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(REVIEW ARTICLE)

Annona squamosa and *Tetracapidium conophorum* exhibited dose ratio dependent additive and synergic combination effects in *in-vitro* and *in-vivo* models of hyperthyroidism

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Abstract

Annona squamosa and *Tetracapidium conophorum* leaf extracts have been documented to possess anti-hyperthyroidal activity and are used together in ethnomedicine; however, their effective combination dose ratios have not been explored. This study investigated the combined therapeutic effects of *Annona squamosa* and *Tetracapidium conophorum* extracts in *in vitro* and in vivo models of hyperthyroidism. *In vitro* studies showed concentration-dependent inhibition of thyroid peroxidase (TPO) enzyme activity by both extracts, with *Tetracapidium conophorum* exhibiting a lower EC50 (224.05 µg/ml) than that of *Annona squamosa* (437.24 µg/ml). combination of half their EC_{50} produced 51% TPO inhibition and a combination index (CI) of 1.0, indicating additivity, while 250 µg/ml *Annona squamosa* combined with 65 µg/ml *Tetracapidium conophorum* resulted in 57% inhibition and a CI of 0.8, suggesting synergism. In an in vivo hyperthyroidism model induced by L-thyroxine, both extracts dose-dependently increased thyroid-stimulating hormone (TSH) levels. The combination dose ratios of 30 mg/kg *Annona squamosa* + 90 mg/kg *Tetracapidium conophorum* and 25 mg/kg *Annona squamosa* + 105 mg/kg *Tetracapidium conophorum* produced a CI of 0.7, indicating synergism. The combination treatment also significantly reduced elevated T3 and T4 levels, liver enzymes, and lipid peroxidation while improving kidney function markers. *Annona squamosa* and *Tetracapidium conophorum* extracts exhibited both additive and synergistic effects at different dose ratios in hyperthyroidism models, highlighting their potential to enhance antithyroid efficacy through strategic combinations.

Keywords: Hyperthyroidism; Drug interaction; Combination therapy; *Annona squamosa*; *Tetracapidium conophorum*; Thyroid peroxidase; Synergism

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1. Introduction

Hyperthyroidism, also known as overactive thyroid, is a clinical condition characterized by excessive production of thyroid hormones (triiodothyronine, T3, and tetraiodothyronine, T4) by the thyroid gland, leading to systemic hypermetabolism and increased oxidative stress [1]. Hyperthyroidism affects a notable proportion of the population, with a higher prevalence in women and younger adults [2]. The thyroid gland is one of the most common endocrine disorders in the world (generally about 4% to 7% of thyroid nodules are symptomatic). Approximately half of the cases of thyroid disease involve hyperthyroidism, and the other half involves hypothyroidism [3]. Untreated hyperthyroidism can lead to cardiovascular dysfunction, atrial fibrillation, embolic events, fragility fractures, weight loss, and osteoporosis [4]. Conventional therapeutic options for hyperthyroidism, including antithyroid medication, radioiodine, and surgery, have not improved over the past 70 years, despite substantial unmet clinical needs and a significant lack of efficacy for many patients [5]. Relapse occurs in over half of the adults after discontinuation of antithyroid drugs, and the lack of functional thyroid tissue following definitive treatment with radioiodine or surgery results in hypothyroidism with a lifelong requirement for levothyroxine replacement and associated clinical and biochemical monitoring [6]. Serious side effects, including agranulocytosis, hepatotoxicity, and vasculitis, have also been associated with current antithyroidal medications [7]. Conventional treatment resistance has been described for the management of Graves' disease-associated hypertyroidism [5]. Such cases are characterized by the persistence of thyrotoxicosis and hyperthyroidism in patients on an optimal dose of antithyroidal drugs for at least 6 months, with *good* patient compliance and at least one substitution of the antithyroidal drug type [6]. Drug resistance complicates the management of hyperthyroidism, especially in Africa where access to other therapies is often difficult. Further exploration of thyrotoxicosis mechanisms has revealed that the complexity of the disease condition requires multiple approaches [8,9]. It is now clear that the future of antithyroidal therapy relies on effective combination therapies that can tackle diverse hyperthyroidal evolution and resistance pathways. With this new understanding, the next problem is to determine the correct combination [10]. Natural products play an important role in the discovery and development of drugs. Tetracarpidium conophorum and Anona squamosa are among the numerous plants with therapeutic potential against hyperthyrothoxicosis are *Tetracarpidium conophorum* and *Anona squamosal* [11].

Annona squamosa L. (Family Annonaceae) is a tropical fruit-bearing tree, also referred to as sugar apple or custard apple. It is highly valued for its many therapeutic applications, including antithyroid, antibacterial, antidiabetic, and anti-inflammatory properties, in addition to its sweet and creamy fruit [12]. Acetogenins found in plants also have anticancer properties, particularly against colon and breast malignancies. It is also used for digestive health and skin conditions, and as a natural sedative. Among these, its use as an antithyroid agent is especially notable [13,14]. According to research on *Annona squamosa*, this plant has great potential as a natural treatment for hyperthyroidism [11]. Its antihyperthyroid properties are mainly supported by research on its seeds and leaves, which has shown that they can regulate thyroid hormone levels and reduce the negative physiological effects of hyperthyroidism [15,16]. *Tertacarpidium conophorum* (Family Euphorbiaceae), commonly known as African walnut, is a medicinal plant that is extensively utilized in West African traditional medicine. Plants have been used to treat oxidative stress, thyroid dysfunction, and inflammatory diseases. In hyperthyroidism, excessive thyroid hormone synthesis causes tissue damage and oxidative stress, and its anti-inflammatory and antioxidant properties are especially important. Additionally, studies have demonstrated its capacity to alter endocrine and metabolic processes, which may contribute to thyroid health. The various pharmacological properties of *Tetracapidium conophorum,* including its historical application in treating thyroid dysfunction symptoms, make it a viable option for additional research on combination treatments for hyperthyroidism [11]. Although individual extracts of *Annona squamosa* and *Tetracarpidium conophorum* have demonstrated effectiveness in blocking thyroid peroxidase (TPO), a crucial enzyme in the synthesis of thyroid hormones, the potential of their combinations has been undermined scientifically and may serve as the right key to unlocking successful antithyroidal combination therapy. This study was designed to evaluate the combined therapeutic effects of *Anona squamosa* and *Tetracapidium conophorum* leaf extracts in in *in-vitro* and *in-vivo* models of hyperthyroidism [11].

2. Material and methods

2.1. Plant materials

Tetracarpidium conophorum and *Anona squamosa* leaves were obtained during the rainy season (July 2022) from Nsukka Enugu State, Nigeria. The plant materials were authenticated by a taxonomist, Mr. Nwafor Felix, of the Department of Pharmacognosy and Environmental Medicine, University of Nigeria, Nsukka. A voucher specimen was deposited in the herbarium of the Department of Pharmacognosy and Traditional Medicine, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Agulu. The voucher number for *Annona squamosa* leaves was PCG/474/A/049,

and the voucher number for the leaves of *Tetracapidium conophorum* was PCG/474/E/022. The leaves were air-dried at room temperature and pulverized using a mechanical grinding machine (GX160 Delmar 5.5HP).

2.2. Animals

Male Swiss Albino rats of 8 weeks old were used for *in vivo* pharmacological evaluations, while albino mice (25 – 30 g) were used for the acute toxicity study. To reduce individual variation in disease induction and drug response, the animals were bred in the Animal House of the Department of Pharmacology, Faculty of Pharmaceutical Sciences, Enugu State University of Science and Technology, under ideal conditions of temperature, humidity, and light. The animals were fed pelletized feed (Vital Feeds, Nigeria) and had access to filtered water *ad libitum*. All animal experiments were conducted in compliance with the NIH Guide for the Care and Use of Laboratory Animals (National Institute of Health (NIH) (2011) Pub No: 85-23). 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. Extraction

Approximately 1kg of pulverized leaves of *Tetracarpidium conophorum* and *Anona squamosa* were separately extracted by cold maceration method as outlined by Ajaghaku *et al.* [17] using 5 litres of 90% methanol. The plant-to-solvent ratio was maintained at 1:5. The mixture was then shaken intermittently for 72 h. The solution was first filtered through a muslin cloth and then through a Whatman filter paper. The filtrate was concentrated to dryness *in vacuo* using a rotary evaporator (RE300 Model, United Kingdom) at 40 °C.

2.4. Total Phenolic Content of the Extracts by Folin Ciocalteu's Assay

Nzekwe et al. [11] reported the total phenolic contents of Annona quamosa and *Tetracapidium conophorum*, and the total phenolic content was estimated from the calibrated curve, which was prepared from gallic acid solution and expressed as milligrams of gallic acid equivalent (GAE) per gram of the extracts.

2.5. Phytochemical Analysis

Nzekwe et al. [11] reported the methanol extract of Annona quamosa yielded 9.42%, whereas *Tetracapidium conophorum* yielded 7.56%. While Qualitative phytochemical analysis of extracts from *Annona squamosa* and *Tetracapidium conophorum* identified various secondary metabolites, the extract from *Annona squamosa* contained alkaloids, saponins, cardiac glycosides, tannins, flavonoids, and steroids/terpenoids, but did not contain reducing sugars. In contrast, the extract from *Tetracapidium conophorum* comprises flavonoids, tannins, and alkaloids but lacks saponins, glycosides, and steroids/terpenoids.

2.6. *In Vitro* Pharmacological Assays

2.6.1. DPPH assay

The free-radical scavenging activity of the plant extracts was evaluated using the method described by Ajaghaku et al. [18]. The inhibitory effects of different dilutions of *Annona squamosa* (7.8, 15.6, 31.5, 62.5, and 125 ug/ml) and *Tetracapidium conophorum* (62.5, 125, 250, 500, and 1000 ug/ml) extracts were calculated using the following relationship:

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

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

A plot of the percentage scavenging activity against concentration was used to determine the median effective concentration (EC_{50}s) of the extracts.

2.6.2. Combination Dose selection for DPPH radical scavenging activity

Using Loewe Additivity combination strategy equation:

$$\left(\frac{a}{A} + \frac{b}{B} = 1\right)$$

Where A and B are the median effective doses of *Annona squamosa* and *Tetracapidium conophorum* extracts, respectively, and a and b represent the doses of these agents (*Annona squamosa* and *Tetracapidium conophorum* extracts, respectively) that were combined.

complemented with isobologram analysis, all the pairs of doses of drugs A (*Annona squamosa* extract) and B (*Tetracapidium conophorum* extract) that could lead to the combined effect of EAB to form additivity were defined, and these were drawn as a line of additivity of negative slope on a graph where the x and y axes represent the doses of drugs A and B.

All experimental points below the line that could give the desired effect (50% scavenging of HPPH) corresponded to a CI < 1 and indicated synergism.

Combination doses were selected based on the predicted combinations that would provide additivity and synergism. The doses included: a^1b^1 – $a^{10}b^{10}$ represented a combination of *Annona squamosa* and *Tetracapidium conophorum* extract in the ratios of 8:8, 6.52:84, 6:32, 5:64, 4.42:159.46, 4:40, 3:104, 2:52, 2:148, and 1:200 µg/ml, respectively.

The DPPD scavenging effects of these combination doses were then determined.

2.6.3. Thyroid peroxidase inhibitory activity

Thyroid Peroxidase enzyme was prepared according to the method described by Habza-Kowalska et al. [19]. Measurements were performed using a plate spectrophotometer (BioTek) in 96 well plates at a wavelength of 470 nm. The assay was conducted as follows: 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 H_2O_2. The total volume of the mixture was 210 µl. In the sample probe, the extracts were replaced with a buffer. Absorbance readings were recorded every minute for a total of 3 min at 37 °C, and the unit of TPO activity was defined as the change in the absorbance per minute. All measurements were performed in triplicate.

The TPO inhibitory activity will be calculated as follows:

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

where ΔA/min is the linear absorbance change per minute of the test material, and ΔA min blank is the linear change in absorbance per minute of blank.

The IC_{50} value was determined by interpolation of the dose-response curves. The IC_{50} values were calculated using fitted models as the concentration of the tested compound that gave 50% of the maximum inhibition based on a dose-dependent mode of action.

2.6.4. Combination Dose selection for Thyroid peroxidase inhibitory activity

Loewe Additivity combination strategy equation as previously stated above was used.

Combination doses were selected based on the predicted combinations that would provide additivity and synergism. The doses included a^1b^1 – $a^{10}b^{10}$ representing a combination of *Annona squamosa* and *Tetracapidium conophorum* extract in the ratios of 437:224, 350:15, 275:15, 250:65, 219:112, 175:45, 150:115, 125:75, 100:60, 50:45, and 50:165 µg/ml, respectively.

The TPO inhibitory effects of these combination doses were then determined as previously described.

2.7. In vivo Pharmacological Assays

2.7.1. Induction of Hyperthyroidism

After acclimatization of the rats for this assay, hyperthyroidism was induced by daily subcutaneous administration of L-thyroxine (T4) (Sigma, USA) at a dose of 600 µg/kg for 12 consecutive days according to the method described by Kim et al. [20].

2.7.2. Treatment protocol for determination of effective dose

For each extract, the animals were randomly divided into five groups of six rats each after 12 days of L-thyroxine (T4) treatment as follows:

Group 1 served as normal control and received 10 ml/kg 5% Tween 80. Group 2 served as the hyperthyroidism control and received distilled water orally for 15 days. Group 3 served as the treatment control and received 10 mg/kg propylthiouracil i. p. (PTU) orally for 15 days. Group 4 served as extract treatment group A and received 100 mg/kg extract orally for 15 days. Group-5 served as extract treatment group B and received 200 mg/kg extract orally for 15 days. Group-6 served as the extract treatment group C and received 400 mg/kg of the extract orally for 15 days. Group 7 served as the extract treatment group D and received 800 mg/kg of the extract orally for 15 days. Group 8 served as extract treatment group E and received 1000 mg/kg of the extract orally for 15 days.

The effective doses of the two plants were determined based on their effect on thyroid-stimulating hormone, which formed the basis of dose selection for the combination study.

2.7.3. Pharmacological assays

After 15 days of treatment, blood was collected from the retro-orbital plexus puncture of all overnight-fasted rats using a microcapillary. Serum was separated to estimate thyroid-stimulating hormone (TSH) levels.

2.7.4. Serum Thyroid Hormones

Serum levels of thyroid-stimulating hormone (TSH) were analyzed by colorimetric competitive enzyme immunoassay using an ELISA kit (Elabscience, USA).

The ED_{50} value was determined by interpolation of the dose-response curves. The ED_{50} values were calculated using fitted models as the concentration of the tested extracts (*Annona squamosa and Tetracapidium conophorum*) that gave 50% increase in TSH.

2.7.5. Combination Dose selection for in vivo anti-hyperthyroid activity

Loewe Additivity combination strategy equation as previously stated above was used.

Combination doses were selected based on the predicted combinations that would provide additivity and synergism. The doses include: a^1b^1 – $a^{10}b^{10}$ represented combination of *Annona squamosa* and *Tetracapidium conophorum* extract in the ratios of 50:45 mg/kg; 45:30 mg/kg; 35:60 mg/kg; 33.5:153.5 mg/kg; 30:90 mg/kg; 25:105 mg/kg; 15:60 mg/kg; 10:120 mg/kg; 10:210 mg/kg; 5:150 mg/kg respectively.

The experimental procedure for the induction and treatment of hyperthyroidism was as previously described. Blood samples were collected into evacuated tubes, and serum was separated by centrifugation at 3000 rpm for 10 min at 4°C. The separated serum was used immediately for analysis. Serum levels of the thyroid hormones T3 and T4 and thyroid-stimulating hormone (TSH) were analyzed by colorimetric competitive enzyme immunoassay using an ELISA kit (Elabscience, USA).

Further toxicity assays were carried out on groups of animals that received combination doses (33.5:153.5 mg/kg – additivity combination dose; 30:90 mg/kg and 25:105 mg/kg – synergistic combination doses). The effects of the treatment on body weight, liver function, kidney function, and lipid peroxidation were tested.

2.7.6. Biochemical Assay of Serum Liver Marker Enzymes

Serum alanine and aspartate aminotransferases were estimated using the method described by [21] using ALT and AST test kits (Span Diagnostics Ltd., India). ALT and AST concentrations were extrapolated from a graph of concentrations against wavelength absorbances of known ALT and AST concentrations.

Alkaline phosphatase was estimated by the method described by [21] using an ALP test kit (Span Diagnostics Ltd., India. ALP concentration was calculated using the following equation:

$$ALP = \frac{\text{Abs of samples x value of standard } \left(\frac{\text{IU}}{\text{L}}\right)}{\text{Abs of standard}}$$

Where Standard value = 50 IU/L

2.7.7. Biochemical assay of kidney parameters

Serum creatinine and blood urea nitrogen (BUN) levels were estimated using the method described by Tietz [22] and Heinegard and Tiderstrom [23], respectively, using BUN and creatinine test kits (Teco Diagnostics, USA).

2.7.8. Malondialdehyde (MDA) Assay

MDA levels in serum were estimated using a modified thiobarbituric acid method as described by Kinsella et al. [24] using a malondialdehyde assay kit (Elabscience Biotechnology Co. Ltd., South Africa).

2.8. Statistical Analysis

Numerical data are presented as mean ± standard deviation (SD). The obtained data were analyzed using a one-way ANOVA test followed by Newmann-Keuls multiple range tests. Statistical analyses were performed using the GraphPad version 3.1. P values were considered statistically significant at $P < 0.05$.

3. Results

3.1. Extraction, yield and phytochemical content

Table 1 Yield and Phytochemical content of *Annona squamosa and Tetracapidium conophorum*

Phytocompounds	***squamosa***	***Tetracapidium conophorum***
Saponins	+	-
Flavonoids	+	+
Tannins	+	+
Alkaloid	+	+
Glycosides	+	-
Steroid/Terpenoids	+	-
Total phenolic (mgGAE/g)	266.27±14.2	100.05±7.7
Yield (%)	9.42	7.56

Annona squamosa yielded greater extract than *Tetracapidium conophorum;* however, both extracts were less than 10% of their powdered samples (Table 1). Higher phenolic content was also recorded for *Annona squamosa* than for *Tetracapidium conophorum. Annona squamosa* contains an abundance of diverse phytocompounds, whereas saponins, glycosides, and steroids are absent from the phytocompounds identified in *Tetracapidium conophorum.*

3.2. Combination effect of *Annona squamosa* and *Tetracapidium conophorum* on thyroid peroxidase activity

Both *Annona squamosa* and *Tetracapidium conophorum* (figure 1 and 2) showed concentration-dependent inhibition of the thyroid peroxidase enzyme activity. A higher inhibitory effect was shown by *Tetracapidium conophorum* compared to *Annona squamosa* as evidenced by the lower concentration (224.05 ug/ml) of *Tetracapidium conophorum* required to produce half-maximal inhibitory effect (EC_{50}) compared to almost double the concentration (437.24 ug/ml) required by *Annona squamosa* to produce the same half-maximal inhibitory effect.

Using the EC_{50}s of the extract, all pairs of both extracts that could lead to an additive combination effect were drawn as a line of additivity of negative slope (isobologram) (figure 3). At EC_{50} of *Tetracapidium conophorum*, no quantity of *Annona squamosa* was required to produce half-maximal inhibition of thyroid peroxidase activity and vice versa. However, increasing the concentration of *Annona squamosa* in combination led to a reduced concentration of *Tetracapidium conophorum* required to produce half the maximal effect. Figure 4 shows that the combination of both extracts at various dose ratios produces different interactive effects. A combination of half their EC_{50}s (219 ug/ml for *Annona squamosa* and 112ug/ml for *Tetracapidium conophorum*) produced 51% inhibitory effect with a combination index of 1.0, an evidence of additive combination interaction. Other combination dose ratios that were tested produced

subthreshold effect (lower than 50%), except 250 ug/ml of *Annona squamosa* combined with 65 ug/ml of *Tetracapidium conophorum,* which produced 57% inhibitory effect and CI of 0.8 – an indication of synergistic interaction.

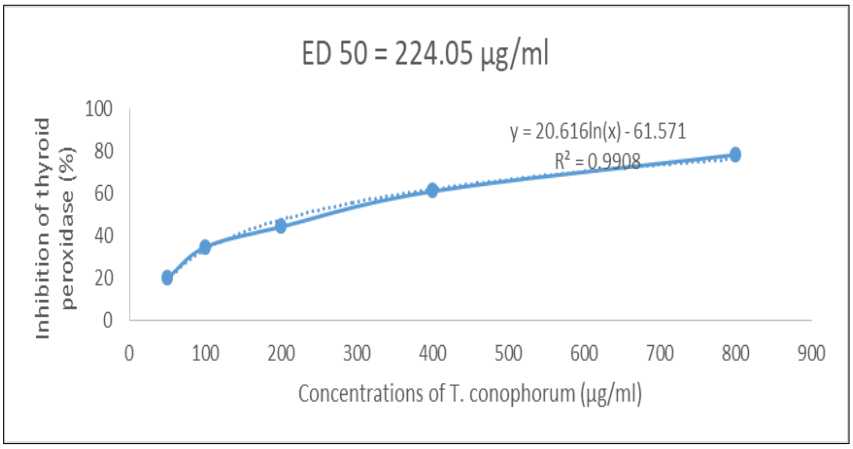


Figure 1 Inhibitory effect of graded concentrations of *Tetracapidium conophorum* on thyroid peroxidase enzyme activity

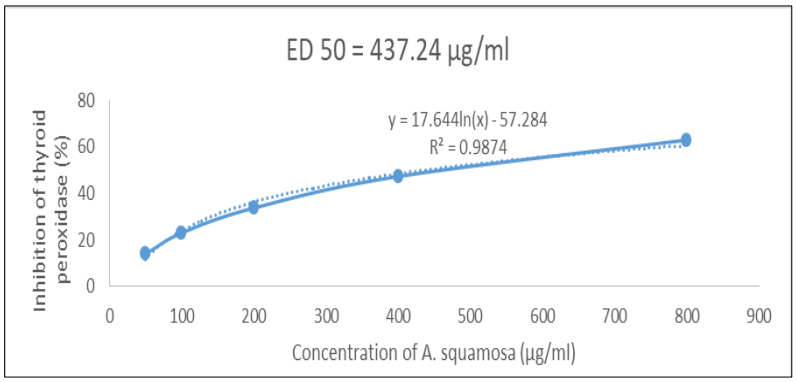
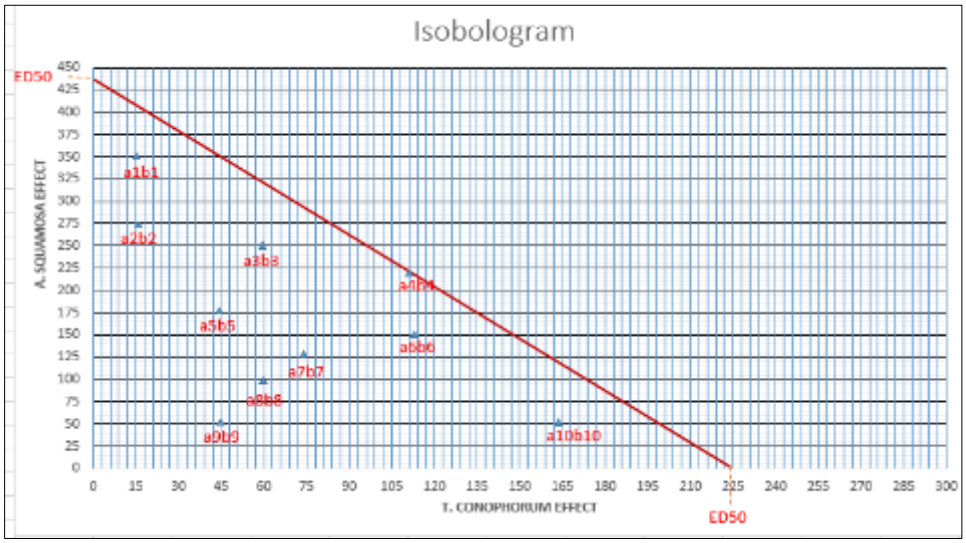
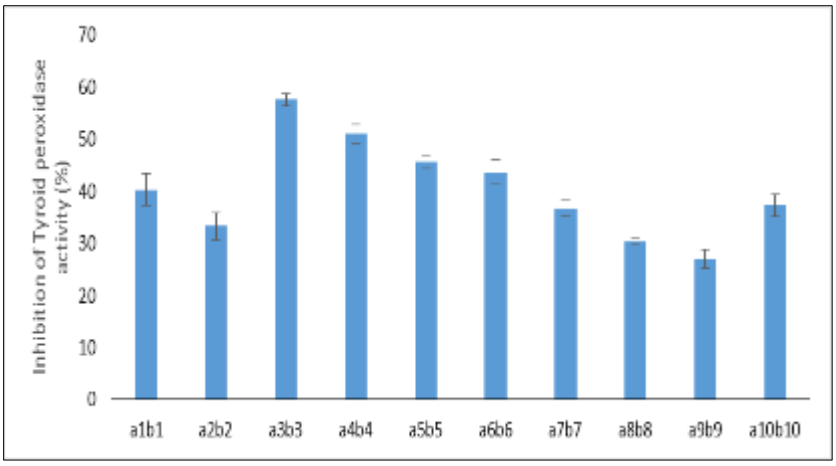


Figure 2 Inhibitory effect of graded concentrations of *Annona squamosa* on thyroid peroxidase enzyme activity



where a and b represent the combination ratios of *Annona squamosa* and *Tetracapidium conophorum* respectively; a1ba = 350:15 ug/ml; a2b2 = 275:15 ug/ml; a3b3 = 250:65 ug/ml; a4b4 219:112 ug/ml; a5b5 = 175:45 ug/ml; a6b6 = 150:115 ug/ml; a7b7 = 125:75 ug/ml; a8b8 = 100:60 ug/ml; a9b9 = 50:45 ug/ml; a10b10 = 50:165 ug/ml.

Figure 3 Isobologram plot of combination strategy of *Tetracapidium conophorum* and *Annona squamosa* on inhibition of thyroid peroxidase enzyme



where a and b represent the combination ratios of *Annona squamosa* and *Tetracapidium conophorum* respectively; a1ba = 350:15 ug/ml; a2b2 = 275:15 ug/ml; a3b3 = 250:65 ug/ml; a4b4 219:112 ug/ml; a5b5 = 175:45 ug/ml; a6b6 = 150:115 ug/ml; a7b7 = 125:75 ug/ml; a8b8 = 100:60 ug/ml; a9b9 = 50:45 ug/ml; a10b10 = 50:165 ug/ml.

Figure 4 Inhibitory effect of combination concentrations of *Annona squamosa* and *Tetracapidium conophorum* on thyroid peroxidase enzyme activity

3.3. Combination inhibitory effect of *Annona squamosa* and *Tetracapidium conophorum* on DPPH radical

Annona squamosa and *Tetracapidium conophorum* inhibited DPPH free radicals in a concentration-dependent manner. *Annona squamosa* has better inhibitory potentials with IC_{50} 8.84 ug/ml (fig. 5) compared to *Tetracapidium conophorum* with IC_{50} 318.92 ug/ml (fig. 6). Using a dose-effect-based strategy of analysis of combination interactions, an isobologram plot was derived using the effective DPPH inhibitory concentrations of both plant extracts, as shown in fig. 7. An additive combination interaction was produced by a 4.42 ug/ml combination of *Annona squamosa* and 159.46 ug/ml of *Tetracapidium conophorum*. Similarly, 6.52 ug/ml of *Annona squamosa* combined with 84 ug/ml of *Tetracapidium conophorum* produced an additive combination interaction. When traced on the isobologram plot, these combination concentrations fell on the line of additivity, and their combination index was calculated to be 1.0, a confirmatory indication of the additivity combination interaction. The concentrations that produced additivity interactions were able to produce up to 50% inhibition of DPPH radical, which was the target/desired effect. No other combination concentrations tested had an effect up to the threshold or target. Combination concentrations of *Annona squamosa* less than 4 ug/ml produced inhibition below 40%, as shown in fig. 8.

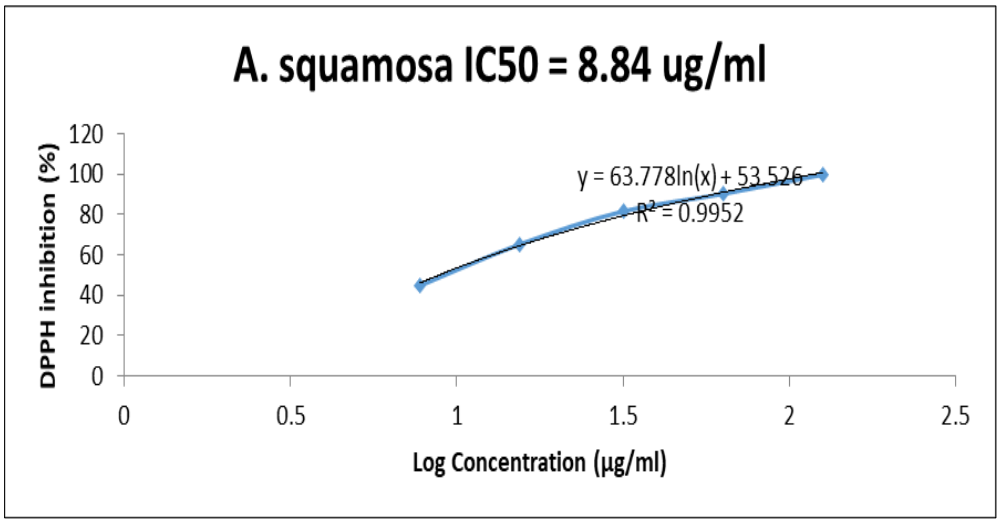


Figure 5 Inhibitory effect of graded concentrations of *Annona squamosa* on DPPH radical

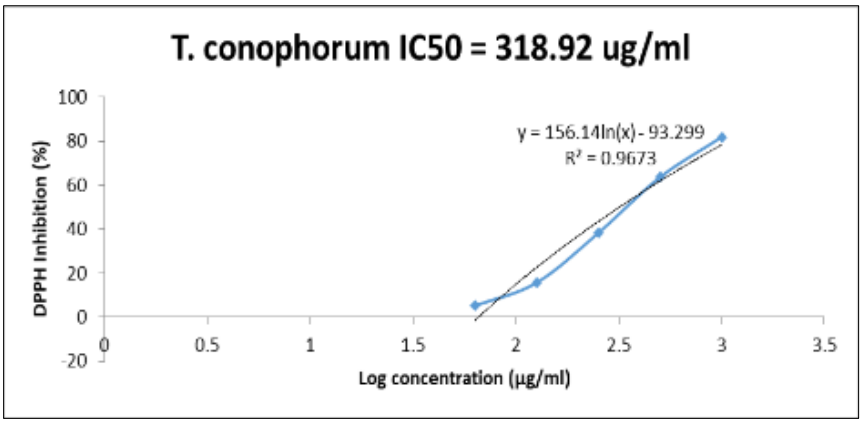
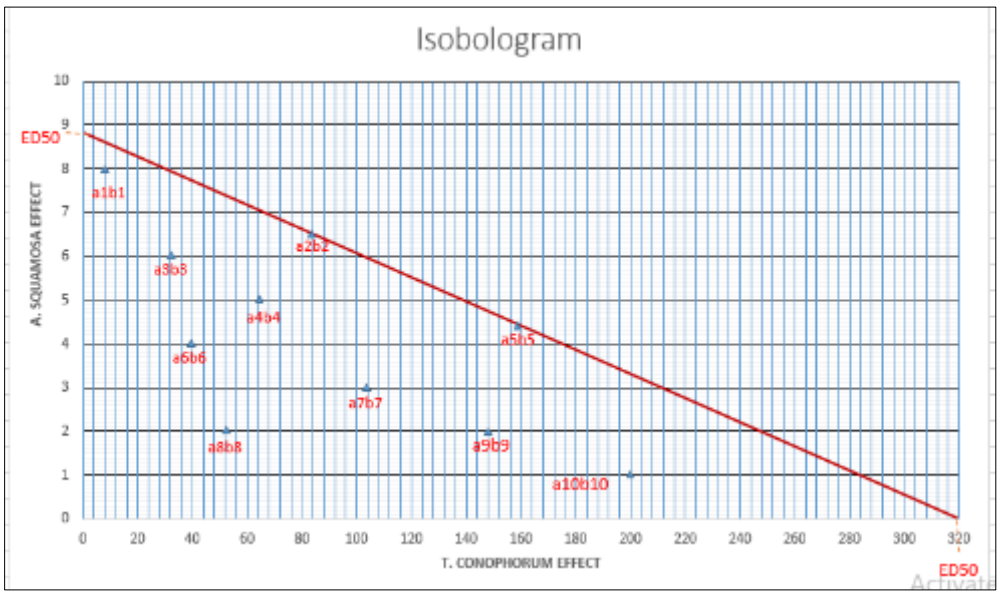
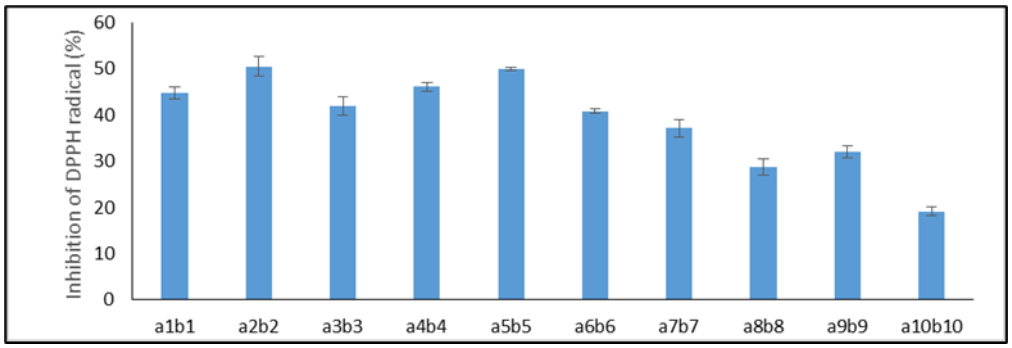


Figure 6 Inhibitory effect of graded concentrations of *Tetracapidium conophorum* on DPPH radical



Where a and b represent combination ratios of *Annona squamosa* and *Tetracapidium conophorum* respectively- a1ba = 8:8 ug/ml; a2b2 = 6.52:84 ug/ml; a3b3 = 6:32 ug/ml; a4b4 5:64 ug/ml; a5b5 = 4.42:159.46 ug/ml; a6b6 = 4:40 ug/ml; a7b7 = 3:104 ug/ml; a8b8 = 2:52 ug/ml; a9b9 = 2:148 ug/ml; a10b10 = 1:200 ug/ml.

Figure 7 Isobologram plot of combination strategy of *Tetracapidium conophorum* and *Annona squamosa* on inhibition of DPPH radical



Where a and b represent combination ratios of *Annona squamosa* and *Tetracapidium conophorum* respectively- a1ba = 8:8 ug/ml; a2b2 = 6.52:84 ug/ml; a3b3 = 6:32 ug/ml; a4b4 5:64 ug/ml; a5b5 = 4.42:159.46 ug/ml; a6b6 = 4:40 ug/ml; a7b7 = 3:104 ug/ml; a8b8 = 2:52 ug/ml; a9b9 = 2:148 ug/ml; a10b10 = 1:200 ug/ml.

Figure 8 Inhibitory effect of combination concentrations of *Annona squamosa* and *Tetracapidium conophorum* on DPPH radical

3.4. Combination effect of *Annona squamosa* and *Tetracapidium conophorum* on hyperthyroidism induced reduction in serum TSH concentration

Annona squamosa and *Tetracapidium conophorum* extracts showed a dose-dependent increase in thyroid-stimulating hormone concentrations. A greater increase in TSH was exhibited by *Annona squamosa* with a median effective dose (ED_{50}) of 67 mg/kg compared to *Tetracapidium conophorum* with an ED_{50} of 307 mg/kg, as shown in figures 9 and 10. Using the median effective doses of both extracts, an isobologram plot (fig. 11) was constructed and possible combination doses that could lead to additivity and synergistic interactions were identified. All dose combinations in the line of additivity were predicted to produce an additive effect. When tested, a4b4 (33.5 mg/kg *Annona squamosa* + 153.5 mg/kg *Tetracapidium conophorum*) produced a 48.8 percent increase in TSH. However, this dose combination was predicted to have an additive effect of 50% increase in TSH. However, the effect was slightly lower than that of the goal/effect set. Among all the dose combinations below the line of additivity in isobologram plot predicted to produce synergistic interaction, only a5b5 (30 mg/kg *Annona squamosa* + 90 mg/kg *Tetracapidium conophorum*) and a6b6 (25 mg/kg *Annona squamosa* + 105 mg/kg *Tetracapidium conophorum*) produced effect above the desired target of 50% increase in TSH with a combination index of 0.7 evidence of synergistic interaction (fig. 12).

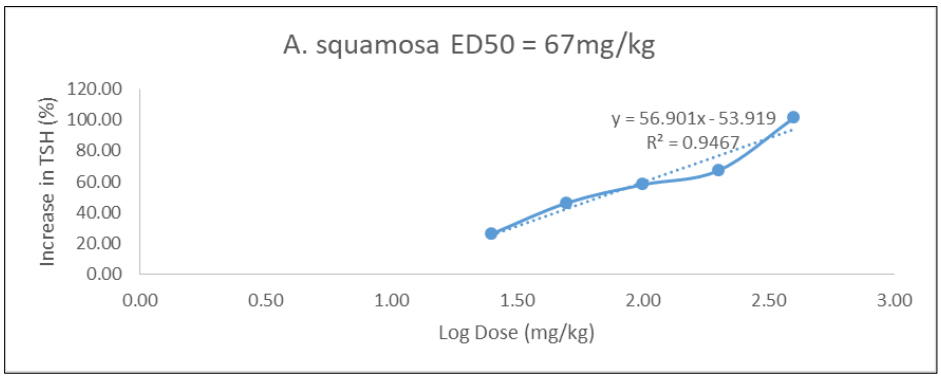


Figure 9 Effect of graded doses of *Annona squamosa* on Thyroid stimulating hormone

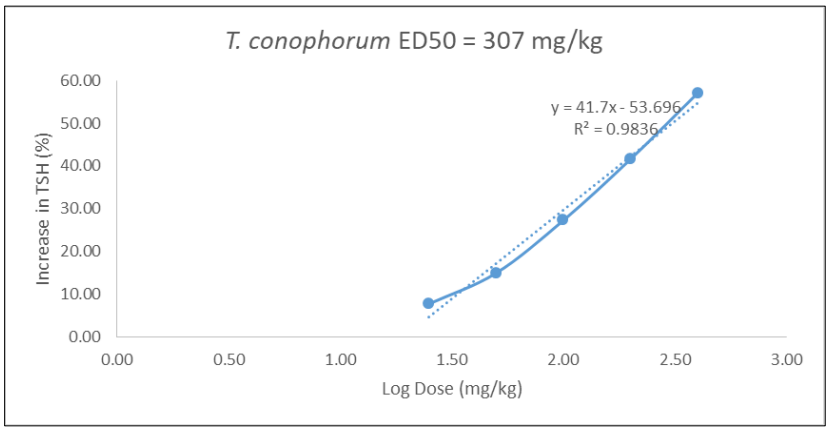
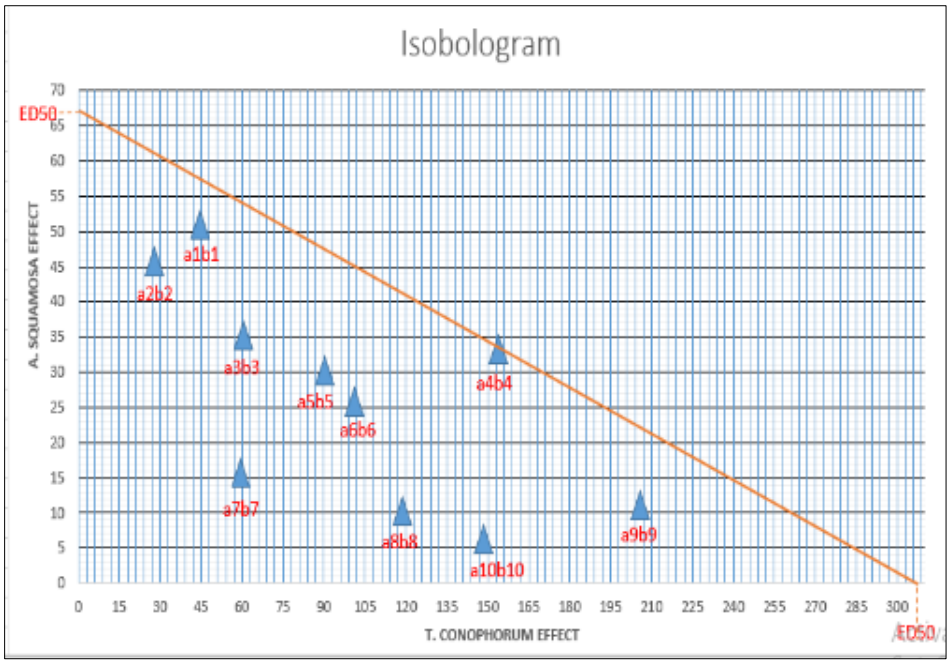
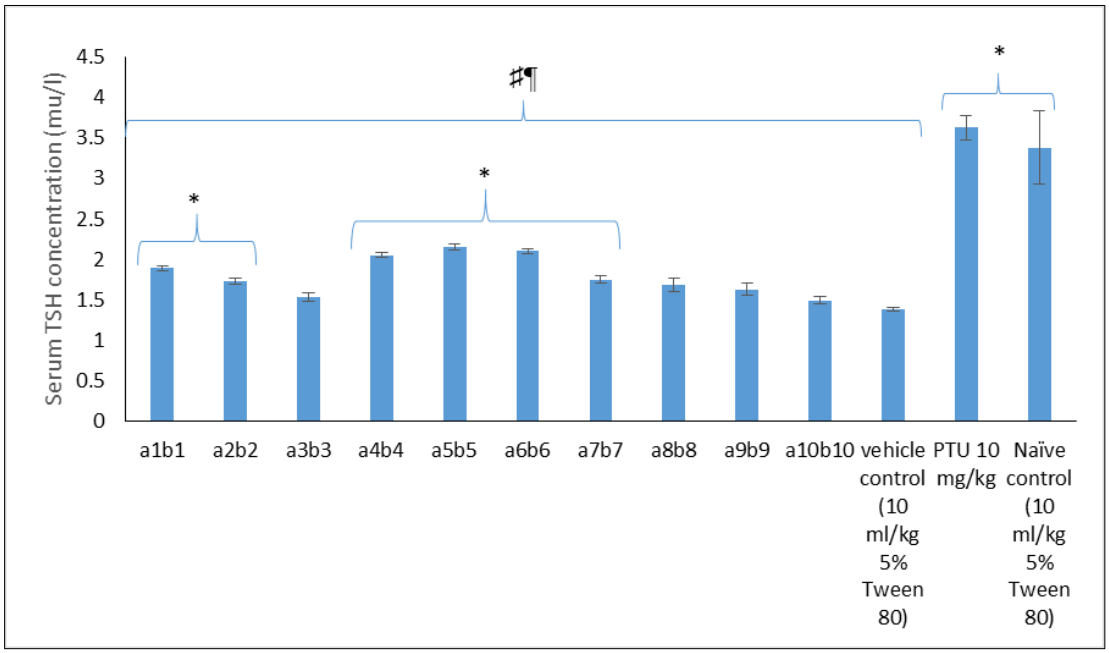


Figure 10 Effect of graded doses of *Tetracapidium conophorum* on Thyroid stimulating hormone



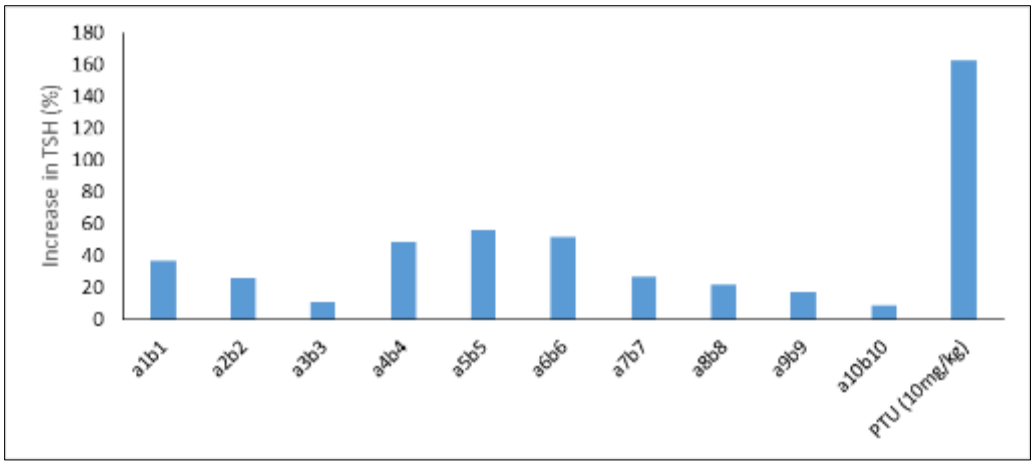
Where a and b represent combination ratios of *Annona squamosa* and *Tetracapidium conophorum* respectively- a1ba = 50:45 mg/kg; a2b2 = 45:30 mg/kg; a3b3 = 35:60 mg/kg; a4b4 33.5:153.5 mg/kg; a5b5 = 30:90 mg/kg; a6b6 = 25:105 mg/kg; a7b7 = 15:60 mg/kg; a8b8 = 10:120 mg/kg; a9b9 = 10:210 mg/kg; a10b10 = 5:150 mg/kg,

Figure 11 Isobologram plot of combination strategy of *Tetracapidium conophorum* and *Annona squamosa* on increase in TSH



where a and b represent the combination ratios of *Annona squamosa* and *Tetracapidium conophorum* respectively; a1ba = 50:45 mg/kg; a2b2 = 45:30 mg/kg; a3b3 = 35:60 mg/kg; a4b4 33.5:153.5 mg/kg; a5b5 = 30:90 mg/kg; a6b6 = 25:105 mg/kg; a7b7 = 15:60 mg/kg; a8b8 = 10:120 mg/kg; a9b9 = 10:210 mg/kg; a10b10 = 5:150 mg/kg, PTU = propylthiouracil. * = P<0.05 compared to vehicle control; ♯ = P<0.05 compared to propylthiouracil; ¶ = P<0.05 compared to Naïve control group

Figure 12 Effect of combination doses of *Annona squamosa* and *Tetracapidium conophorum* on serum TSH concentration



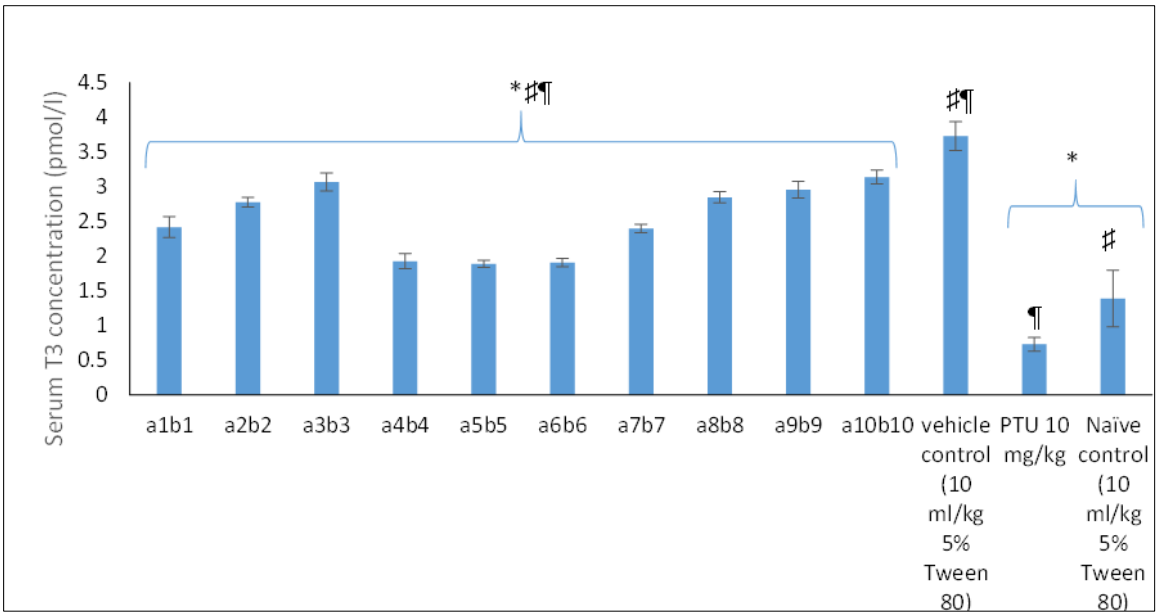
where a and b represent the combination ratios of *Annona squamosa* and *Tetracapidium conophorum* respectively; a1ba = 50:45 mg/kg; a2b2 = 45:30 mg/kg; a3b3 = 35:60 mg/kg; a4b4 33.5:153.5 mg/kg; a5b5 = 30:90 mg/kg; a6b6 = 25:105 mg/kg; a7b7 = 15:60 mg/kg; a8b8 = 10:120 mg/kg; a9b9 = 10:210 mg/kg; a10b10 = 5:150 mg/kg, PTU = propylthiouracil.

Figure 13 Effect of combination doses of *Annona squamosa* and *Tetracapidium conophorum* on percentage increase in serum TSH concentration

The reduction in TSH following the induction of hyperthyroidism was evidenced by the significant ($P<0.05$) reduction in serum TSH in the induced vehicle-treated control group compared to the naïve uninduced control group. Treatment with combined doses of *Annona squamosa* and *Tetracapidium conophorum* produced a dose-ratio combination-dependent effect. Of the combination doses, a1b1 (50 mg/kg *Annona squamosa* + 45 mg/kg *Tetracapidium conophorum*), a2b2 (45 mg/kg *Annona squamosa* + 30 mg/kg *Tetracapidium conophorum*), a4b4 (33.5 mg/kg *Annona squamosa* + 153.5 mg/kg *Tetracapidium conophorum*), a5b5 (30 mg/kg *Annona squamosa* + 90 mg/kg *Tetracapidium conophorum*), a6b6 (25 mg/kg *Annona squamosa* + 105 mg/kg *Tetracapidium conophorum*) and a7b7 (15 mg/kg *Annona squamosa* + 60 mg/kg *Tetracapidium conophorum*) produced significant ($P<0.05$) improvement in serum TSH compared to vehicle control while other combination doses a3b3 (35 mg/kg *Annona squamosa* + 60 mg/kg *Tetracapidium conophorum*), a8b8 (10 mg/kg *Annona squamosa* + 120 mg/kg *Tetracapidium conophorum*), a9b9 (10 mg/kg *Annona squamosa* + 210 mg/kg *Tetracapidium conophorum*) and a10b10 (5 mg/kg *Annona squamosa* + 150 mg/kg *Tetracapidium conophorum*) produced non-significant improvement in serum TSH compared to vehicle control group. Combination doses that produced significant ($P<0.05$) improvement recorded percentage increases above 25%, while those that produced non-significant ($P>0.05$) effects recorded below 25% increase. The effect produced by the combination dose ratios was lower than that of the reference standard propylthiouracil (PTU 10 mg/kg), as seen by significant ($P<0.05$) differences between these groups' serum TSH levels (fig. 13). Similarly, these combination dose ratios were unable to restore serum TSH to basal levels observed in uninduced naïve controls. The reference drug at 10 mg/kg produced an increase in TSH beyond the level recorded in the naïve uninduced control group, although the difference between both groups was not statistically significant.

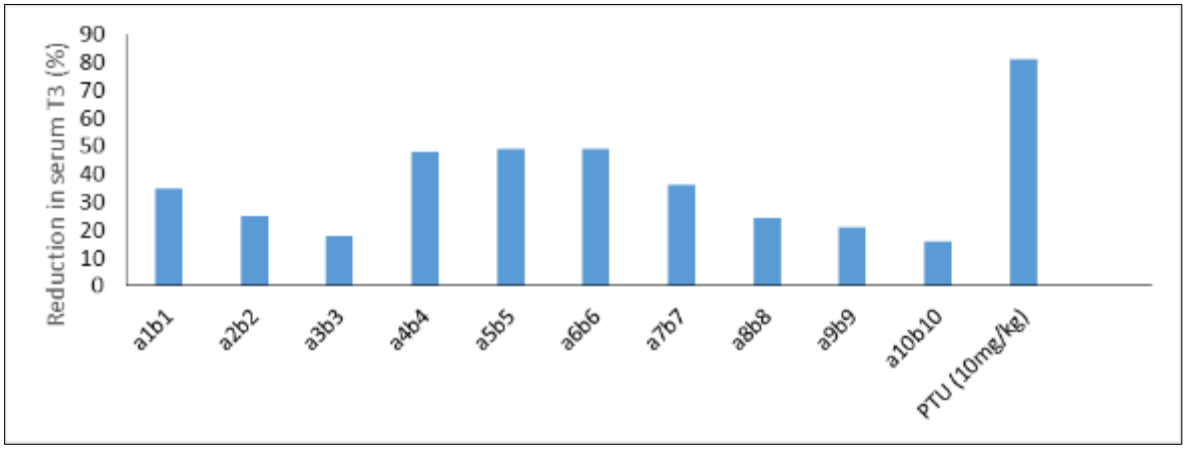
3.5. The combined effect of *Annona squamosa* and *Tetracapidium conophorum* on hyperthyroidism increased serum T3 and T4 concentrations

Hyperthyroidism-induced increases in serum T3 and T4 levels reduced following treatment with various combination dose ratios of *Annona squamosa* and *Tetracapidium conophorum*. Compared with the vehicle control group, all combination doses tested produced a significant ($P<0.05$) reduction in serum T3 concentration (fig. 14). The effects produced by a4b4, a5b5, and a6b6 combination doses (48%, 49 %, and 49%, respectively) were highest compared to other combination doses, which produced percentage reductions lower than 40%. Similarly, all tested combination dose ratios, except a3b3, produced a significant reduction in T4 serum concentrations compared to the vehicle control group. In both T3 and T4, the reference standard (PTU 10 mg/kg) produced a significantly higher effect than the combination dose (fig. 16). The reduction produced by PTU was also significantly higher than that of the naïve uninduced control for serum T3 and non-significantly higher than that of the naïve control for T4 (fig. 17).



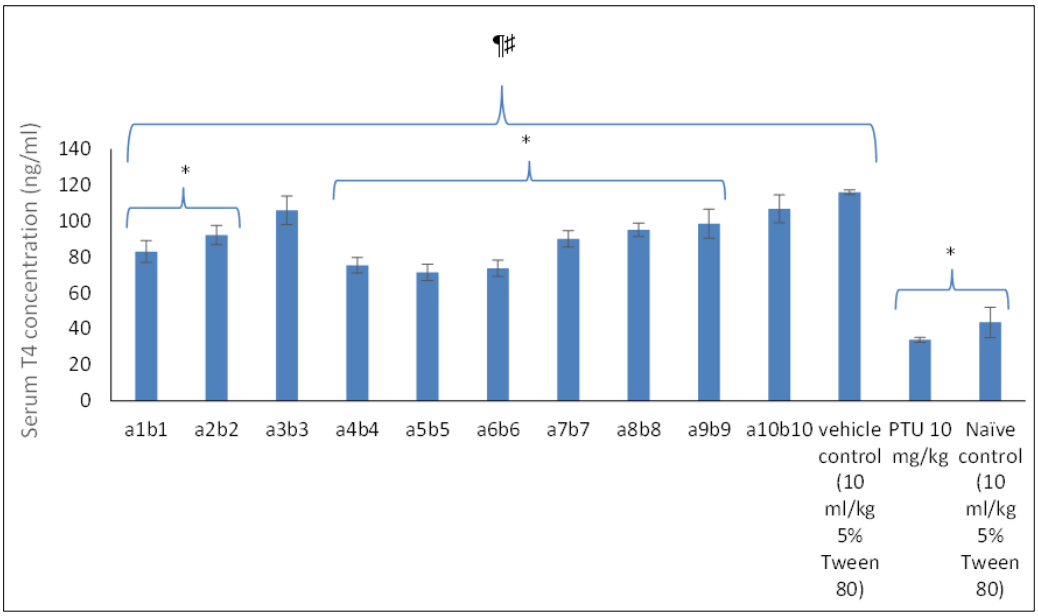
where a and b represent the combination ratios of *Annona squamosa* and *Tetracapidium conophorum* respectively; a1ba = 50:45 mg/kg; a2b2 = 45:30 mg/kg; a3b3 = 35:60 mg/kg; a4b4 33.5:153.5 mg/kg; a5b5 = 30:90 mg/kg; a6b6 = 25:105 mg/kg; a7b7 = 15:60 mg/kg; a8b8 = 10:120 mg/kg; a9b9 = 10:210 mg/kg; a10b10 = 5:150 mg/kg, PTU = propylthiouracil. * = P<0.05 compared to vehicle control; ♯ = P<0.05 compared to propylthiouracil; ¶ = P<0.05 compared to Naïve control group

Figure 14 Effect of combination doses of *Annona squamosa* and *Tetracapidium conophorum* on serum T3 concentration



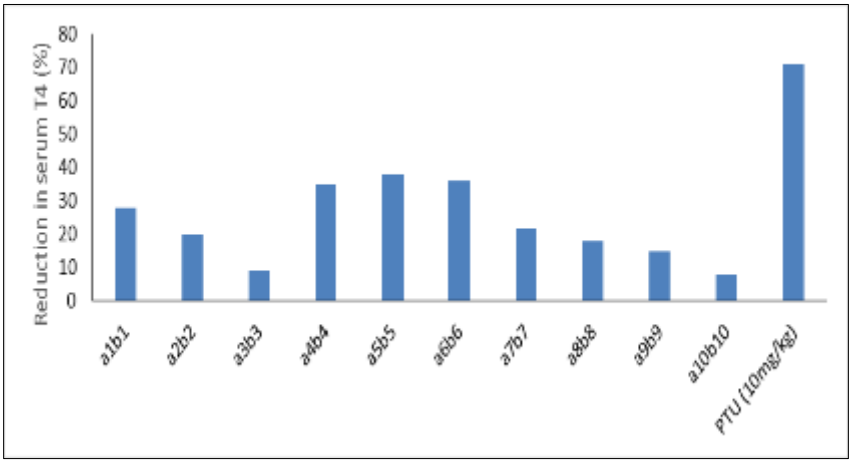
Where a and b represent combination ratios of *Annona squamosa* and *Tetracapidium conophorum* respectively- a1ba = 50:45 mg/kg; a2b2 = 45:30 mg/kg; a3b3 = 35:60 mg/kg; a4b4 33.5:153.5 mg/kg; a5b5 = 30:90 mg/kg; a6b6 = 25:105 mg/kg; a7b7 = 15:60 mg/kg; a8b8 = 10:120 mg/kg; a9b9 = 10:210 mg/kg; a10b10 = 5:150 mg/kg, PTU = propylthiouracil

Figure 15 Effect of combination doses of *Annona squamosa* and *Tetracapidium conophorum* on percentage reduction of serum T3 concentration



where a and b represent the combination ratios of *Annona squamosa* and *Tetracapidium conophorum* respectively; a1ba = 50:45 mg/kg; a2b2 = 45:30 mg/kg; a3b3 = 35:60 mg/kg; a4b4 33.5:153.5 mg/kg; a5b5 = 30:90 mg/kg; a6b6 = 25:105 mg/kg; a7b7 = 15:60 mg/kg; a8b8 = 10:120 mg/kg; a9b9 = 10:210 mg/kg; a10b10 = 5:150 mg/kg, PTU = propylthiouracil. * = P<0.05 compared to vehicle control; ♯ = P<0.05 compared to propylthiouracil; ¶ = P<0.05 compared to Naïve control group

Figure 16 Effect of combination doses of *Annona squamosa* and *Tetracapidium conophorum* on serum T4 concentration



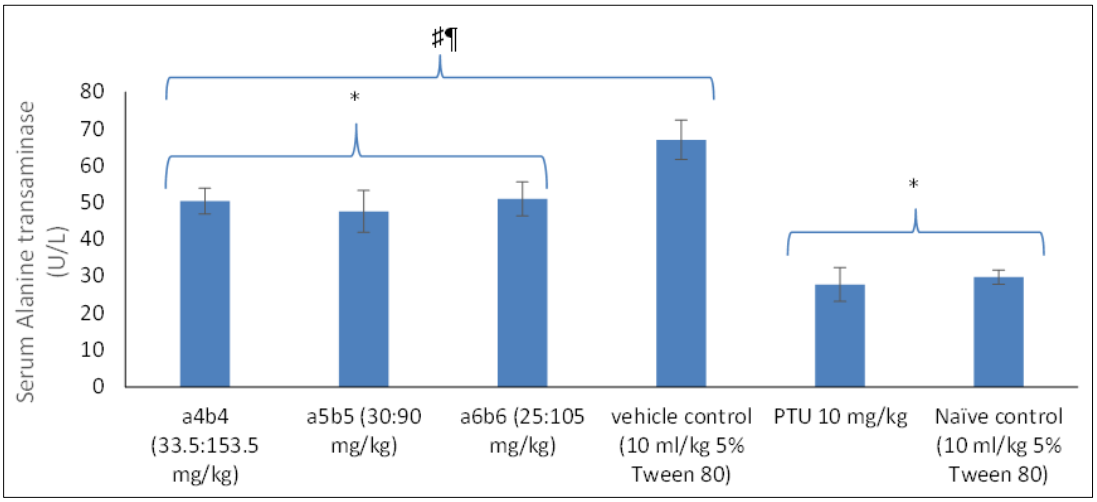
Where a and b represent combination ratios of *Annona squamosa* and *Tetracapidium conophorum* respectively- a1ba = 50:45 mg/kg; a2b2 = 45:30 mg/kg; a3b3 = 35:60 mg/kg; a4b4 33.5:153.5 mg/kg; a5b5 = 30:90 mg/kg; a6b6 = 25:105 mg/kg; a7b7 = 15:60 mg/kg; a8b8 = 10:120 mg/kg; a9b9 = 10:210 mg/kg; a10b10 = 5:150 mg/kg, PTU = propylthiouracil

Figure 17 Effect of combination doses of *Annona squamosa* and *Tetracapidium conophorum* on percentage reduction of serum T3 concentration

3.6. Combination effect of *Annona squamosa* and *Tetracapidium conophorum* on liver function enzymes in hyperthyroidism-induced animals

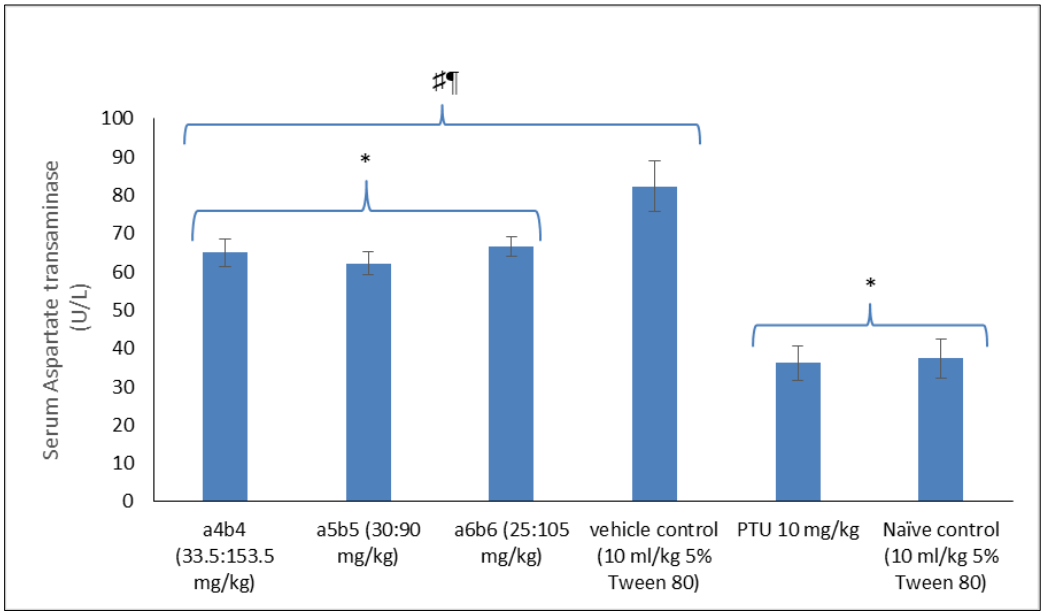
Induction of hyperthyroidism led to elevated serum concentrations of liver function enzymes, alanine transaminase, aspartate transaminase, and alkaline phosphatase. Compared to the uninduced naïve control group, the vehicle-treated control group showed a significant (P<0.05) elevation in the serum concentration of all liver function marker enzymes. Treatment with the combination dose ratios of *Annona squamosa* and *Tetracapidium conophorum* (that produced near additive and synergic combination interactive effect in TSH in hyperthyroid experimental model) produced significant (P<0.05) reduction in these elevated enzymes compared to the vehicle control group. Similarly, a significant (P<0.05) reduction was observed in the reference drug-treated group compared to the vehicle control group. The reduction produced by the combination dose ratio was not sufficient to return the elevated serum enzyme concentration to the basal values seen in the naïve control group. Only the reference standard was able to restore serum concentrations of

these liver marker enzymes to levels similar to those of the naïve control, without significant (P>0.05) differences between these groups, as shown in fig. 18, 19, and 20.



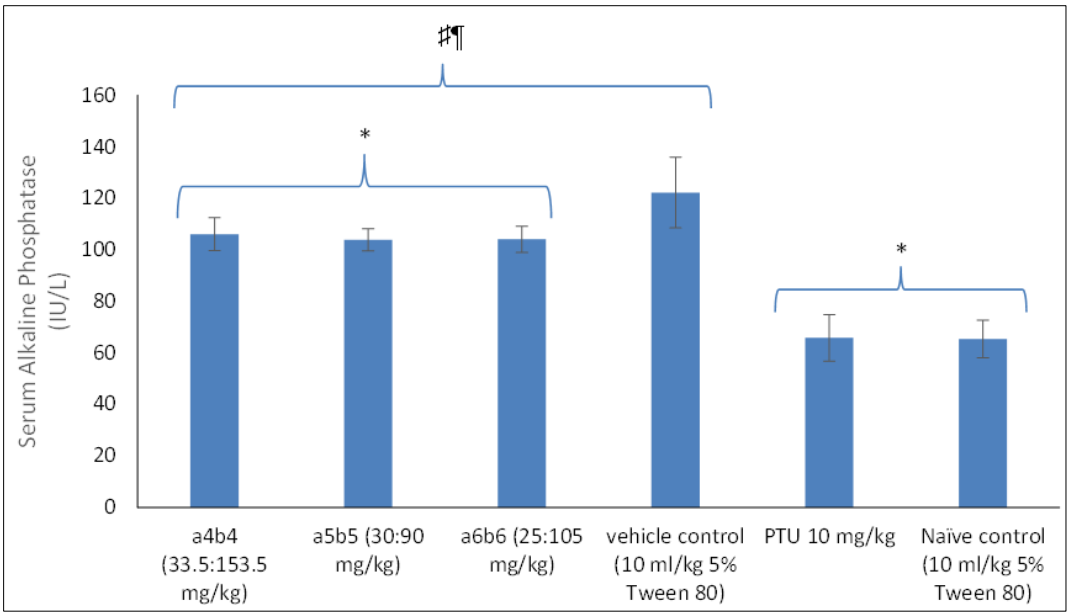
Where a and b represent combination ratios of *Annona squamosa* and *Tetracapidium conophorum* respectively- a4b4 33.5:153.5 mg/kg; a5b5 = 30:90 mg/kg; a6b6 = 25:105 mg/kg; PTU = propylthiouracil. * = P<0.05 compared to vehicle control; ♯ = P<0.05 compared to propylthiouracil; ¶ = P<0.05 compared to Naïve control group

Figure 18 Effect of combination doses of *Annona squamosa* and *Tetracapidium conophorum* on serum Alanine transaminase concentration



Where a and b represent combination ratios of *Annona squamosa* and *Tetracapidium conophorum* respectively- a4b4 33.5:153.5 mg/kg; a5b5 = 30:90 mg/kg; a6b6 = 25:105 mg/kg; PTU = propylthiouracil. * = P<0.05 compared to vehicle control; ♯ = P<0.05 compared to propylthiouracil; ¶ = P<0.05 compared to Naïve control group

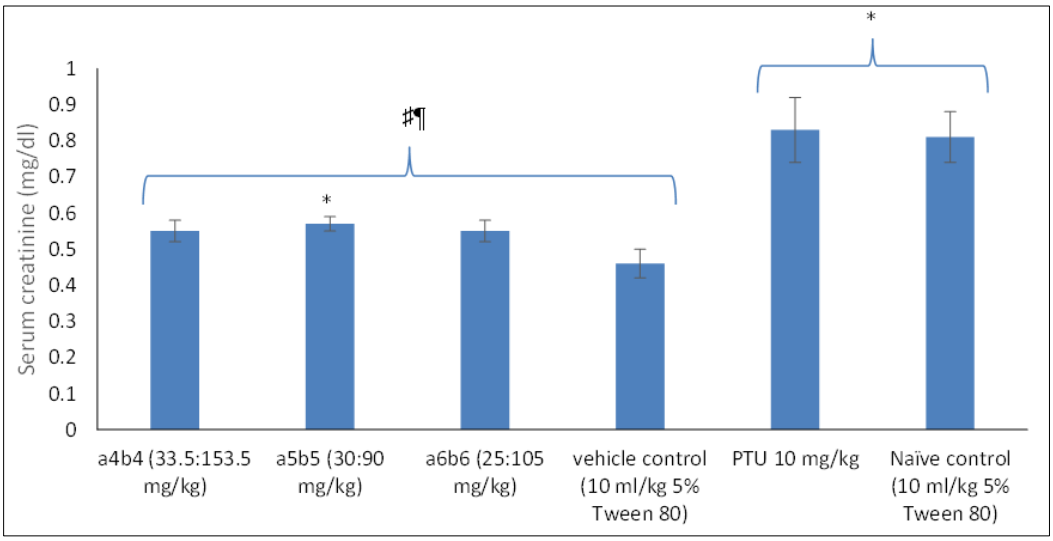
Figure 19 Effect of combination doses of *Annona squamosa* and *Tetracapidium conophorum* on serum Aspartate transaminase concentration



Where a and b represent combination ratios of *Annona squamosa* and *Tetracapidium conophorum* respectively- a4b4 33.5:153.5 mg/kg; a5b5 = 30:90 mg/kg; a6b6 = 25:105 mg/kg; PTU = propylthiouracil. * = P<0.05 compared to vehicle control; ♯ = P<0.05 compared to propylthiouracil; ¶ = P<0.05 compared to Naïve control group.

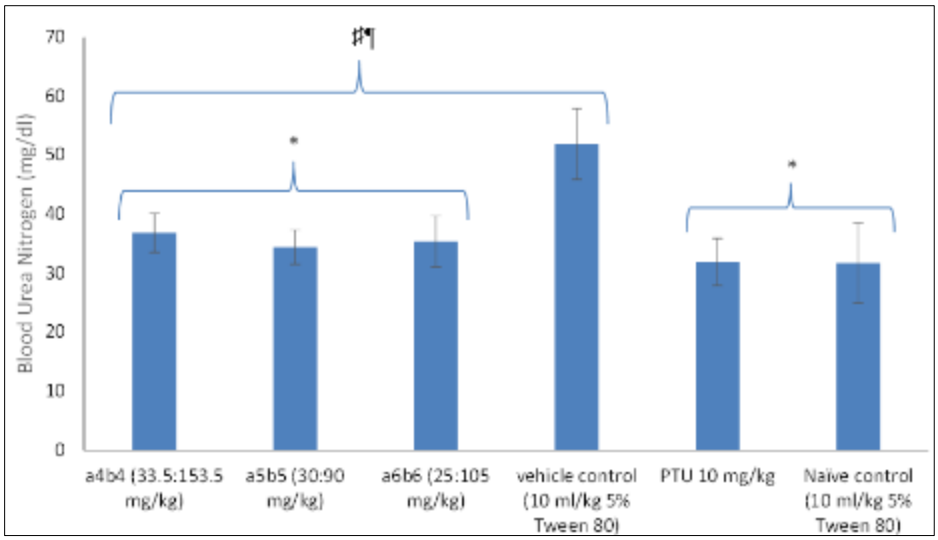
Figure 20 Effect of combination doses of *Annona squamosa* and *Tetracapidium conophorum* on serum Alkaline phosphatase concentration

3.7. Combination effect of *Annona squamosa* and *Tetracapidium conophorum* on kidney function markers in hyperthyroidism-induced animals



Where a and b represent combination ratios of *Annona squamosa* and *Tetracapidium conophorum* respectively- a4b4 33.5:153.5 mg/kg; a5b5 = 30:90 mg/kg; a6b6 = 25:105 mg/kg; PTU = propylthiouracil. * = P<0.05 compared to vehicle control; ♯ = P<0.05 compared to propylthiouracil; ¶ = P<0.05 compared to Naïve control group

Figure 21 Effect of combination doses of *Annona squamosa* and *Tetracapidium conophorum* on serum creatinine concentration



Where a and b represent combination ratios of *Annona squamosa* and *Tetracapidium conophorum* respectively- a4b4 33.5:153.5 mg/kg; a5b5 = 30:90 mg/kg; a6b6 = 25:105 mg/kg; PTU = propylthiouracil. * = P<0.05 compared to vehicle control; ♯ = P<0.05 compared to propylthiouracil; ¶ = P<0.05 compared to Naïve control group.

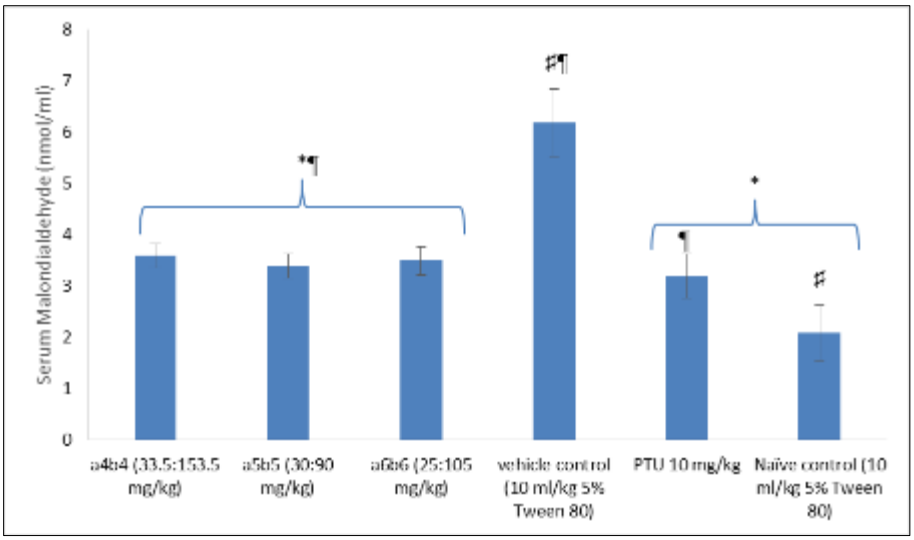
Figure 22 Effect of combination doses of *Annona squamosa* and *Tetracapidium conophorum* on serum Blood urea nitrogen concentration

Figure 21 shows that hyperthyroidism was accompanied by a significant (P<0.05) reduction in serum creatinine concentration and a significant (P<0.05) increase in blood urea nitrogen, as evidenced by the statistical comparison between the naïve uninduced control and vehicle control groups. Although the combination doses did not differ significantly (P>0.05) from each other in terms of their effect on these kidney function biomarkers, only a5b5 produced a significant (P<0.05) increase in serum creatinine concentration compared with the vehicle control group. The effect produced by the reference standard was similar to that of the naïve control group and was significantly higher than that produced by the combination dose ratio of *Annona squamosa* and *Tetracapidium conophorum*.

For the blood urea nitrogen as shown by fig. 22, treatment with the combination doses produced a significant (P<0.05) reduction in BUN concentration compared to the vehicle control group. The reductive effect of these treatments was able to reduce BUN concentration to a concentration similar to that found in the naïve uninduced control group, similar to the reference drug-treated group. No significant (P>0.05) difference existed between BUN concentration in the combination extract-treated groups and the naïve control group, as well as the reference drug-treated group.

3.8. Combination effect of *Annona squamosa* and *Tetracapidium conophorum* lipid peroxidation in hyperthyroidism induced animals

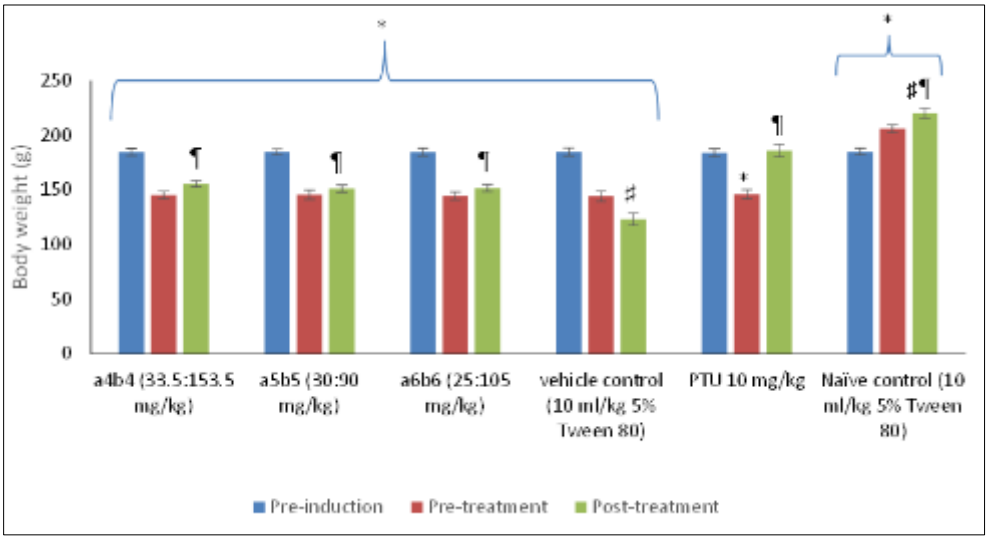
As shown in figure 23, hyperthyroidism was marked by an increase in serum malondialdehyde, a product of lipid peroxidation. The increase in serum malondialdehyde in the vehicle control animals was significant (P<0.05) compared to that in the naïve uninduced control. Treatment with the combined doses of *Annona squamosa* and *Tetracapidium conophorum* produced a significant (P<0.05) reduction in serum malondialdehyde levels, comparable (similar) to the effect produced by the reference standard PTU 10 (mg/kg). The inhibition of lipid peroxidation by the combined doses did not differ significantly (P>0.05) across the different dose ratios and compared to the reference drug. However, when compared to the naïve uninduced control, both the combination doses and the reference standard showed significantly (P<0.05) higher serum malondialdehyde levels.



Where a and b represent combination ratios of *Annona squamosa* and *Tetracapidium conophorum* respectively- a4b4 33.5:153.5 mg/kg; a5b5 = 30:90 mg/kg; a6b6 = 25:105 mg/kg; PTU = propylthiouracil. * = P<0.05 compared to vehicle control; ♯ = P<0.05 compared to propylthiouracil; ¶ = P<0.05 compared to Naïve control group.

Figure 23 Effect of combination doses of *Annona squamosa* and *Tetracapidium conophorum* on serum Blood urea nitrogen concentration

3.9. Combination effect of *Annona squamosa* and *Tetracapidium conophorum* on body weight in hyperthyroidism induced animals



Where a and b represent combination ratios of *Annona squamosa* and *Tetracapidium conophorum* respectively- a4b4 33.5:153.5 mg/kg; a5b5 = 30:90 mg/kg; a6b6 = 25:105 mg/kg; PTU = propylthiouracil. * = P<0.05 compared to vehicle control; ♯ = P<0.05 compared to propylthiouracil; ¶ = P<0.05 compared to Naïve control group.

Figure 24 Effect of combination doses of *Annona squamosa* and *Tetracapidium conophorum* on body weight

Induction of hyperthyroidism resulted in body weight reduction, as evidenced by the significant (P<0.05) difference between the pre-induction and pre-treatment body weights across all groups. Although the control group showed a significant (P<0.05) reduction in body weight between the pre-treatment and post-treatment intervals, treatment with the combination doses resulted in a further reduction in body weight, with a non-significant increase in body weight when compared to the pre-treatment values. Compared to the post treatment body weight of the vehicle control group, all the combination dose ratios produced significant (P<0.05) increases in body weight, except for the reference standard treated group. All other treatment groups failed to restore body weight to the pre-induction value, as evidenced by the significant (P<0.05) difference that exists between the pre-induction and post-treatment body weight, as shown in figure 24.

4. Discussion

in recent years, there has been a growing interest in the exploration of natural sources for the development of novel therapeutic agents [25] Several plants have been used in folk medicine throughout the world [26]. Plants have been a source of medicine for the treatment of numerous diseases owing to their diverse phytocompounds. This diversity in plants is better harnessed when used in crude unfractionated forms and combinations [27]. However, it is too simplistic to assume a positive synergy from any two or more herbs combined in traditional practice, as some may lead to an unexpected decrease in activity due to competition for the same site of action or interference with the pharmacokinetics of each other [28]. Similarly, when combined, two plants can exhibit both antagonism and synergism, depending on the dose pair used [29]. The benefit of combination therapy is not simply attributed to the properties of the plants but could also depend on the dose ratio [30]. As the cells do not differentiate between a single dose and a combination, two drugs combined at a given dose ratio could be considered as a third agent with a dose-effect relationship [31]. The establishment of an optimal dose-level pair for each plant extract intended for use in combination therapy is therefore indispensable for maximizing the treatment efficacy of combination therapy. The use of optimal dose pairs in combination therapies increases the chances of improved efficacy and decreases adverse effects [32]. Owing to these advantages, combination therapies have become a standard treatment for several diseases [33]. As such, they continue to represent a promising approach for indications of unmet medical needs. However, combination therapy for the treatment of hyperthyroidism has not been well explored. This motivated the assessment of the combined effects of two antithyroid plants, *Annona squamosa* and *Tetracapidium conophorum*, to establish their nature of interaction and effective combination dose ratio. This study investigated the combined therapeutic effects of *Annona squamosa* and Tetracarpidium conophorum extracts in *in vitro* and in vivo models of hyperthyroidism. The results demonstrated that these plant extracts exhibit both additive and synergistic effects in inhibiting thyroid peroxidase activity and ameliorating hyperthyroidism, depending on the specific dose ratios used. In vitro studies showed that both *Annona squamosa* and *Tetracapidium conophorum* extracts inhibited thyroid peroxidase (TPO) enzyme activity in a concentration-dependent manner. *Tetracapidium conophorum* exhibited more potent TPO inhibition, with a lower EC50 (224.05 µg/ml) than *Annona squamosa* (437.24 µg/ml). This suggests that *Tetracapidium conophorum* may contain compounds with strong direct inhibitory effects on TPO. When combined at certain dose ratios, the extracts produced additive and synergistic inhibition of TPO activity. For example, combining 250 µg/ml *Annona squamosa* with 65 µg/ml *Tetracapidium conophorum* resulted in 57% TPO inhibition and a combination index of 0.8, indicating synergism. This highlights the potential for enhanced antithyroid effects through the strategic combination of these plant extracts. The antioxidant activity of the extracts, as measured by DPPH radical scavenging, also showed dose-dependent effects. Interestingly, *Annona squamosa* demonstrated more potent antioxidant activity than *Tetracapidium conophorum* despite its weaker TPO inhibition. This suggests that the antithyroid mechanisms of these extracts likely involve multiple pathways beyond direct TPO inhibition, including the modulation of oxidative stress. The combination of extracts at certain ratios produces additive and synergistic antioxidant effects, which may contribute to their overall antithyroid activity. In an in vivo hyperthyroidism model, both extracts showed dose-dependent increases in thyroid-stimulating hormone (TSH) levels, with *Annona squamosa* exhibiting greater potency. This is in contrast with the *in vitro* TPO inhibition results, in which *Tetracapidium conophorum* was more potent. This discrepancy may be due to differences in bioavailability, metabolism, or additional mechanisms of action in vivo. Importantly, certain combinations of extracts produced synergistic increases in TSH levels. For instance, combining 30 mg/kg of *Annona squamosa* with 90 mg/kg of *Tetracapidium conophorum* resulted in a combination index of 0.7, indicating synergism. This demonstrated the potential for enhanced in vivo antithyroid effects through strategic extract combinations. Combination treatment also effectively reduced elevated T3 and T4 levels in hyperthyroid animals. All tested combinations significantly lowered T3 levels compared to the vehicle control, with some combinations achieving nearly 50% reduction. Similar effects were observed for the T4 levels. These results further support the antithyroid efficacy of the extract combinations. Beyond thyroid hormone modulation, extract combinations showed beneficial effects on other hyperthyroidism-associated parameters. They significantly reduced elevated liver enzymes (ALT, AST, and ALP), improved kidney function markers, and decreased lipid peroxidation as measured by malondialdehyde levels. These findings suggest that combinations of extracts may help mitigate the systemic effects of hyperthyroidism on multiple organ systems. The observed additive and synergistic effects of *Annona squamosa* and *Tetracapidium conophorum* extracts likely stem from their diverse phytochemical composition. Both extracts contain phenolic compounds, which are known for their antioxidant properties and potential to modulate thyroid function. The presence of alkaloids, particularly in *Annona squamosa*, may contribute to additional antithyroid mechanisms. The combination of these diverse compounds from two different plant sources appears to enhance overall antithyroid efficacy through multiple complementary pathways. This study highlights the importance of dose-ratio selection for achieving optimal combination effects. Different ratios of *Annona squamosa* and *Tetracapidium conophorum* extracts produced varying degrees of additivity and synergism across the different parameters. This underscores the need for careful optimization of herbal combinations to maximize therapeutic benefits.

5. Conclusion

This study provides evidence for the potential use of *Annona squamosa* and *Tetracapidium conophorum* extract combinations as a novel approach for managing hyperthyroidism. The observed additive and synergistic effects, both *in vitro* and in vivo, suggest that these plant extracts may offer enhanced antithyroid efficacy compared to their individual use. Furthermore, the multi-target effects on thyroid hormones, oxidative stress, and organ function markers indicate a holistic therapeutic approach. Future research should focus on optimizing extract combinations, elucidating specific bioactive compounds and mechanisms, and evaluating long-term safety and efficacy in clinical settings.

Compliance with ethical standards

Disclosure of conflict of interest

The authors declare that they have no competing interests.

Data availability

The materials used in this study are described in the references section. In addition, any other information related to this study will be provided upon reasonable request by the corresponding author

Statement of ethical approval

Ethical approval was obtained from the Animal Ethics Committee of Enugu State University of Science and Technology.

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