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Comparative analysis of the antithyroid activity of *Annona squamosa* and *Tetracarpidium conophorum* leaf extracts in *in-vitro* and animal model of hyperthyroidism.

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Abstract

Annona squamosa and *Tetracarpidium conophorum* are medicinal plants that are traditionally used to treat hyperthyroidism. This study comparatively investigated the antithyroid activities of methanol leaf extracts of *A. squamosa* and *T. conophorum* in in-vitro and animal model of hyperthyroidism. The phytochemical constituents and total phenolic contents of both extracts were determined. The effective inhibitory concentrations (IC50) of both extracts on thyroid peroxidase enzyme activity and DPPH radical were determined. In vivo hyperthyroidism was induced with 600 ug/kg L-thyroxin for 12 days and the effect of both extracts on thyroid stimulating hormone (TSH), serum thyroid hormones (T3, T4), liver and kidney function biomarkers and body weight were determined. Propylthiouracil (PTU) 10 mg/kg was used as reference drug. *A. squamosa* extract showed a higher total phenolic content and DPPH radical scavenging activity than *T. conophorum*. Both extracts exhibited dose-dependent inhibition of thyroid peroxidase enzyme, with *T. conophorum* being more potent. In L-thyroxine-induced hyperthyroid rats, the extracts caused dose-dependent reductions in serum T3, T4, and liver enzymes, with *A. squamosa* exhibiting superior activity. Serum TSH levels increased in a dose-dependent manner in both extract-treated groups. *A. squamosa* extract (400 mg/kg) restored hyperthyroidism-induced alterations in body weight, kidney function, and lipid peroxidation to normal levels. These results suggest that *A. squamosa* and *T. conophorum* possess antithyroid activity, with *A. squamosa* showing more potent effects. These findings highlight the potential of *A. squamosa* and *T. conophorum* as adjunctive therapies for managing hyperthyroidism.

Keywords: Hyperthyroidism; *Annona squamosa*; *Tetracarpidium conophorum*; Antithyroid activity; Thyroid hormones; Propylthiouracil; Oxidative stress

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1. Introduction

In Nigeria, thyroid disorders are the second most prevalent endocrine issue, following diabetes mellitus (Okafor *et al*., 2019). These disorders are often referred to as "great masqueraders" because of their diverse clinical manifestations (Revathy *et al*. 2015), which include hypothyroidism, goiter, and hyperthyroidism. Hyperthyroidism is characterized by excessive production and release of thyroid hormones by the thyroid gland (Lee and Ananthakrishnan, 2022). The overall incidence is estimated to range from 0.05% to 1.3%, primarily involving subclinical conditions (Schraga, 2022). Some researchers have noted that hyperthyroidism is less common in Africa than in Europe or North America (Olurin, 1972). Nevertheless, Ogbera *et al*. (2007) argued that thyroid disorders, especially hyperthyroidism, are not as rare as previously thought, attributing this to significant underreporting owing to inadequate diagnostic and treatment facilities. Ogbera and Kuku (2011) suggested that underreporting might result from delayed presentation, financial limitations, missed diagnoses, and absence of documented medical records. Recent research has indicated an increasing incidence of hyperthyroidism (Famuyiwa *et al*. 1990; Okafor *et al*. 2019; Jimoh *et al*. 2020). If left untreated, hyperthyroidism can lead to severe complications (Lee, 2022), such as thyroid storms, cardiovascular problems, and pregnancy-related issues. Diagnosis typically occurs when these complications are significant health concerns (Revathy *et al*., 2015). Treatment options include antithyroid medication, thyroidectomy, and radioactive iodine ablation. Before the 1940s, surgery was the only available treatment (Malboosbaf & Azizi, 2020). Edwin Astwood transformed this treatment by discovering thiourea derivatives, with thiouracil being the most effective (Degner, 1947). Methimazole and Propylthiouracil were later developed as less toxic alternatives (Sawin & Cooper, 2023). Antithyroid drugs are generally the first choice, whereas ablation and surgery are reserved for persistent or recurrent cases. In Nigeria, doctors prefer antithyroid medications or thyroidectomies to manage hyperthyroidism (Ogunjobi *et al*., 2015). Radioactive iodine is not widely used in Africa (Ogbera *et al*., 2008) and may pose an oncogenic risk (Song *et al*., 2021). It is not recommended for children and women of childbearing age (Ogunjobi *et al*., 2015). The management of hyperthyroidism in Africa is complicated by antithyroid drug resistance, and adverse effects are exacerbated by prolonged treatment. This has led to the use of medicinal herbs in African countries, particularly Nigeria (Revathy *et al*., 2015). *Anonna squamosa* and *Tetracapidium conophorum* are used in traditional medicine to treat hyperthyroidism, with patients and healers claiming their effectiveness. *Annona squamosa* L, belonging to the Annonaceae family, is a small, well-branched tree or shrub known to produce edible fruits called sugar-apples or sweetsops (United States Department of the Army 2009; Global Plants 1993). It thrives more effectively in tropical lowland climates than its relatives *Annona reticulata* and *Annona cherimola*, making it the most extensively cultivated species (AgroForestryTree Database, 2011; Morton, 1987). This plant is used in traditional medicine where crushed leaves are applied to the nose to treat fainting (Datiles & Acevedo – Rodriguez, 2015). Leaves, fruits, and seeds possess vermicidal and insecticidal properties. According to Kumar *et al* (2021), *A. squamosa* leaves are traditionally used to treat cancerous tumors, abscesses, insect bites, and skin issues. Root bark scrapings are used to relieve toothaches (Zahid *et al*., 2018). Powdered seeds are effective against head lice and fleas; however, caution is advised to prevent eye contact. *Annona squamosa* is also used in culinary applications, with its pulp serving as a flavoring agent in ice creams (Kumar *et al*., 2021). Its medicinal benefits include anticancer, antidiabetic, antioxidant (Fang *et al*., 2020), antimicrobial (Al-Ghazzawi, 2019), and hepatoprotective properties (Rajeshkumar *et al*., 2015). *Tetracarpidium conophorum* (Mull. Arg., Hutch, & Dalz) from the Euphorbiaceae family is a tropical plant native to West Africa, commonly referred to as the African walnut, black walnut, and Nigerian walnut (Ayeni and Nuhu, 2018). In Nigeria, it is called awusa or asala by the Yoruba, ukpa or okpokirinya by the Igbo, and gawudi bairi by the Hausa. Walnut trees typically climb to other trees for support (Oke *et al*. 2020; Oyinloye, 2021). All parts of *T. conophorum* are used in traditional medicine (Ayeni & Nuhu, 2018). Oyinloye (2021) noted the ethnobotanical use of *T. conophorum* seeds in treating fibroids, and boiled seeds were consumed to enhance sperm count. The bark serves as a mild laxative (Ayeni and Nuhu 2018). Leaf juice is used to boost female fertility and regulate the menstrual cycle (Oyinloye, 2021). *T. conophorum*'s pharmacological activities of *T. conophorum* include enhancement of male fertility (Dada and Aguda, 2015; Ikpeme *et al*., 2014), antioxidant effects (Amaeze *et al*., 2011), anti-ulcer and wound healing properties (Anosike *et al*., 2015), anti-inflammatory effects (Olaniyi *et al*., 2016), antimalarial properties (Akinwande, 2015), antidiabetic effects (Ogbonna *et al*., 2013), and antilipidemic activity (Nwaichi *et al*., 2017). Hyperthyroidism is linked to increased mortality; however, the prognosis can be improved with rapid and sustained control. Therefore, antithyroid medications that are potent, affordable, accessible, and with minimal side effects are invaluable.

2. Material and methods

2.1. Plant material

In July 2022, fresh leaves of *Tetracarpidium conophorum* and *Annona squamosa* were collected from Nsukka, Enugu State, Nigeria. Mr. Nwafor Felix, a taxonomist from the Department of Pharmacognosy and Environmental Medicine at

the University of Nigeria, Nsukka, verified the plant material. The voucher specimen was placed in the herbarium of the Department of Pharmacognosy and Traditional Medicine, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Agulu. The voucher number assigned to the *Annona squamosa* leaves was PCG/474/A/049, while the *Tetracapidium conophorum* leaves were given the voucher number PCG/474/E/022. The leaves were then air-dried at room temperature and ground into powder using a mechanical grinding machine (GX160 Delmar 5.5HP).

2.2. Ethical approval

The Ethics Committee at Enugu State University of Science and Technology (ESUT), located in Agbani, Enugu State, approved the study. (Ref No: ESUT/AEC/2024/0356/AP217).

2.3. Animals

For in vivo pharmacological assessments, male Swiss Albino rats aged 8 weeks were used, while albino mice weighing 25–30 g were used for the acute toxicity analysis. To minimize variability in disease induction and drug response, the animals were raised in the Animal House of the Department of Pharmacology at the Faculty of Pharmaceutical Sciences, Enugu State University of Science and Technology, under optimal conditions of temperature, humidity, and lighting. They were provided pelletized feed (Vital Feeds, Nigeria) and had unrestricted access to filtered water. All experimental procedures were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals (of NIH, 2011, Pub No: 85-23).

2.4. Extraction

Approximately 1 kg of crushed leaves from *Tetracarpidium conophorum* and Anona squamosa was individually subjected to cold maceration with 5 liters of 90% methanol. The plant material to solvent ratio was maintained at 1:5. The mixture was agitated periodically for 72 hours. Afterward, the solution was filtered using a muslin cloth and Whatman filter paper. The resulting filtrate was then concentrated to dryness under reduced pressure using a rotary evaporator (RE300 Model, United Kingdom) set at 40°C.

2.5. Total phenolic content of the extracts by folin ciocalteu's assay

The method outlined by Mbagwu *et al*. (2021) was used to assess the total phenolic content of the extracts. The total phenolic content was calculated from a standard curve derived from gallic acid solution and expressed as milligrams of gallic acid equivalent (GAE) per gram of extract.

2.6. Phytochemical Analysis

Qualitative phytochemical analyses of the extracts were performed using standard methods described by Odoh *et al*. (2019).

2.7. *In vitro* Pharmacological Assays

2.7.1. DPPH assay

The method outlined by Ajaghaku *et al*. (2017) was employed to assess the free radical scavenging capability of the plant extracts. The free radical scavenging activities of the extracts were calculated using the following relationship:

$$\text{DPPH scavenging activity} = 100\ [(AC - AS)/\ AC]$$

Where

- AC = Absorbance of control
- AS = Absorbance of sample

2.8. Thyroid peroxidase inhibitory activity

2.8.1. Thyroid Peroxidase Preparation

The assay followed the method outlined by Habza-Kowalska *et al*. (2019) with some adjustments. Frozen thyroid glands were sectioned and homogenized in a buffer solution composed of 0.25 M sucrose, 2 mM Tris-HCl, 100 mM KCl, 40 mM NaCl, and 10 mM $MgCl_2$ at pH 7.4. The thyroid gland underwent two rounds of centrifugation at 4000 RPM for 15 min at 4 °C. The enzyme precipitated at a rate of 60%. The supernatant was reserved for further analysis and stored at −20

°C. Enzyme activity was measured using a guaiacol assay. The reaction mixture included 33 mM guaiacol, 0.27 mM H2O2, and 33 mM sucrose, and the components were incubated at 37 °C before the assay. The absorbance was determined using a Shimadzu spectrophotometer (Model UV-1280, Shimadzu Corporation, Kyoto, Japan) at a wavelength of 470 nm. The assay procedure involved mixing 180 µL buffer, 100 µL guaiacol, and 40 µL TPO in a cuvette to reach a total volume of 420 µL. The cuvette was then placed in a spectrophotometer, and the reaction was initiated by adding 100 µL of H2O2. Absorbance was recorded every minute for 3 min. Thyroid peroxidase activity was confirmed by a linear correlation between concentration and absorbance readings.

2.8.2. Thyroid Peroxidase Inhibitory Assay

The assay was performed following the procedure outlined by Habza-Kowalska *et al*. (2019) with certain adjustments. Measurements were taken using a plate spectrophotometer (BioTek) in 96-well plates at a wavelength of 470 nm. The assay procedure involved combining 50 µL of buffer, 40 µL of pure substance solution, methanol extract solution, or in vitro digested solution, 50 µL of guaiacol, 20 µL of thyroid peroxidase enzyme, and 50 µL of H2O2, resulting in a total mixture volume of 210 µL. The extracts were substituted with buffer in the sample probe. Absorbance was measured every minute for a duration of 3 min at 37 °C, with the TPO activity unit defined as the absorbance change per minute. All measurements were performed in triplicate.

The TPO inhibitory activity was determined using the formula:

$$\%\text{inhibition} = (1 - (\Delta A/〖\min〗_{\text{test}})/(\Delta A〖\min〗_{\text{blank}})) \times 100\%$$

where ΔA/min represents the linear absorbance change per minute for the test material, and ΔA min blank is the linear absorbance change per minute for the blank. The IC^{50} value was derived by interpolating the dose-response curves. IC^{50} values were calculated using fitted models as the concentration of the tested compound that achieved 50% maximum inhibition based on a dose-dependent mechanism. The mode of enzyme inhibition was assessed using a Lineweaver–Burk plot.

2.9. *In vivo* Pharmacological Assays

2.9.1. Acute Toxicity Studies

Animals were randomly selected, marked for individual identification, and kept in cages for 7 days before dosing to acclimatize them to the cage conditions. Nulliparous, non-pregnant female rats were used because females are generally more sensitive to toxic effects. Animals of similar weight (25 – 27 g) and 8 weeks of age were selected. Acute toxicity analyses of the extracts were performed using the Lorke's method as described by Ihekwereme *et al*. (2023).

LD50 was calculated using the following formula:

$$\text{LD50} = \sqrt{(\text{D0} \times \text{D100})}$$

Where D_0 = Highest dose that gave no mortality,

D_{100} = Lowest dose that produced mortality.

2.10. Induction of hyperthyroidism

Following the acclimatization period for the rats in this experiment, hyperthyroidism was induced by subcutaneous administration of L-thyroxine (T4) (Sigma, USA) at a dosage of 600 µg/kg each day for 12 consecutive days, as outlined by Kim *et al*. (2012).

- Preparation of Drugs: Methanol extracts of *T. conophonium* and *A. squamosal* leaves were dissolved in 5% Tween 80 in sterile distilled water. Propyl thiouracil tablets were weighed, powdered, and dissolved in saline. L-thyroxine tablets were weighed and dissolved in saline solution.

2.11. Treatment Protocol

After 12 days of L-thyroxine (T4) administration, the animals were randomly assigned to five groups, each consisting of six rats, as detailed below. Group 1 served as the normal control, receiving 10 ml/kg of 5% Tween 80. Group 2 served as the hyperthyroidism control, with distilled water administered orally for 15 days. Group 3 was the treatment control,

administered 10 mg/kg propylthiouracil (PTU) orally for 15 days. Group 4, designated as extract treatment group A, received 25 mg/kg *A. squamosa* leaf extract orally for 15 days. Group 5, known as extract treatment group B, was orally administered 50 mg/kg of *A. squamosa* leaf extract for 15 days. Group 6, referred to as extract treatment group C, received 100 mg/kg *A. squamosa* leaf extract orally for 15 days. Group 7, labeled as extract treatment group D, was orally administered 200 mg/kg of *A. squamosa* leaf extract for 15 days. Group 8, termed extract treatment group E, received 400 mg/kg *A. squamosa* leaf extract orally for 15 days. Group 9, identified as extract treatment group A1, was orally administered 25 mg/kg of *T. conophorum* leaf extract for 15 days. Group 10, called the extract treatment group B1, received 50 mg/kg of *T. conophorum* leaf extract orally for 15 days. Group 11, known as extract treatment group C1, was orally administered 100 mg/kg of *T. conophorum* leaf extract for 15 days. Group 12, referred to as the extract treatment group D1, received 200 mg/kg of *T. conophorum* leaf extract orally for 15 days. Group 13, labeled as extract treatment group E1, was orally administered 400 mg/kg of *T. conophorum* leaf extract for 15 days.

2.12. Pharmacological Assay

Following a 15-day treatment period, blood samples were drawn from the retro-orbital plexus of all rats that had fasted overnight using a microcapillary tube. Serum was isolated to measure thyroid hormones (TSH, T3, and T4), liver enzymes (AST, ALT, and ALP), and antioxidant activity. The animals were euthanized by decapitation and the thyroid gland was promptly removed, rinsed in ice-cold saline to eliminate blood, and preserved in 10% formalin for histopathological examination.

2.13. Estimation of Biochemical Parameters

2.13.1. Serum Thyroid Hormones

Blood samples were drawn into vacuum-sealed tubes, and the serum was isolated by centrifugation at 3000 rpm for 10 min at 4°C. The isolated serum was promptly used for testing. Serum levels of T3, T4, and thyroid-stimulating hormone (TSH) were measured using a colorimetric competitive enzyme immunoassay with a specific ELISA kit from Elabscience, USA.

2.13.2. Biochemical Assay of Serum Liver Marker Enzymes

Serum alanine transaminase (ALT), Aspartate transaminase (AST) and Alkaline phosphatase (ALP) levels were determined following the procedure outlined by Colville (2002) using an ALT, AST and ALP test kits respectively from Span Diagnostics Ltd., India.

2.13.3. Biochemical assay of kidney parameters

The levels of serum creatinine and blood urea nitrogen (BUN) were determined using the techniques outlined by Tietz (1976) and Heinegard and Tiderstrom (1973), respectively, with the aid of BUN and creatinine test kits (Teco Diagnostics, USA).

2.13.4. Malondialdehyde (MDA) Assay

Serum MDA levels were determined using a modified thiobarbituric acid method, as described by Draper and Hadley (1990), employing a malondialdehyde assay kit from Elabscience Biotechnology Co. Ltd., South Africa. MDA levels in each sample were calculated using the following formula:

$$MDA\ (nmol/ml) = \frac{Absorbance\ of\ Sample - Absorbance\ of\ Blank}{Absorbance\ of\ Standard - Absorbance\ of\ Blank} \times 10$$

2.14. Statistical Analysis

Data are expressed as mean ± standard deviation (SD). The data were analyzed using one-way ANOVA, followed by Newmann-Keuls multiple range tests. Statistical evaluations were conducted using GraphPad version 3.1, and P values were deemed statistically significant when $P < 0.05$.

3. Results

3.1. Extraction and phytochemical analysis

The methanol extract of Annona quamosa yielded 9.42%, while *Tetracapidium conophorum* yielded 7.56%, as detailed in Table 1. Qualitative phytochemical analysis of extracts from *A. squamosa* and *T. conophorum* identified various secondary metabolites, as shown in Tables 1 and 2. The extract from *A. squamosa* contains alkaloids, saponins, cardiac glycosides, tannins, flavonoids, and steroids/terpenoids, but does not contain reducing sugars. In contrast, the extract from *T. conophorum* comprises flavonoids, tannins, and alkaloids but lacks saponins, glycosides, and steroids/terpenoids.

Table 1 Phytochemical constituents of the leaf extracts

PHYTOCOMPOUNDS	***Anonna squamosa.***	***Tetracapidium conophorum***
Alkaloid	+	+
Saponins	+	-
Cardiac glycosides	+	-
Tannins	+	+
Flavonoids	+	+
Steroids and terpenoids	+	-
Percentage Yield (%)	9.42[a]	7.56 [a]

KEY: + = Present, - = Absent, a = Calculated from 1000 g of pulverized leaves.

3.2. ANTIOXIDANT ANALYSIS

3.2.1. Total Phenolic Content

Phenolic compounds, known for their hydroxyl groups, are more soluble in polar organic solvents; therefore, methanol was chosen as the extraction solvent. The phenolic content was measured using Folic Ciocalteu's phenol reagent, with results calculated from a calibration curve (Y = 19.371x – 0.0413, R2 = 0.9951) based on gallic acid concentrations ranging from 0.01 0.06 mg/ml. The total phenolic content was reported as gallic acid equivalents (GAE) per gram of dry extract. A comparative study indicated that *A. squamosa* had a notably higher phenolic content than *T. conophorum*, with average total phenolic contents of 266.27 ± 14.24 mgGAE/g and 100.05 ± 7.67 mgGAE/g, respectively, as presented in table 2.

Table 2 Comparative Analysis of Total Phenolic Content and Absorbance (at λ =760nm) in *Annona squamosa and Tetracapidium conophorum*

Treatment (100µg/ml)	**Absorbance1**	**Absorbance2**	**Mean Absorbance**	**Total phenolic content Abs1**	**Total phenolic content Abs2**	**Mean Total phenolic content (mgGAE/g)**	**STDev**
A. squamosal	0.494	0.455	0.4745	276.3409	256.2077	266.2743	14.23631
T. conophorum	0.163	0.142	0.1525	105.4669	94.62599	100.0465	7.665708

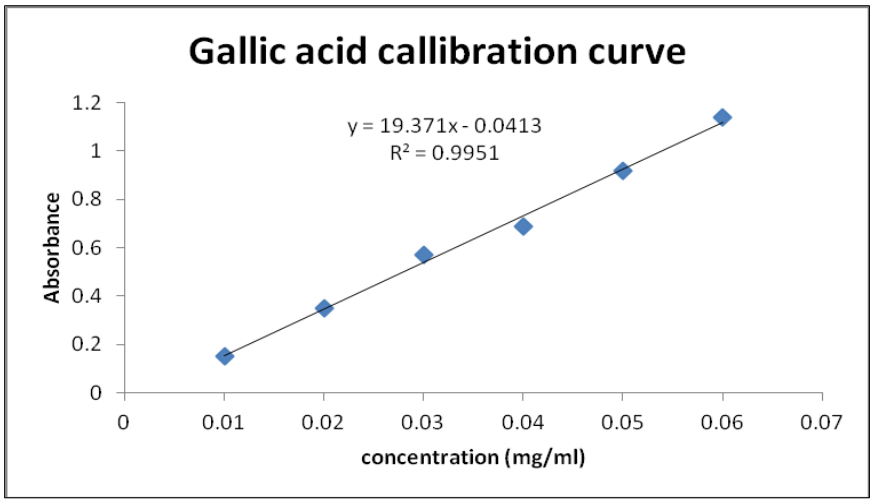


Figure 1 Gallic Acid Caliberation Curve

3.2.2. DPPH Radical Scavenging Activity

The extracts from *A. squamosa* and *T. conophorum* plants showed concentration-dependent increases in radical-scavenging ability. This was demonstrated by the change in color from purple to yellow in DPPH, indicating the presence of antioxidants. The color shift was more evident in solutions with higher extract concentrations and ascorbic acid standards. A comparison indicated that the methanolic extracts of *A. squamosa* had a more potent scavenging effect, with an IC50 value of 8.84ug/ml, compared to the IC50 value of 318.92ug/ml for *T. conophorum* extracts. Remarkably, the extract from *A. squamosa* was comparable to that of the standard ascorbic acid (IC50 = 8.06ug/ml), highlighting its strong antioxidant capabilities. Consequently, *A. squamosa* is considered to be a more effective radical scavenger than *T. conophorum*, suggesting its superior antioxidant activity.

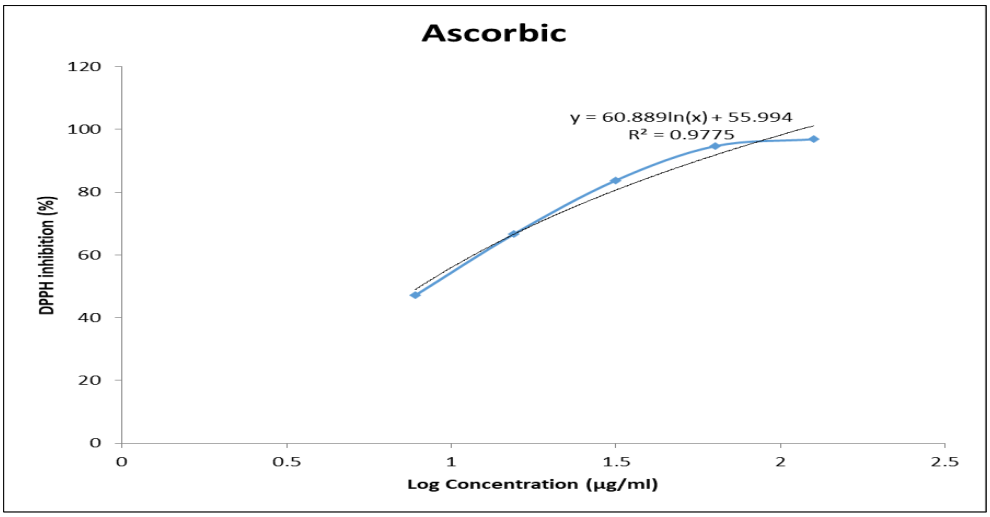


Figure 2 Dose response curve of DPPH radical inhibition against log concentration of ascorbic acid

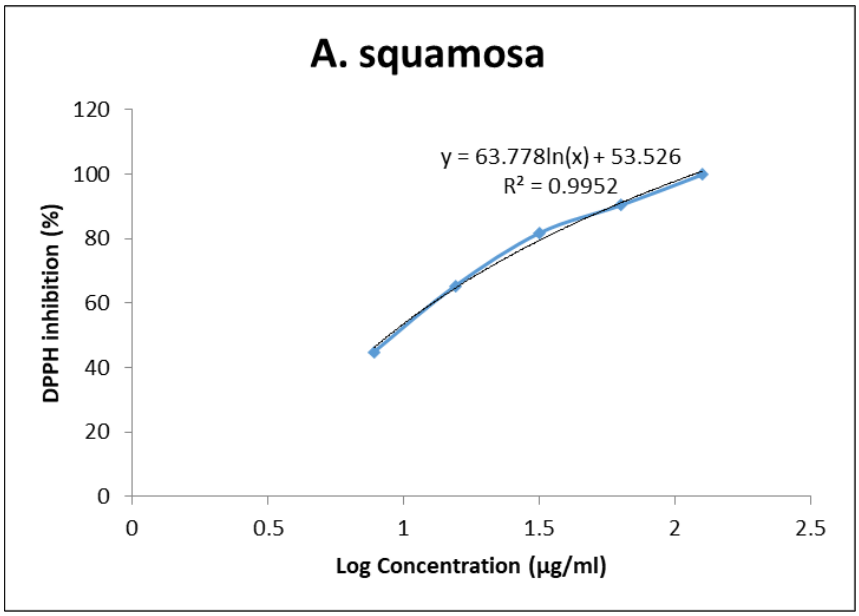


Figure 3 Dose response curve of DPPH inhibition against log concentration of *Annona squamosa* extract

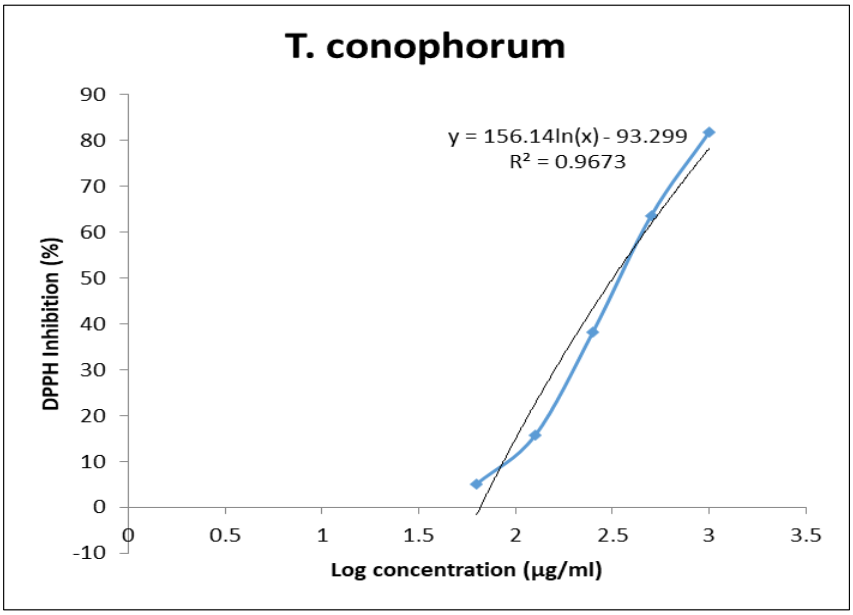


Figure 4 Dose response curve of DPPH inhibition against log concentration of *Tetracapidium conophorum* extract

3.3. Effects of treatment on thyroid peroxidase activity

Guaiacol assay was employed to confirm the activity of thyroid peroxidase (TPO) in the thyroid gland, revealing a direct relationship between TPO levels and absorbance measurements. The potential of *A. squamosa* and *T. conophorum* extracts to inhibit TPO was examined, and both extracts demonstrated inhibitory effects that increased with increasing dosage. The methanolic extract, ranging from 50ug/ml to 800ug/ml, showed an increased TPO inhibition from 14% to 63%. Specifically, the methanolic extract of *T. conophorum* showed 20% inhibition at 50ug/ml and 78% at 800ug/ml. The greatest inhibition of TPO was recorded with the 800ug/ml *T. conophorum* extract, whereas the lowest was observed with the 50ug/ml *A. squamosa* extract, as depicted in Fig 5 and 6. When isolated, TPO indicated that the methanolic extract of *T. conophorum* was more effective, with an ED50 value of 224.05ug/ml, compared to the *A. squamosa* methanolic extract, which had an ED50 value of 437.24ug/ml.

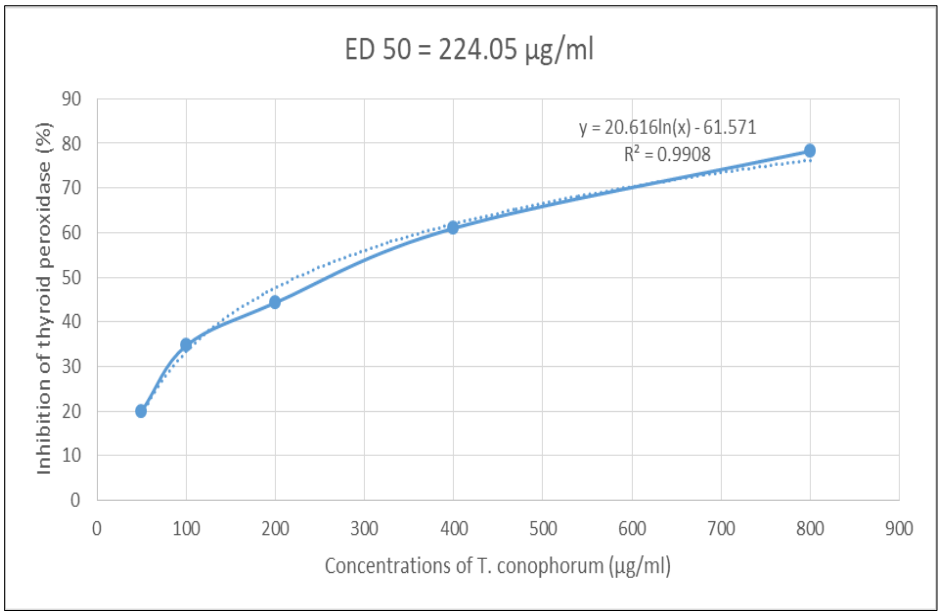


Figure 5 Dose response curve of percentage inhibition of thyroid peroxidase against concentration of *Tetracapidium conophorum* extract

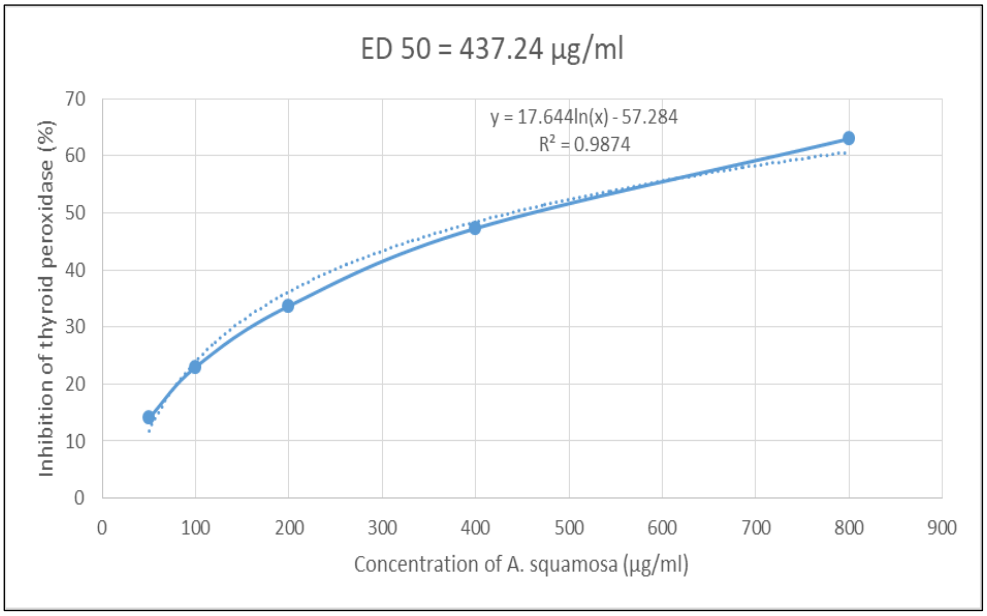


Figure 6 Dose response curve of percentage inhibition of thyroid peroxidase against concentration of *Annona squamosa* extract.

3.4. Acute Toxicity Studies

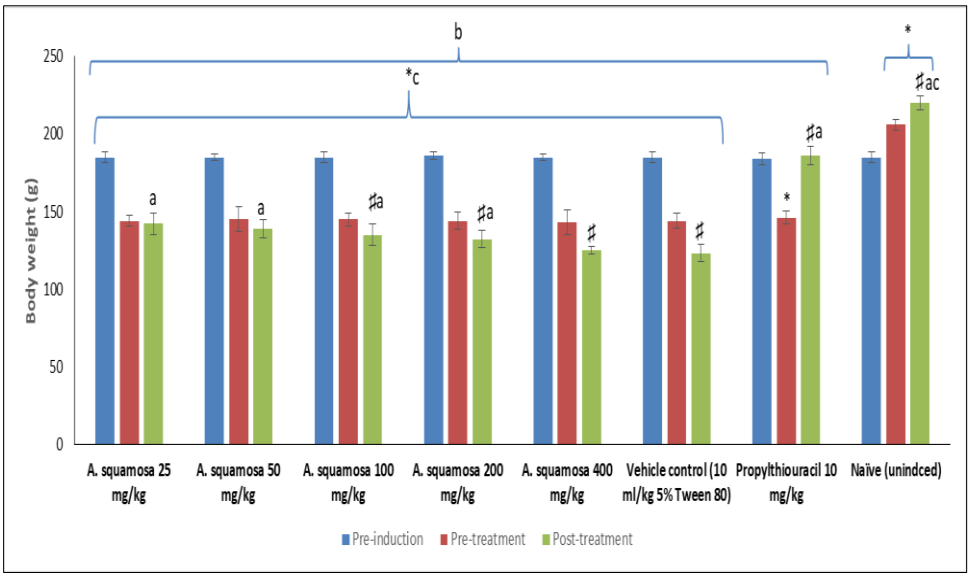
During the initial 30 min and then at 2, 6, 12, and 24 h, the animals were monitored for signs of toxicity and death. The mice were also checked daily for delayed toxic effects and mortality. No deaths occurred during either phase of the acute toxicity tests for either extract. Behavioral assessments indicated normal motor function with no signs of coma, seizures, tremors, or itching. Observations of parameters, such as eye condition, breathing, fecal consistency, fur and skin condition, saliva, urine color, sleep, and feeding habits, were all normal throughout the study. However, animals in phase II that received higher doses of the extract showed reduced physical activity. Normal activity levels returned 2 h after administering 5000 mg/kg of *A. squamosa* extracts, whereas it took up to 4 h for T. conophorium extracts. The LD50 for both extracts was determined to be > 5000 mg/kg, which informed the dosage selection for further animal studies using these extracts.

3.5. Effect of treatment on body weight

Administration of L-thyroxine (T4) led to hyperthyroidism, which significantly reduced body weight (P<0.05), as evidenced by the weight differences before and after induction (Figures 7 and 8). The methanolic extract of *A. squamosa* further decreased body weight in a dose-dependent manner, whereas *T. conophorum* extracts caused a dose-dependent increase in weight (Figure 9). The negative control group lost weight, whereas the propylthiouracil treatment resulted in weight gain. Despite the weight reduction from *A. squamosa* extract, animals receiving doses from 25 to 200 mg/kg had significantly (P<0.05) higher weights than the vehicle control group. In contrast, *T. conophorum* extract significantly increased the body weight at all tested doses. *A. squamosa* caused a notable weight reduction starting at 100 mg/kg, similar to that in the vehicle control group. Conversely, *T. conophorum* methanol extract led to significant weight gain at 50 mg/kg, similar to propylthiouracil, likely due to treatment-induced hypothyroidism (Kyriacou *et al*. 2019). Compared with the reference drug group, all doses of *A. squamosa* and lower doses of *T. conophorum* (25 and 50 mg/kg) resulted in significantly (P<0.05) higher weights. However, higher doses of *T. conophorum* (100–400 mg/kg) led to significantly higher body weights than the reference drug group. Notably, 400 mg/kg *T. conophorum* was the only treatment that normalized the hyperthyroidism-induced weight reduction, as indicated by the non-significant difference (P>0.05) between post-treatment body weight and the naïve control group.

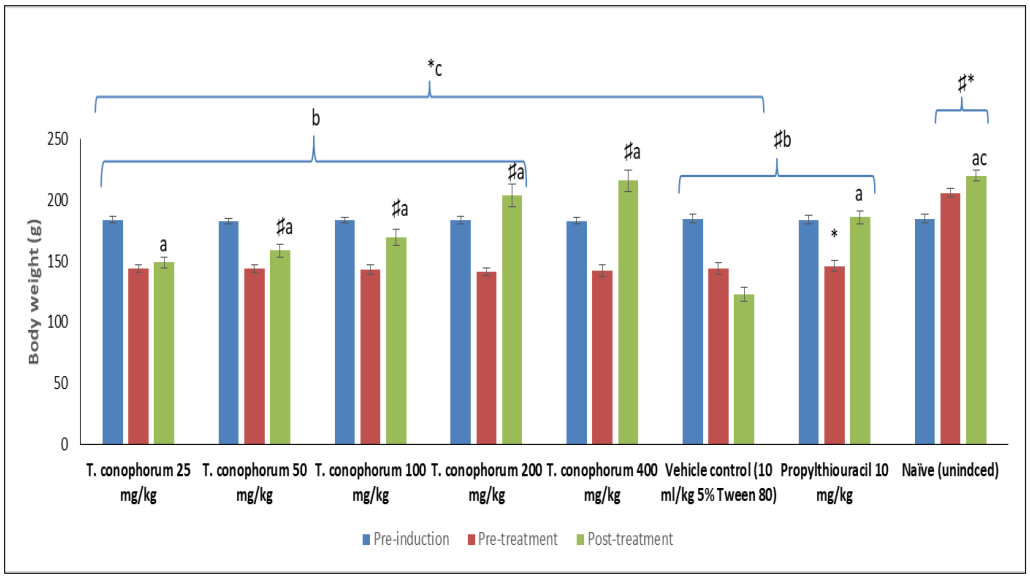
Analysis of body weight changes before and after treatment showed that *A. squamosa* methanolic extract significantly (P<0.05) reduced body weight at a dose of 100 mg/kg, similar to that in the vehicle control group. Conversely, a significant (P<0.05) weight increase was observed in animals treated with *T. conophorum* methanol extract, similar to the propylthiouracil-treated group. This may be due to treatment-induced hypothyroidism (Kyriacou *et al*. 2019), which is often associated with weight gain in most patients.

Compared with the reference drug-treated group, significantly (P<0.05) higher weights were recorded for all tested doses of *A. squamosa* extract and lower doses of *T. conophorum* extract (25 and 50 mg/kg). However, higher doses of *T. conophorum* extract (100–400 mg/kg) resulted in significantly (P<0.05) higher body weights than the reference drug-treated group. *T. conophorum* methanolic extract at 400 mg/kg was the only treatment that restored hyperthyroidism-induced weight reduction to normal levels, as evidenced by a non-significant (P>0.05) difference between the post-treatment body weight of animals treated with this dose and the naïve (uninduced) control group.



Where * P<0.05 compared to pre-induction; ♯ P<0.05 compared to pre-treatment; a P<0.05 compared to vehicle control; b P<0.05 compared to Naïve; c P <0.05 compared to propylthiouracil (reference standard).

Figure 7 Effect of *A. squamosa* on body weight



Where * P<0.05 compared to pre-induction; ♯ P<0.05 compared to pre-treatment; a P<0.05 compared to vehicle control; b P<0.05 compared to Naïve; c P <0.05 compared to propylthiouracil (reference standard)

Figure 8 Effect of *T. conophorum* methanol body weight

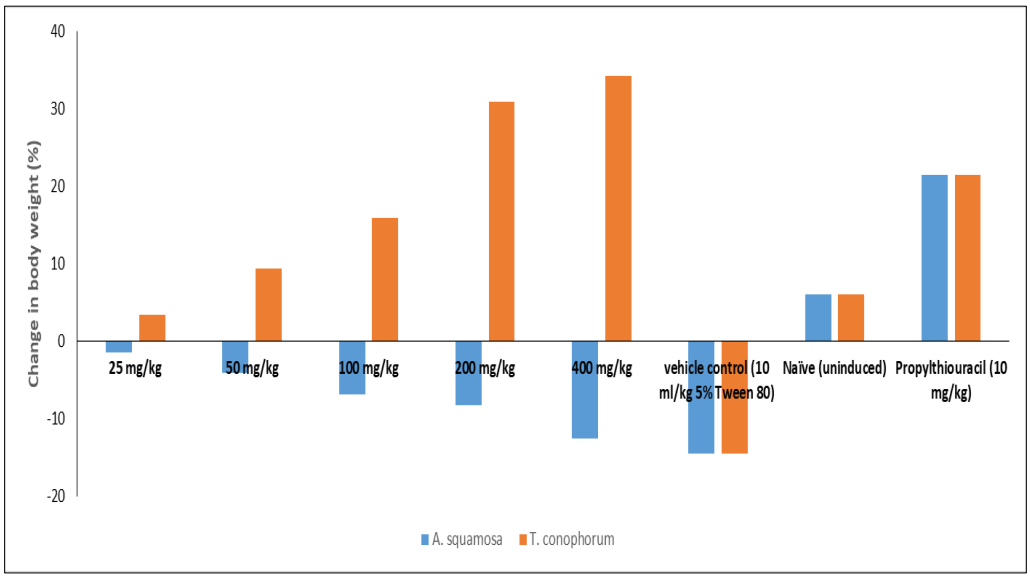
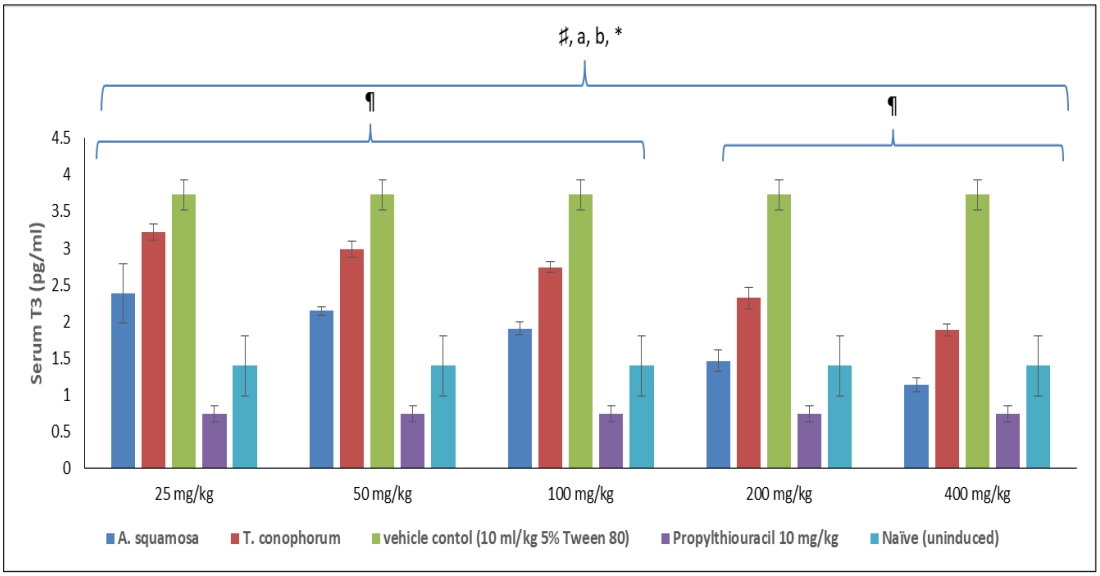


Figure 9 Comparative effect on *A. squamosa* and *T. conophorum* on body weight

3.6. Effect of treatment on thyroid hormones and thyroid stimulating hormone

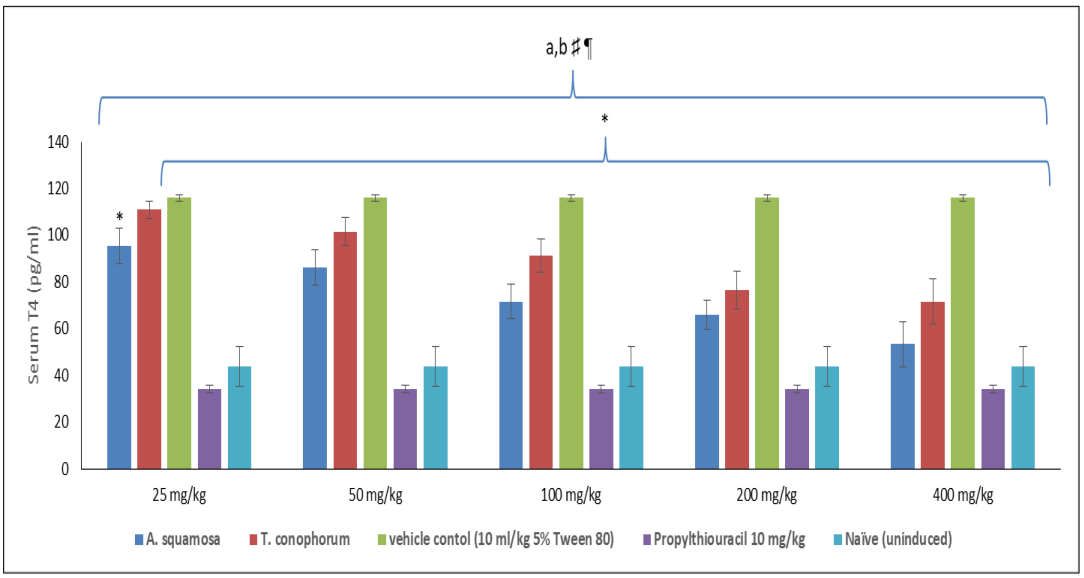
The successful induction of hyperthyroidism through thyroxine was demonstrated by a notable (P<0.05) increase in serum triiodothyronine (T3) and thyroxine (T4) levels, along with a significant decrease in serum thyroid-stimulating hormone (TSH) levels in the vehicle control group compared with the naïve (un-induced) control group (figures 10, 11, and 12). Extracts from *A. squamosa* and *T. conophorum* led to dose-dependent decreases in serum T3 and T4 levels. These decreases were statistically significant (P<0.05) at all tested doses of *A. squamosa* methanol extract for both hormones. The *T. conophorum* extract showed a significant (P<0.05) reduction in T3 at all doses (25–400 mg/kg) and from 50 to 400 mg/kg for T4 compared with the vehicle control group. Although both plant extracts demonstrated antihyperthyroid activity, significant (P<0.05) differences in activity were observed at all tested doses. The *A. squamosa* extract exhibited superior activity compared with the *T. conophorum* extract. However, both extracts were significantly less effective than the reference standard (PTU). The reduction in serum T3 and T4 levels caused by PTU was also significantly (P<0.05) lower than that in the naïve control group. At all tested doses, *T. conophorum* extract was unable to reduce serum T3 and T4 concentrations to the levels observed in the naïve control group. Similar effects were noted for lower doses of *A. squamosa* extract (25 – 100 mg/kg) for T3. At 200 mg/kg, no significant (P>0.05) difference was

found between the serum T3 concentrations of hyperthyroidism-induced animals treated with *A. squamosa* extract and naïve animals. At 400 mg/kg, *A. squamosa* extract showed a significant (P<0.05) reduction in T3 compared with the naïve control group. These differences in T3 levels were not observed for T4. Significantly higher serum concentrations of T4 were recorded at all doses compared with the naïve control group for both extracts (P < 0.05). Treatment with *A. squamosa* and *T. conophorum* extracts resulted in a dose-dependent increase in the serum TSH levels. *A. squamosa* showed a significant increase in TSH at all tested doses compared with the vehicle control group, whereas for *T. conophorum*, a significant effect was recorded at 50 mg/kg. The increase recorded by both extracts was significantly lower than that of both the reference standard and naïve control groups.



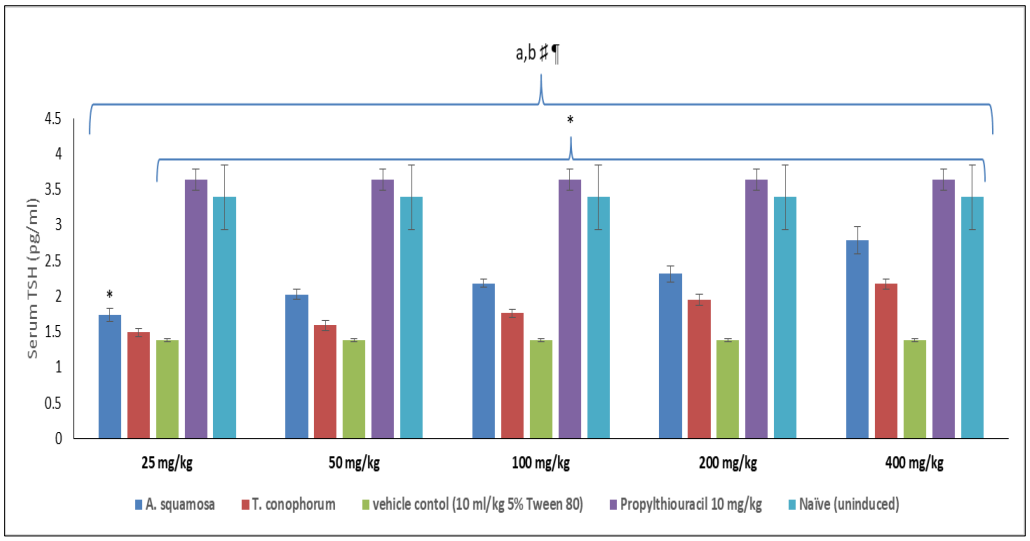
Different letters (a, b) represent significant (P<0.05) differences between treatments (A squamosa and *T. conophorum*; * P<0.05 compared to vehicle control; ♯ P<0.05 compared to reference standard (propylthiouracil); ¶ P<0.05 compared to naïve (uninduced) control group.

Figure 10 Comparative effect on *A. squamosa* and *T. conophorum* on serum Triiodothyronine



Different letter alphabets (a, b) represents significant (P<0.05) difference between treatments; * P<0.05 compared to vehicle control; ♯ P<0.05 compared to reference standard (propylthiouracil); ¶ P<0.05 compared to Naïve (uninduced) control group.

Figure 11 Comparative effect on *A. squamosa* and *T. conophorum* on serum Thyroxine

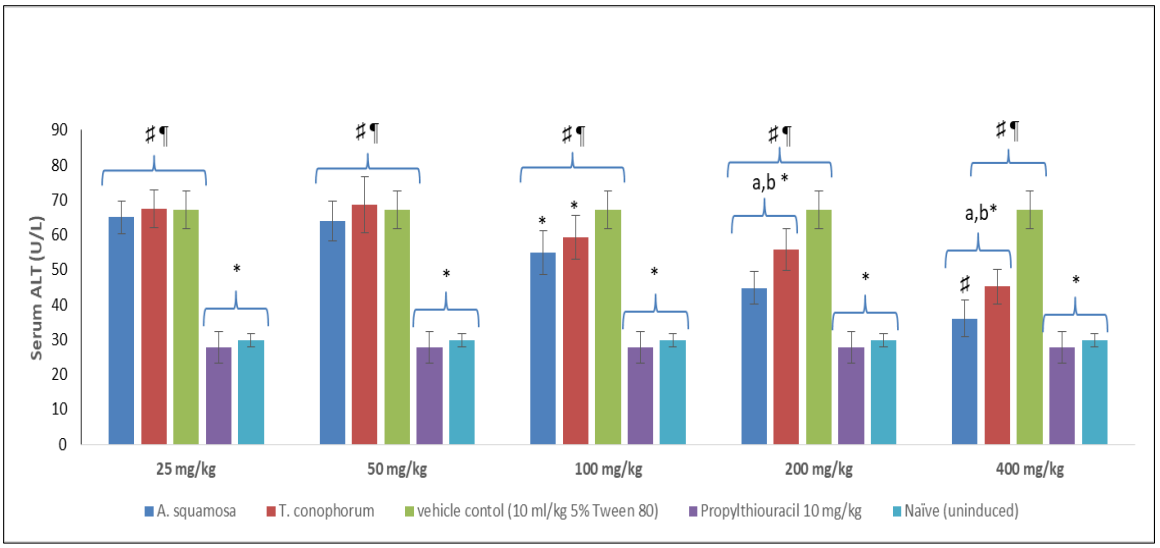


Different letter alphabets (a, b) represents significant (P<0.05) difference between treatments; * P<0.05 compared to vehicle control; ♯ P<0.05 compared to reference standard (propylthiouracil); ¶ P<0.05 compared to Naïve (uninduced) control group.

Figure 12 Comparative effect on *A. squamosa* and *T. conophorum* methanol extracts on serum Thyroid stimulating hormone

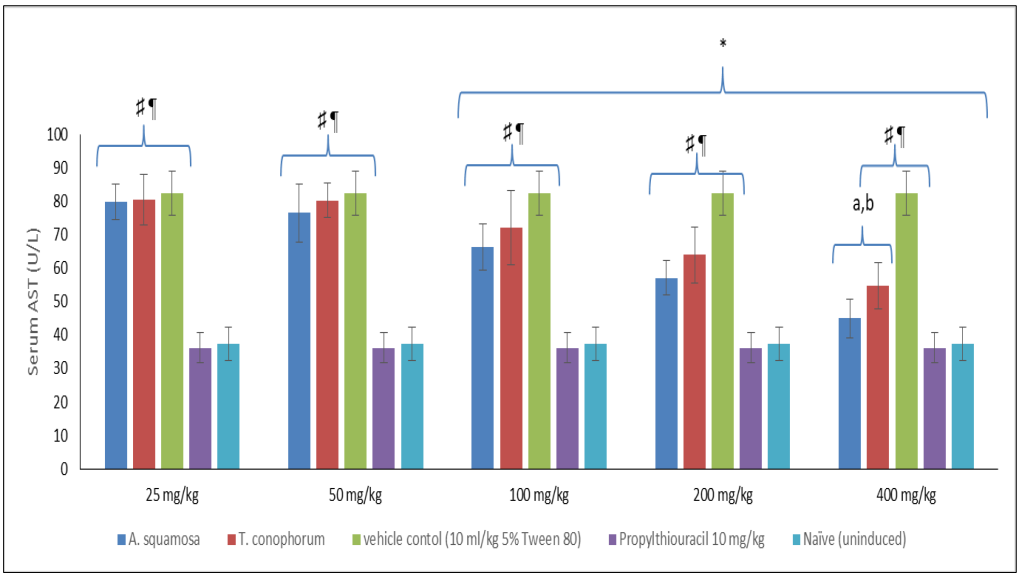
3.7. Effect of treatment on liver function enzyme

In experiments where hyperthyroidism was induced, there was a notable (P<0.05) increase in serum liver marker enzymes compared to un-induced control subjects. Extracts from *A. squamosa* and *T. conophorum* were found to lower serum ALT, AST, and ALP levels (figures 13, 14, and 15). This decrease was statistically significant (P<0.05) at a dose of 100 mg/kg compared with that in the vehicle control group. The *A. squamosa* extract demonstrated greater efficacy than the *T. conophorum* extract, although the differences between the two were significant at doses of 200 and 400 mg/kg for serum ALT and 400 mg/kg for serum AST, with no significant (P>0.05) difference observed for serum ALP. Compared to the reference standard and un-induced control groups, *T. conophorum* extract resulted in significantly (P<0.05) elevated serum liver enzyme levels at all doses tested, similar to the vehicle control group. At a dose of 400 mg/kg, animals treated with *A. squamosa* extract had ALT enzyme levels comparable to those of the un-induced control animals, with no significant difference between these groups (P>0.05). Additionally, this dosage had a similar impact on serum AST, akin to that of the un-induced and reference drug-treated groups.



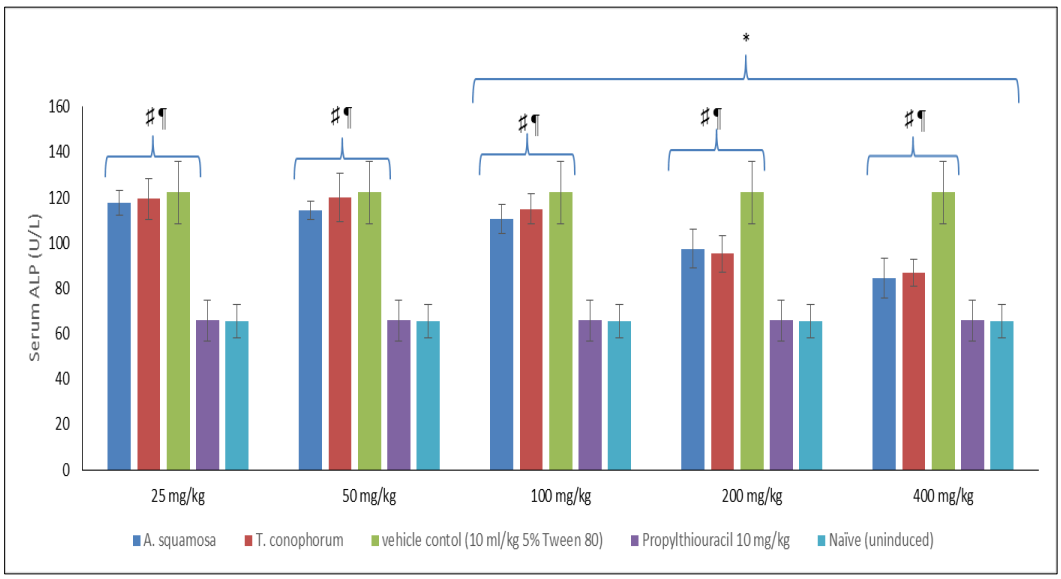
Different letter alphabets (a, b) represents significant (P<0.05) difference between treatments; * P<0.05 compared to vehicle control; ♯ P<0.05 compared to reference standard (propylthiouracil); ¶ P<0.05 compared to Naïve (uninduced) control group.

Figure 13 Comparative effect on *A. squamosa* and *T. conophorum* on serum Alanine transaminase



Different letter alphabets (a, b) represents significant (P<0.05) difference between treatments; * P<0.05 compared to vehicle control; ♯ P<0.05 compared to reference standard (propylthiouracil); ¶ P<0.05 compared to Naïve (uninduced) control group.

Figure 14 Comparative effect on *A. squamosa* and *T. conophorum* methanol extract on serum Aspartate transaminase



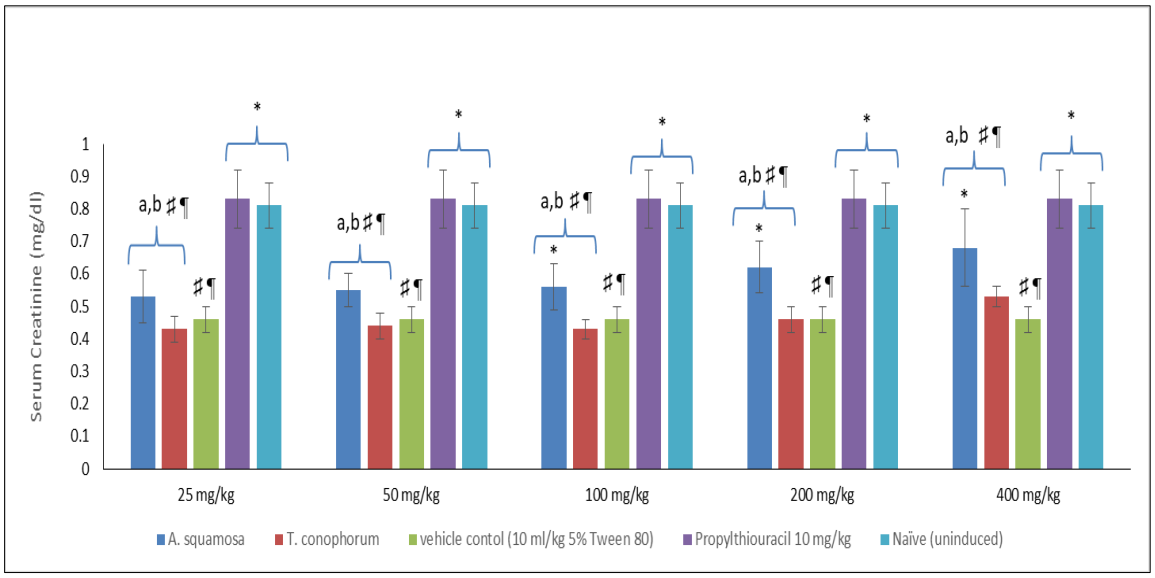
Different letter alphabets (a, b) represents significant (P<0.05) difference between treatments; * P<0.05 compared to vehicle control; ♯ P<0.05 compared to reference standard (propylthiouracil); ¶ P<0.05 compared to Naïve (uninduced) control group.

Figure 15 Comparative effect on *A. squamosa* and *T. conophorum* on serum Alkaline Phosphatase

3.8. Effect of treatment on the kidney

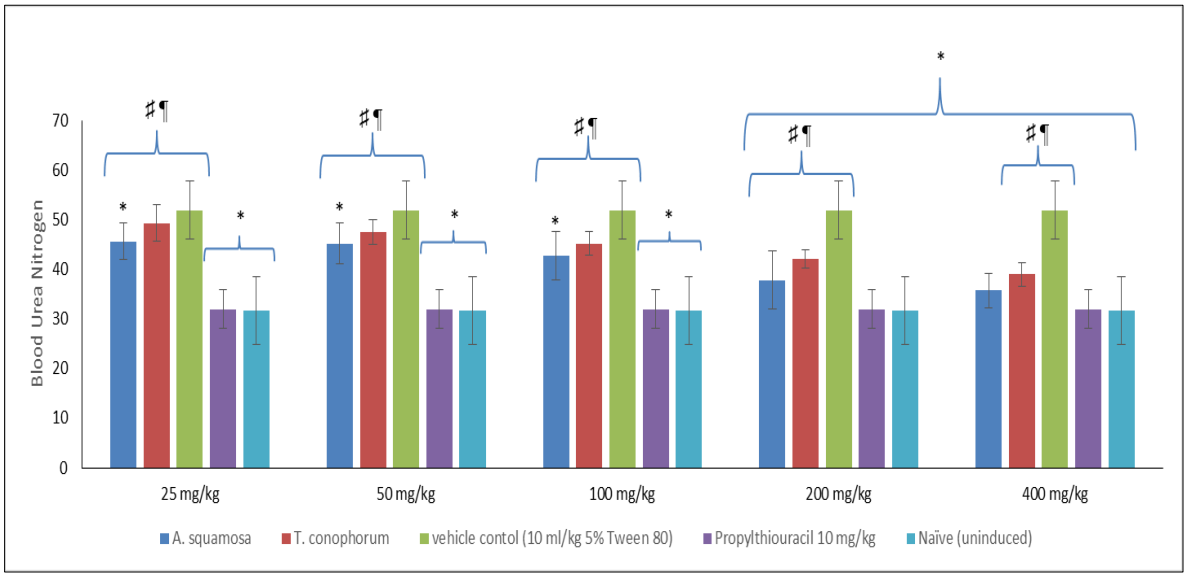
Following the induction of hyperthyroidism, a decrease in creatinine and an increase in blood urea nitrogen (BUN) were observed, as indicated by significant (P<0.05) changes in serum creatinine and BUN levels (figures 16 and 17) in naïve control animals compared to those in the vehicle control group. The methanolic extract of *T. conophorum* did not show a significant (P>0.05) improvement in serum creatinine levels compared with the vehicle control group. In contrast, the methanolic extract of *A. squamosa* at doses of 100–400 mg/kg significantly (P<0.05) enhanced serum creatinine levels compared with the vehicle control, similar to the effects observed in the reference drug-treated group. Significant (P<0.05) differences were noted between the effects of *A. squamosa* and *T. conophorum* extracts at all the tested doses. Both extracts resulted in significantly (P<0.05) lower serum creatinine activity than that of the reference drug. The significant (P<0.05) increase in BUN caused by hyperthyroidism was mitigated by treatment with *A. squamosa* and *T. conophorum* extract. No significant (P>0.05) differences were observed in the effects of either plant extract at any dose tested. The *A. squamosa* extract significantly (P<0.05) reduced BUN across the tested doses (25 – 400 mg/kg) compared to the vehicle control group, whereas the *T. conophorum* extract showed significant (P<0.05) effects at doses of 200 and

400 mg/kg. At a dose of 400 mg/kg, *A. squamosa* extract produced an effect comparable to that of the reference drug treatment, with no significant (P>0.05) differences in BUN concentration between the two groups. Furthermore, a 400 mg/kg dose of *A. squamosa* extract restored serum BUN to the level observed in the naïve control group, with no significant difference between the groups (P>0.05).



Different letter alphabets (a, b) represents significant (P<0.05) difference between treatments; * P<0.05 compared to vehicle control; ♯ P<0.05 compared to reference standard (propylthiouracil); ¶ P<0.05 compared to Naïve (uninduced) control group.

Figure 16 Comparative effect on *A. squamosa* and *T. conophorum* on serum Creatinine

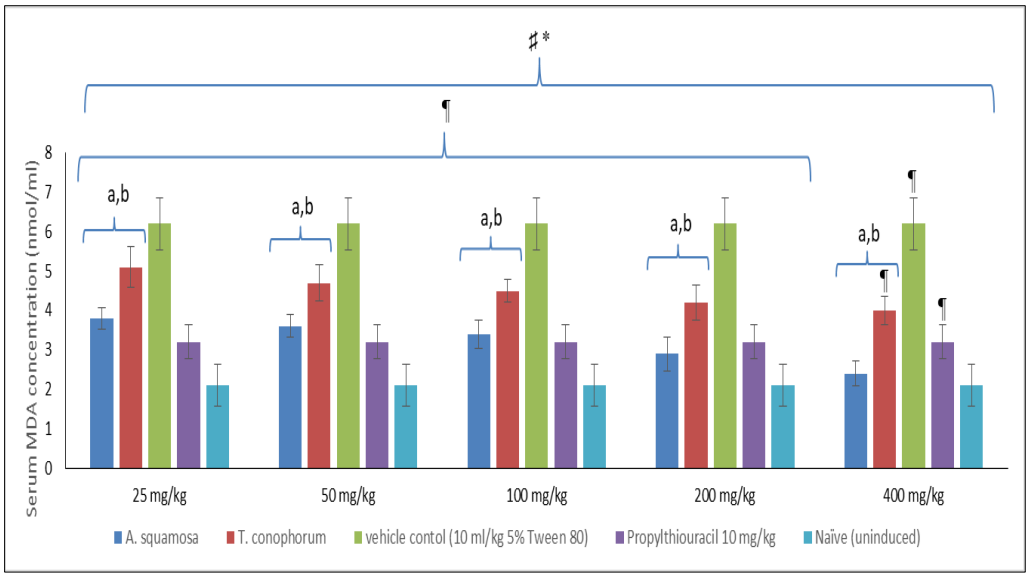


Different letter alphabets (a, b) represents significant (P<0.05) difference between treatments; * P<0.05 compared to vehicle control; ♯ P<0.05 compared to reference standard (propylthiouracil); ¶ P<0.05 compared to Naïve (uninduced) control group.

Figure 17 Comparative effect on *A. squamosa* and *T. conophorum* on Blood urea nitrogen

3.9. Effect of treatment on lipid peroxidation

In the vehicle control group, hyperthyroidism led to a marked (P<0.05) increase in serum malondialdehyde levels, indicating lipid peroxidation, compared to the naïve uncontrolled group (figure 18). *A. squamosa* and *T. conophorum* extracts significantly (P<0.05) decreased serum MDA levels at all doses relative to the vehicle control. The *A. squamosa* extract demonstrated a significantly (P<0.05) greater effect at all tested doses. Compared to the reference standard, the *A. squamosa* extract exhibited significantly (P<0.05) lower activity at doses of 25–100 mg/kg but higher activity at 200 and 400 mg/kg. At a dose of 400 mg/kg, the *A. squamosa* extract restored serum MDA levels back to those of the naïve control group, with no significant (P>0.05) difference observed between the two groups.



Different letter alphabets (a, b) represents significant (P<0.05) difference between treatments; * P<0.05 compared to vehicle control; ♯ P<0.05 compared to reference standard (propylthiouracil); ¶ P<0.05 compared to Naïve (uninduced) control group.

Figure 18 Comparative effect on *A. squamosa* and *T. conophorum* on lipid peroxidation

4. Discussion

The methanolic leaf extracts of *A. squamosa* and *T. conophorum* contain alkaloids, flavonoids, and tannins, which may play a role in their medicinal properties. The alkaloids present in these plants can disrupt enzymatic activities, including those related to thyroid hormones. For instance, nicotine, an alkaloid, induces central hypothyroidism in lactating rats (Miranda et al., 2020). Piperine is known to reduce serum thyroxine and triiodothyronine (Panda & Kar, 2003). Flavonoids exhibit antithyroid properties by inhibiting thyroid peroxidase enzyme activity (Gaitan et al., 1989) and the iodination of tyrosine (Divi and Doerge, 1966). Quercetin and Rutin are known to suppress TPO activity and tyrosine iodination, suggesting their potential as treatments for hyperthyroidism (Habza-Kowalska et al., 2019). These compounds likely contribute to the pharmacological effects of these plant extracts. Hyperthyroidism is characterized by an increased metabolic rate, which often results in weight loss. However, treatment with drugs, such as propylthiouracil, can unexpectedly cause hypothyroidism, which is usually associated with weight gain (Amisha and Rehman, 2023; Kyriacou et al., 2019). In an animal model of induced hyperthyroidism, the administration of leaf extracts from *Annona squamosa* and Tetracapidium conophorum resulted in notable weight gain, indicating effects similar to those of hypothyroidism. No fatalities were observed for either extract during either phase of acute toxicity testing. Animals in phase II that received higher doses showed reduced physical activity, but normal activity levels returned 2 h after receiving 5000 mg/kg of *A. squamosa* leaf extract, whereas it took up to 4 h for those administered T. conophorium leaf extract. The LD50 values for both extracts were determined to be > 5000 mg/kg, implying that they might be safe for consumption. Proper functioning of the thyroid gland is closely linked to the antioxidant system and oxidative metabolism (Sabatino, 2023). The synthesis of thyroid hormones involves the production of hydrogen peroxide, which can cause oxidative stress and cellular damage. Therefore, an effective antioxidant system is necessary to prevent oxidative stress in the thyroid gland. Studies indicate that combining antithyroid medications with antioxidants can enhance hyperthyroidism treatment of hyperthyroidism (Guerra et al., 2001; Song and Ji, 2023). Antioxidant supplementation can help reduce thyroid hormone levels in patients with hyperthyroidism (Sultana et al., 2021). The *A. squamosa* and *T. conophorum* leaf extracts exhibited significant antioxidant activity, which may contribute to their pharmacological effects and therapeutic activity. Iodide is transported into the follicular lumen, oxidized, and covalently bound to thyroglobulin by thyroid peroxidase (TPO) to form thyroid hormones (Sellitti & Suzuki, 2014). The methanolic leaf extracts of *A. squamosa* and *T. conophorum* exhibited potent in vitro inhibitory activities against TPO. As TPO inhibitors, such as Propylthiouracil and Methimazole, are established therapeutic agents for hyperthyroidism (Awosika et al, 2023; Habza-kowalska et al, 2019), the TPO-inhibiting properties of plant extracts may contribute to their pharmacological effects in treating hyperthyroidism. By inhibiting TPO, these plants may decrease thyroid hormone synthesis, thus restoring normal thyroid function, similar to the effects of Propylthiouracil and Methimazole. Thyroid-Stimulating Hormone (TSH), released from the pituitary gland, stimulates the thyroid gland to increase hormone synthesis and release. In patients with hyperthyroidism, TSH levels are generally reduced, whereas T3 and T4 levels are elevated. Compounds that can elevate TSH serum levels while reducing T3 and T4 levels are potential treatments for hyperthyroidism. This study revealed that extracts from *A. squamosa* and *T. conophorum* leaves led to

dose-dependent decreases in serum T3 and T4 hormones, with *A. squamosa* extract showing greater efficacy. Both extracts also raised serum TSH levels, with *A. squamosa* extract having a more pronounced effect. The antithyroid effects of *A. squamosa* extract were consistent with previous findings, including the identification of an antithyroid compound from *A. squamosa* (Panda et al., 2015). Although Propylthiouracil resulted in a greater reduction in serum T4 and T3 levels and a higher increase in serum TSH, the ability of the plant extract to influence TSH, T4, and T3 levels indicates its potential for hyperthyroidism treatment. This study is the first to explore the antithyroid properties of *T. conophorum*, marking a novel discovery. Patients with hyperthyroidism often exhibit elevated liver function; however, treatment can also enhance liver function. *A. squamosa* and *T. conophorum* extracts reduced serum ALT, AST, and ALP levels, suggesting improved liver function. Antithyroid medications, such as PTU and MMI, can raise liver biomarker levels in the serum, possibly indicating liver damage (Qingfeng et al., 2024). This increase may appear weeks after starting treatment, with asymptomatic ALT elevation reported in 14–28% of patients on PTU within the first two months (Kubota et al., 2008). PTU has been associated with acute hepatitis and a higher risk of severe hepatotoxicity in African-Americans. In our 15-day study, *A. squamosa* methanolic leaf extract was more effective than *T. conophorum* methanolic extract in enhancing serum liver markers, although PTU was more effective than both the plant extracts. Nonetheless, the plant extract improved liver function in a hyperthyroidism-induced animal model, indicating its potential therapeutic benefits. Blood Urea Nitrogen (BUN) levels are crucial indicators of kidney function. Hyperthyroidism is linked to increased BUN, suggesting possible kidney injury or impaired renal function (Dieterle et al., 2010). This increase was attributed to the increased urea production from retrograde protein catabolism. Hyperthyroidism treatment has been shown to lower BUN levels. Treatment with leaf extracts of *A. squamosa* and *T. conophorum* effectively reduced the elevated BUN levels associated with hyperthyroidism. At a dosage of 400 mg/kg, the *A. squamosa* leaf extract restored serum BUN levels back to those seen in the naïve control group, showing no significant difference between the two. This result underscores the potential of these plants to address kidney dysfunction linked to hyperthyroidism and supports their use in hyperthyroidism treatment. Malondialdehyde (MDA), a marker of oxidative stress-induced lipid peroxidation, is involved in organ dysfunction caused by sepsis (Tsikas et al., 2023). Increased serum MDA levels in patients with hyperthyroidism indicate oxidative stress, which contributes to organ damage. Treatment with the leaf extracts of *A. squamosa* and *T. conophorum* significantly lowered serum MDA levels compared with the vehicle control at all doses. The *A. squamosa* extract showed a more pronounced effect than the *T. conophorum* extract at all the tested doses. At 400 mg/kg, *A. squamosa* extract restored serum MDA levels to those of the naïve control group, suggesting its potential benefit in hyperthyroidism treatment. These findings highlight the antioxidant properties of both the extracts and their potential to reduce oxidative stress-related organ damage in hyperthyroidism. This study indicates that methanol leaf extracts of *A. squamosa* and *T. conophorum* could serve as valuable adjunct therapies for hyperthyroidism, with *A. squamosa* leaf extract showing more significant effects

5. Conclusion

This research indicates that methanolic leaf extracts from *A. squamosa* and *T. conophorum* could serve as beneficial supplementary treatments for hyperthyroidism, with *A. squamosa* extract showing more pronounced effects. The plants' capacity to lower serum T3 and T4 levels, enhance liver and kidney functions, and reduce oxidative stress-induced lipid peroxidation underscores their potential therapeutic advantages. Importantly, *A. squamosa* leaf extract exhibited a stronger impact in restoring BUN concentration and alleviating lipid peroxidation in the model used, which may aid in reducing organ damage associated with hyperthyroidism.

Compliance with ethical standards

Disclosure of conflict of interest

The authors declare that they have no conflicts of interest.

Statement of ethical approval

Ethical approval was obtained from the Ethics Committee at Enugu State University of Science and Technology (ESUT), located in Agbani, Enugu State

Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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