40).

Analysis of the data from groups G3 and G4 indicates that *H.sabdariffa* extract exhibits antioxidant activity comparable to that of vitamin C, effectively mitigating oxidative stress induced by chlorpyrifos (CPF). Hormonal measurements of TSH, T3, and T4 in these two groups show no significant differences when compared to the G5 group, which received vitamin C. Notably, the *H. sabdariffa* extract appears to normalize hormone levels in G3 and G4, bringing them closer to the baseline values observed in the control group (G1). This effect may be attributed to the presence of various bioactive constituents in the extract—such as phenolic acids, flavonoids, and organic acids—which are known for their potent antioxidant properties, like those of vitamin C.

These results align with the previous findings regarding the effectiveness of *H. sabdariffa* as a powerful antioxidant against oxidative stress (41). The findings related to hormone measurement indicate that there are no significant differences observed between G3 and G4 when the dosage is increased from 250 mg/kg to 750 mg/kg, which indicates the effectiveness of the extract even in low concentrations.

The above hormone analyses are consistent with the findings obtained from the histological examination of the thyroid gland. The G2 group exhibits considerable destruction of the gland's functional tissues, attributable to oxidative stress induced by CPF. This also occurred when CPF was used by El-Kerdasy and caused oxidative damage to the thyroid gland structure (42).

The administration of a powerful antioxidant, specifically vitamin C, markedly mitigates the damage linked to CPF in Group G5. Subsequent analyses reveal a significant improvement in the histological evaluation of the thyroid gland. Similar enhancements in thyroid tissue were also

noted in Groups 3 and 4, which were treated with *H. sabdariffa* extract, known for its antioxidant properties and protective effects akin to those of vitamin C, thereby providing defense to thyroid gland tissues against oxidative stress induced by CPF. The protective effect of Hibiscus extract on the thyroid gland is likely due to its richness in bioactive compounds, which confer strong antioxidant and anti-inflammatory properties. These properties enable it to counteract the oxidative damage induced by CPF as a pro-oxidant agent (43).

Conclusion

The findings of this study demonstrate that *H sabdariffa* extract possesses significant antioxidant properties capable of mitigating chlorpyrifos-induced oxidative stress and protecting thyroid function in rats. The traditional preparation method of *H. sabdariffa* tea effectively yields a solution rich in active compounds, particularly anthocyanins, which contribute to its therapeutic potential. The extract supported the rats' endogenous antioxidant defense system. It preserved normal thyroid hormone levels and histological structure, comparable to the effects observed with vitamin C. Considering the increasing prevalence of environmental oxidative stressors and the well-established safety of *H. sabdariffa* as a herbal remedy, its inclusion in the daily diet may offer a low-risk, natural strategy for supporting thyroid health and overall physiological balance.

Conflict of interest

The author reported no potential conflict of interest.

Ethical Clearance

This work is approved by The Research Ethical Committee.

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الدور الوقائي لمستخلص كؤوس نبات الكركدية (*Hibiscus sabdariffa* L.) ضد الخلل الوظيفي للغدة الدرقية المستحث بالكلوربايرفوس: دراسة مقارنة لفعالية التأثيرات المضادة للأكسدة

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الخلاصة

تُعد الكؤوس الزهرية لنبات الكركدية (*Hibiscus sabdariffa* L.) مصدرًا غنيًا بالمركبات الفعالة، لاسيما مضادات الأكسدة، وقد استُخدمت منذ القدم وحتى اليوم كمشروب عشبي طبي شائع في مختلف أنحاء العالم. تهدف هذه الدراسة إلى تقييم الدور الوقائي لمستخلص الكركدية في حماية وظيفة الغدة الدرقية تحت تأثير الإجهاد التأكسدي الناتج عن التعرض لمبيد الحشري الكلوربايرفوس(CPF) . تم تقسيم ثلاثين جرذًا إلى خمس مجموعات، المجموعة الأولى G1 هي المجموعة الضابطة، بينما تم إعطاء المجموعة G2 CPF فقط بجرعة 10 ملغ/كغ/يوم، وتلقت المجموعتان (G3) و (G4) مستخلص الكركدية بجرعات 250 ملغ/كغ و750 ملغ/كغ على التوالي، وتم إعطاء المجموعة (G5) فيتامين C بجرعة 100 ملغ/كغ. تم إعطاء CPF للمجاميع G3 ,G4 وG5 نفس الجرعة التي تم إعطاؤها للمجموعةG2 . جميع الجرعات كانت عن طريق الفم لمدة 28 يومًا. في نهاية التجربة، تم التضحية بالفئران لتحليل مستوياتTSH ، T3 وT4 في المصل وكذلك تم جمع عينات من نسيج الغدة الدرقية للفحص النسيجي. تشير النتائج إلى عدم وجود تغيير في مستويات هرمونات الغدة الدرقية T3 , T4وTSH في المجموعتين G3وG4 مقارنةً بالمجموعة الضابطة والمجموعة G5, على النقيض، هناك انخفاض كبير في مستويات هذه الهرمونات في المجموعةG2 . الفحص النسيجي لنماذج من الغدة الدرقية للجرذان تدعم نتائج التحليل الهرموني. الخاتمة: تشير النتائج الهرمونية والنسيجية إلى أن مستخلص الكؤوس الزهرية لنبات الكركدية قد يقدم تأثيرات وقائية ضد الضرر التأكسدي الناتج عن الكلوربايرفوس على الغدة الدرقية، مماثلة لتلك التي يتمتع بها فيتامينC ، وهو مضاد أكسدة معروف. كذلك اشارت النتائج الى عدم وجود فرق بفعالية المستخلص ذو دلالة إحصائية عند زيادة التركيز من 250 الى 750 ملغ/كغم من وزن الجرذ.

الكلمات المفتاحية: الكركدية، الغدة الدرقية، الاجهاد التأكسدي

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