Clinical Phytoscience

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Thyrogrit, supplemented with a sub-optimal dose of levothyroxine, restores thyroid function in rat model of propylthiouracil-induced hypothyroidism

Acharya Balkrishna[1,2,3,4], Ritu Paliwal[1], Madhulina Maity[1], Yash Varshney[1], Sandeep Sinha[1] and Anurag Varshney[1,2,5*]

Abstract

Background Hypothyroidism is a common endocrine ailment, whose current standard of care is hormonal replacement therapy with levothyroxine (LT4). There is a medical need for alternative and safer therapies as LT4 is associated with special treatment considerations and adverse effects. Thyrogrit (THY) is a polyherbal formulation indicated for the treatment of hypothyroidism. The present study, describes the characterization of the phytocompounds present in THY and its in-vivo efficacy in rat model of hypothyroidism, in combination with a sub-optimal dose of LT4.

Methods Ultra High Performance Liquid chromatography was employed for the identification of the phytocompounds present in THY. For the evaluation of its in-vivo efficacy, female Wistar rats were administered THY orally, 15-days prior to disease induction, and continued throughout the experiment. Subsequently, hypothyroidism was induced by oral administration of propylthiouracil (PTU). From day 45 onwards, animals were administered orally with a sub-optimal dose of LT4 (2 µg/kg) till the end of the study. On day 79, animals were euthanized, blood was collected for measurement of thyroid hormones and other clinical chemistry parameters. Weights of liver, kidney and thyroid were recorded. Finally, the thyroid was subjected to histopathological evaluation through hematoxylin and eosin (H&E staining), immunohistochemistry as well as immunofluorescence.

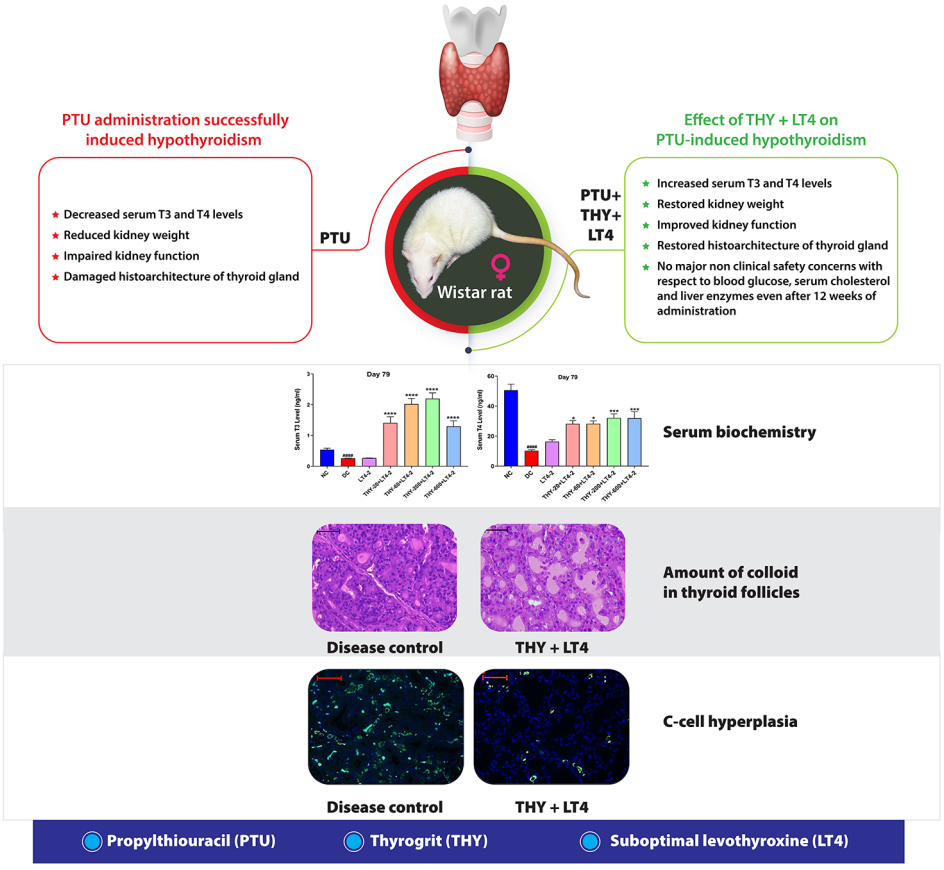
Results The principal phyto-components detected in THY by Ultra High Performance Liquid Chromatography included gallic acid, protocatechuic acid, corilagin, ellagic acid, piperine, guggulsterone E and Z, which are documented to exerted beneficial effects on thyroid function. In the in-vivo study, THY when supplemented with a low dose of levothyroxine restored the PTU-induced reduction in the serum levels of T3 and T4 and improved PTU-induced renal impairment. THY treatment ameliorated the hallmark histopathological changes associated with hypothyroidism and C-cell hyperplasia. Further, co-administration of THY and LT4 did not show any major non-clinical safety concerns even after the administration for more than twelve weeks.

*Correspondence:
Anurag Varshney
anurag@patanjali.res.in

Full list of author information is available at the end of the article

Conclusion This study has demonstrated that co-administration of THY and LT4 improves the PTU-evoked alterations in the thyroid ultrastructure and function, abrogates hypothyroidism-associated renal impairment and exhibits an acceptable basic safety profile.

Graphical abstract



Keywords Thyrogrit, Hypothyroidism, PTU (6-propyl-2-thiouracil), Levothyroxine, Ayurveda

Introduction

Hypothyroidism is amongst the most prevalent hormonal disorders, adversely affecting the health of people worldwide. Hypothyroidism is characterized by fatigue, constipation, dry skin, weight gain, reduced metabolism, muscle weakness, hoarseness, elevated blood cholesterol levels, muscle fatigue, depression, enlarged thyroid gland, impaired memory, and intolerance to cold temperatures, among the other signs and symptoms. Hypothyroidism can be of primary, secondary, or tertiary type. Primary hypothyroidism occurs due to a decreased production of thyroid hormones as a result of the inability of the thyroid gland to produce them. Due to the pituitary gland's inability to produce Thyroid-stimulating hormone (TSH), secondary hypothyroidism develops as a result of a decrease in TSH production, while tertiary hypothyroidism may result due to decreased production of Thyrotropin releasing hormone from the hypothalamus [1]. Thyroid gland is responsible for many vital functions of our body. It releases three hormones namely: triiodothyronine (T3), thyroxine (T4), and a peptide hormone, calcitonin, which coordinate growth and development, metabolism, and protein synthesis. It has been documented that 5% of the general population suffers from hypothyroidism out of which 99% of cases belong to primary hypothyroidism [2]. The prevalence of hypothyroidism has been reported to be 11% in India, 2% in the UK, and 4.6% in the USA [3, 4]. According to a study, in India, out of the total 108 million people suffering from endocrine and metabolic disorders, 42 million people are sufferers of various thyroid disorders [5]. A prevalence of 10.9% of hypothyroidism in eight different cities was reported which was found

to be greater in the inland cities than the coastal ones [6]. Presently, thyroid hormone replacement therapy is used widely for the treatment of hypothyroidism but with several side effects like heat intolerance, nervousness, chest pain, headache, vomiting, increased heart rate and weak bones. Further, in order to maintain euthyroidism throughout the lifetime of a patient, dose adjustments are frequently necessitated [7]. In contrast, ethnobotanicals have an ancient history of usage as a therapeutic modality with negligible side effects. Many studies have been conducted to evaluate the efficacy of herbal drugs for the treatment of hypothyroidism and in addition various herb-based remedies have been described for this disorder [8–10].

THY is an Ayurvedic prescription drug, formulated for the treatment of thyroid disorders like goitre, hypopituitarism, and other thyroid-related abnormalities, by Divya Pharmacy, Haridwar. The composition of THY formulation has been mentioned in Table 1. THY contains nine herbs, which have been traditionally used by Ayurvedic physicians in the management of thyroid disorders for more than five centuries. To exemplify, in the 16th century classical medicinal text, Bhavprakash Nighantu, the bark of *Bauhinia variegata* L. (Kachnar) has been explicitly mentioned for its therapeutic use in hypothyroidism-associated goitre [11]. Additionally, in Bhavprakash Nighantu, the other components of THY namely, *Coriandrum sativum* L. (Dhaniya), *Trapa natans var. bispinosa* (Roxb.) Makino (Singhada), *Terminalia bellirica* (Gaertn.) Roxb. (Baheda), *Piper nigrum* L. (Marich), *Piper longum* L. (Pippali), *Zingiber officinale* Roscoe (Sunthi), *Boerhavia diffusa* L. (Punarnava) and *Commiphora mukul* Engl. (Shuddh Guggul) have also been described for the management of hypothyroidism, due to their anti-inflammatory and metabolism-stimulant properties [11]. To further elaborate, *Coriandrum sativum* L. (Dhaniya) has been traditionally used for treatment of thyroid disorders [12]. *Bauhinia variegata* L.(Kachnar) has been known to modulate thyroid function and accordingly has been used in the treatment of hypothyroidism [13]. Shuddha Guggul is a dry gum resin obtained from the stem of *Commiphora mukul* Engl. Its beneficial activity on the thyroid gland has been identified and accordingly well documented. Recent scientific studies have proven that the administration of the Z-guggulsterone (a ketosteroid), isolated from Shuddha Guggul, to rats led to a significant increase in uptake of iodine by the thyroid, enzymes involved in the synthesis of thyroid hormones, and tissue oxygen

Table 1 Composition of Thyrogrit (THY)

Sr. No.	Components/ Classical Name	Botanical Name/ Chemical Name	Part Used	Voucher Number	Form Used	Classical Text Reference	Page No.	Quantity (mg)
1	Dhaniya Extract	*Coriandrum sativum* L.	Fruit	NISCAIR/RHMD/Consult/2019/3453-54-50	Powder	B.P.N.	34	50
2	Kachnar Chhal Extract	*Bauhinia variegata* L.	Bark	NISCAIR/RHMD/Consult/2019/3453-54-96	Powder	B.P.N.	323–324	100
3	Singhada Extract	*Trapa natans var. bispinosa* (Roxb.) Makino	Fruit	NISCAIR/RHMD/Consult/2018/3134-83-76	Powder	B.P.N.	567	50
4	Baheda Extract	*Terminalia bellirica* (Gaertn.) Roxb.	Fruit Rind	NISCAIR/RHMD/Consult/2019/3453-54-21	Powder	B.P.N.	9	50
5	Marich	*Piper nigrum* L.	Fruit	NISCAIR/RHMD/Consult/2019/3453-54-93	Powder	B.P.N.	19	33.3
6	Pippali	*Piper longum* L.	Fruit	NISCPR/RHMD/Consult/2022/3988-89-138	Powder	B.P.N.	19	33.3
7	Sunthi	*Zingiber officinale* Roscoe	Rhizome	NISCAIR/RHMD/Consult/2019/3453-54-173	Powder	B.P.N.	19	33.3
8	Punarnava Extract	*Boerhavia diffusa* L.	Root	NISCAIR/RHMD/Consult/2019/3453-54-149	Powder	B.P.N.	408	50
9	Shuddh Guggul	*Commiphora mukul* Engl.	Gum resin	NISCPR/RHMD/Consult/2022/3988-89-65	Powder	B.P.N.	205	100
Excipients:								
8	Gum Acacia	*Acacia arabica* (Lam.) Willd.	Resin	Not Applicable	Powder	I.P-Vol. III	3172	8
9	Talcum	Hydrated magnesium silicate	Chemical	Not Applicable	Powder	I.P-Vol. III	2821	8
10	MCC	Microcrystalline cellulose	Chemical	Not Applicable	Powder	I.P-Vol. II	2229	16
11	Croscarmellose sodium	Sodium carboxymethyl cellulose	Chemical	Not Applicable	Powder	I.P-Vol. II	1469	8

Note: B.P.N., Bhavprakash Nighantu, Edition – 2006 & 2010; I.P., Indian Pharmacopoeia-2014

uptake, revealing its thyroid stimulatory action [14]. It has also been observed that oral administration of the extract of *Commiphora mukul* Engl. to mice could increase the serum levels of Tri-iodothyronine, the functionally important thyroid hormone with a greater biological activity when compared to thyroxine [15]. Trikatu with the goodness of its three components namely *Piper nigrum* L. (Marich), *Piper longum* L. (Pippali) and *Zingiber officinale* Roscoe (Sunthi), works for the improvement of digestion of food, boosts immunity and relieves inflammation [16].

With a substantial background information about the traditional use and the scientifically validated efficacy of the herbal components of THY in the treatment of hypothyroidism, the present study was conceptualized to test the efficacy of THY in PTU (6-propyl-2-thiouracil)-induced experimental hypothyroidism in female Wistar rats in combination with an ineffective sub-optimal dose of levothyroxine. In the current study, THY was administered prophylactically, 15-days prior to disease induction following which, PTU was orally administered to the animals for 78-concecutive days. On day 0, the baseline serum levels of T3 and T4 were measured. Further, on day 15, T3 and T4 measurements were again estimated in the serum, to establish the development of hypothyroidism. THY treatment was continued for the entire experimental duration. From day 45 onwards, the animals started to receive a suboptimal dose of levothyroxine (2 µg/kg) by oral route, in addition to THY. Finally, on day 79, the rats were humanely sacrificed and blood was collected to estimate the levels of T3 and T4. Further, calculated blood urea nitrogen, creatinine and uric acid were also measured in the serum with an objective of assessing the effect of THY on a possible renal dysfunction, which might have developed due to a prolonged hypothyroid state. Since THY was administered to the animals for more than 90-days, the serum levels of total cholesterol, aspartate transaminase, alanine transaminase and blood glucose levels were additionally measured with an objective of evaluating the impact of THY on these important non-clinical safety parameters. Subsequently, thyroid glands were excised from the animals for histological analysis, which encompassed routine histology using hematoxylin and eosin staining as well as immunohistochemical and immunofluorescence studies for evaluating the effects of treatments on C-cell hyperplasia. Additionally, the liver and kidney were harvested from the animals and their wet weights were recorded. In addition to evaluation of its in-vivo efficacy, the phytochemical analysis of THY was also conducted to characterize and quantify the potential bioactive phytocompounds, with an objective of explaining the observations of the preclinical in-vivo efficacy study.

Materials and methods

Test article, chemicals and reagents

Thyrogrit (THY) (Batch# ATGT200003, date of expiry Nov 2023) was sourced from Divya Pharmacy, Haridwar, Uttarakhand, India. The names of the plants used in formulating THY have been verified using the online flora of known Plants - http://www.worldfloraonline.org and their voucher numbers are mentioned in Table 1.

PTU (6-Propyl-2-Thiouracil) was purchased from TCI Chemicals, India; levothyroxine (LT4) tablets of a standard brand (Eltroxin, Glaxo SmithKline Pharmaceuticals Ltd), was purchased from a local vendor; methylcellulose (MC) was purchased from Loba Chemie, India; paraffin wax, eosin, haematoxylin and formaldehyde were sourced from Merck India Pvt. Ltd.; whereas, ethanol was procured from Changshu Hongsheng Fine Chemical Co. Ltd, China. Acetic acid (LR Grade), acetonitrile (HPLC grade) and methanol (HPLC grade) were obtained from Rankem, India. Gallic acid was purchased from Loba Chemie, India; protocatechuic acid, guggulsterone E and guggulsterone Z were purchased from Natural Remedies, India; corilagin was procured from Cayman Chemicals, USA whereas ellagic acid and piperine were procured from Sigma-Aldrich, USA.

Compositional analysis of THY

The identification and quantification of marker phytocompounds in Thyrogrit (THY) were detected through Ultra-High-Performance Liquid Chromatography (UHPLC) using a Photodiode Array (PDA) detector, and were identified and quantified against known reference standards, available commercially. UHPLC-PDA analysis was performed on a Prominence-XR UHPLC system (Shimadzu, Japan) equipped with a Quaternary pump (NexeraXR LC-20ADXR), PDA detector (SPD-M20 A), auto-sampler (Nexera XR SIL-20 AC XR), degassing unit (DGU-20 A 5R), and column oven (CTO-10 AS VP). Separation of marker phyto-compounds was carried out on a Shodex C18-4E (5 µm, 4.6×250 mm) (Shodex, Japan) column at 35 °C. For separation of phyto-chemicals, 0.05% acetic acid in water as solvent A and acetonitrile as solvent B, were used as mobile phase. The elution gradient program of solvent B was, 5% from 0 to 10 min, change to 20% from 10 to 20 min, continue 20% from 20 to 30 min, further change to 70% from 30 to 40 min, again to 80% from 40 to 50 min, then 90% from 50 to 55 min, continue 90% from 55 to 60 min and return back to 5% in 61 min and continue 5% for 65 min. 10 µL of standard

and test solution was injected and the flow rate was set at 1.0 mL/min.

Stock solutions of phytochemical standards (1000 ppm) were prepared by dissolving accurately weighed reference compounds in methanol. The stock solutions were mixed and diluted with methanol to prepare the appropriate concentrations of working standard solutions. The standards employed were gallic acid, protocatechuic acid, corilagin, ellagic acid, piperine, guggulsterone E and guggulsterone Z. For phytochemical analysis, THY test solution was prepared by transferring 253.5 mg of THY powder to a 5 mL volumetric flask followed by addition of about 3 mL of Hydro-methanol and sonication for 30 min. The solution was further cooled and diluted with Hydro-methanol and mixed well. This solution was then filtered using a 0.45 µm nylon filter and used for the analysis. Wavelength was set at 270 nm and 250 nm for phytochemical analysis.

Animals and ethical statement

The experimental procedure on laboratory animals was conducted according to the guidelines prescribed by the Committee for Control and Supervision of Experiments on Animals (CCSEA), Ministry of Fisheries, Animal Husbandry and Dairying, Govt. of India [17]. and with the approval of the Institutional Animal Ethics Committee (IAEC) of Patanjali Research Foundation, vide protocol number PRIAS/LAF/IAEC-116, on female, specific-pathogen-free (SPF) Wistar rats aged 6–7 weeks. The animals were procured from Hylasco Biotechnology Pvt. Ltd., Telangana, India, a Charles River Laboratory licensed laboratory animal supplier. Animals were housed in the Laboratory Animal Facility of Patanjali Research Foundation, Haridwar, India. Feeding was done *ad libitum* with gamma-irradiated standard pelleted laboratory animal diet purchased from Hylasco Biotechnology Pvt. Ltd., Telangana, India (Manufactured by Purina 5L79 Rodent Lab diet, USA).

Development of experimental hypothyroidism and treatment protocol

Forty-nine female Wistar Rats were quarantined for a period of 7 days. Thereafter the animals were randomized by following the randomization within blocks strategy. The healthy animals were randomized on the basis of their respective body weights by using a Microsoft Excel sheet. Accordingly, animals were randomly allocated to seven groups to ensure no significant difference between the average body weights of the animals in each of the seven experimental groups, prior to the initiation of the study. The sample size was determined on the basis of a previously reported study [18], which evaluated the effect of an essential oil on PTU-induced hypothyroidism. Further, the sample size employed in the present study was also recommended by the IAEC of Patanjali Research Foundation as this is the first study directed at evaluating the pharmacological effects of THY on PTU-induced hypothyroidism. Following randomization, animals were acclimatized for a period of 5 days. The study design is depicted in Fig. 1. To summarize, the animals were allocated into seven groups: NC (normal control), DC (disease control), LT4-2, THY-20 + LT4-2, THY-60 + LT4-2, THY-200 + LT4-2, THY-600 + LT4-2. Animals of groups THY-20 + LT4-2, THY-60 + LT4-2, THY-200 + LT4-2, THY-600 + LT4-2 were administered only THY at the doses of 20, 60, 200, and 600 mg/kg, once daily in the morning, orally for 15 days, prior to the induction of the disease and continued till the end of the experiment (Day 78). Animals allocated to the groups NC and DC were administered 0.5% MC by gavage, respectively.

The rat equivalent dose of THY were calculated based on the differences in the body surface areas of human and rats. The recommended therapeutic dose of THY in humans is 2000 mg/day. Consequently, the human therapeutic dose for a 60 kg individual will be 2000/60 i.e. 33.33 mg/kg/day. The equivalent dose (in mg/kg) for rats was calculated by multiplying the human equivalent dose (in mg/kg) by factor of 6.2 [19]. The resultant rat equivalent dose was hence calculated to be 206.67 mg/kg. Rounding off to the nearest hundred, 200 mg/kg was considered to be the rat equivalent dose. For capturing a dose-response relationship the other doses chosen were from $1/10^{th}$ to 3 times the recommended therapeutic dose i.e. 20, 60 and 600 mg/kg.

After 15 days of prophylactic administration, hypothyroidism induction procedure was initiated wherein, propylthiouracil (PTU), was administered orally at the dose of 8 mg/kg, once-daily, one hour after the administration of test articles to all animals, except those allocated to the NC group and was continued for the entire duration of the experiment (Day 78). Animals allocated to the group LT4 and all the THY treated groups started receiving LT4 at the dose of 2 µg/kg, once-daily by oral route from Day 45 till the end of the experiment (Day 78). The induction of hypothyroidism was confirmed by estimation of serum T3 and T4 from the blood collected on day 15 of the experiment.

Blood collection and serum separation

Blood from rats was withdrawn from the retro-orbital plexus under transient isoflurane anaesthesia and dispensed in plain vials (without anticoagulant) and kept for 1 h at room temperature. Serum was separated

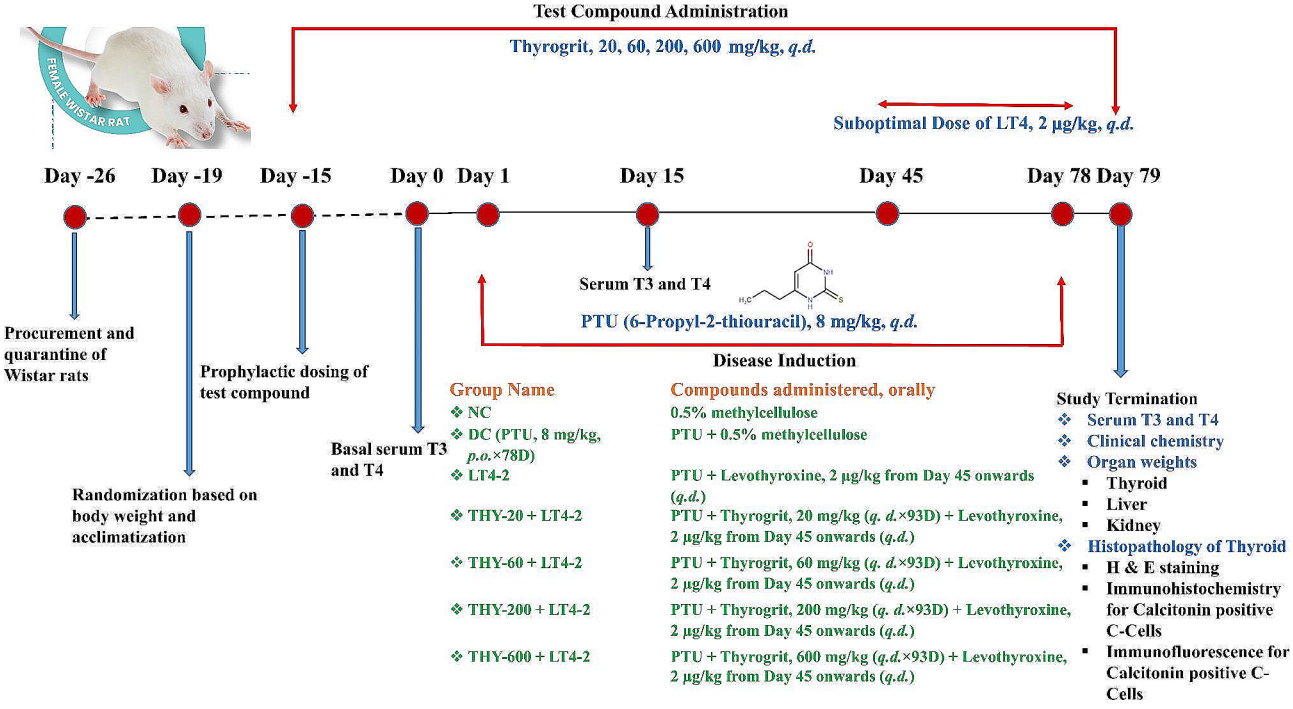


Fig. 1 Schematic diagram of the in-vivo experiment. Subsequent to quarantine and acclimatization, Thyrogrit in four different doses (20, 60, 200 and 600 mg/kg, q.d.) was administered 15 days prior to disease induction which continued till the end of the experiment. Basal readings of the serum T3 and T4 were recorded for all animals prior to disease induction on day 0 of experiment. Afterwards, PTU (8 mg/kg, q.d.) was administered orally to all animals except those in the NC which also continued till the end of experiment. After 15 days of PTU administration, animals were assessed for the development of hypothyroidism by recording serum T3 and T4 levels. On day 45 onwards all animals except NC and DC, were administered with suboptimal dose of LT4 (2 µg/kg, q.d.) till the end of study. On day 79, animals were euthanized and blood was collected for measurement of the serum T3 and T4 and for clinical chemistry. Organ weights of liver, kidney and thyroid were recorded. Thereafter thyroid was harvested for histopathological evaluation through H&E staining and immunohistochemical and immunofluorescence analysis for C-cell hyperplasia

by centrifugation (SORVALL legend micro 21R centrifuge, Thermo scientific, USA) at 3000 × g for 10–15 min at 4 °C.

Estimation of thyroid hormones

T3 and T4 levels were determined in serum using the T3 and T4 enzyme-linked fluorescent immunoassay kits (VIDAS®) respectively, on days 0, 15, and the last day of the experiment. The analysis was performed on a compact multiparametric immunoanalyzer (Model: mini VIDAS®, Biomerieux, SA).

Clinical chemistry

For clinical chemistry analysis, blood samples were collected from overnight fasted rats, and serum was separated by centrifugation (SORVALL legend micro 21R centrifuge, Thermo scientific, USA) at 3000 × g for 15 min at 4°C. Further, the samples were processed using Erba EM-200 (Erba Mannheim, Transasia, Germany) for clinical chemistry analysis. The following parameters were evaluated: Aspartate Transaminase (AST), Alanine Transaminase (ALT), Calculated Blood Urea Nitrogen (CBUN), Creatinine (CREAT), Uric Acid, Total Cholesterol (CHOLE), and Blood glucose (BGL).

Organ weight

At the end of the experiment, animals from each group were euthanized with a high dose of thiopentone sodium (150 mg/kg) injected intraperitoneally. The thyroid gland, pituitary gland, liver, and kidneys were collected and weighed. Relative organ weights were calculated for each animal by using the following formula:

$$\text{Relative organ weights (g)} = (\text{Organ weight(g)}) / (\text{Terminal body weight(g)}) \times 100)$$

Histopathology

Methodology used for hematoxylin and eosin staining

The preserved samples of the thyroid gland were processed by using a tissue processor (Leica biosystems; Model: TP 1020, India). They were then embedded in paraffin wax by using an embedding station (Leica biosystems; Model: Histocore Arcadia H-C, India) Subsequently sections of 3–5 µm were obtained by using a

microtome (Leica biosystems; Model: RM 2245, India), and allowed to float in a tissue floatation bath (Thermo Fisher Scientific; Model: Tsgp-10, USA). Subsequently, the sections were placed on glass slides, deparaffinized, and stained with hematoxylin-eosin. The stained sections were then examined microscopically by using a compound light microscope (AxioScope-A1, Carl Zeiss, Germany) and imaging was performed using Axiovision software. Scoring was done on the basis of the presence of the colloid in the follicles like 0 (absence of colloid), 1 (scanty amount of colloid), 2 (mild to moderate amount of colloid) and 3 (moderate to completely filled follicles with colloid) [20].

Methodology for immunohistochemistry analysis for C-cell hyperplasia

Tissues were sectioned in 2-2.5-µm size and mounted on poly-l-lysine coated slides. Then sections were deparaffinized, rehydrated and washed as mentioned in the H&E staining methodology. Tissues were subjected to heat-induced epitope retrieval using sodium citrate buffer (pH 6.0). The slides were kept 30 min for cooling and were followed by washing in deionized water for 5 min and blocking of non-specific binding using blocking buffer (Master Polymer Plus Detection System, Vitro Master Diagnostica, Spain). Then the sections were immunolabelled with primary antibody (anti-Calcitonin Antibody: Mouse Calcitonin Monoclonal Antibody, Mybiosource Inc., USA, Catalog number: MBS2090546, 1:2500 dilution) and incubated at room temperature for 1 h followed by staining with secondary antibody [Master Polymer Plus Detection System (Peroxidase), Vitro Master Diagnostica, Spain, Catalog Number: MAD-000237QK-R, 1:500 dilution] at room temperature for 1 h. The sections were washed in PBS for 5–10 min followed by staining with diaminobenzidine (DAB), which was provided along with the secondary antibody and finally mounted with Dibutylphthalate Polystyrene Xylene (DPX, Merck Life Science Pvt. Ltd., India). Then positive immunolabelling was evaluated and imaging was carried out using a Zeiss Axioscope microscope. Subsequently, quantitative scoring (% area of immunoreactive cells) was performed using ImageJ software (NIH, USA).

Table 2 Phytocompounds Identified and Quantified in Thyrogrit by UHPLC as Depicted in Fig. 2

S.N	Name	RT (min.)	Content in Thyrogrit (µg/mg)
1	Gallic acid	7.133	5.297
2	Protocatechuic acid	13.921	0.070
3	Corilagin	22.068	0.345
4	Ellagic acid	27.404	1.530
5	Piperine	42.989	3.361
6	Guggulsterone E	45.323	0.116
7	Guggulsterone Z	46.847	0.213

Note: RT: retention time

Methodology for immunofluorescence of C-cell hyperplasia

Methods employed for immunofluorescence were similar to those described for immunohistochemistry, up to the addition of primary antibody. Subsequently, one-hour post-addition of primary antibody, the sections were stained with secondary antibody (Alexa Fluor Plus 488, Invitrogen, Thermo Fisher Scientific, USA, Catalog Number A32723, 1:500 dilution) at room temperature for 1 h. The sections were washed in PBS for 5–10 min followed by staining and mounting with ProLong Diamond Antifade Mountant with DAPI (Invitrogen, Thermo Fisher Scientific, USA, Catalog Number P36962). Further, positive fluorescence signal was evaluated using an Olympus BX43 fluorescent microscope and imaging was carried out using Mantra Imaging System (PerkinElmer, USA). A semi quantitative scoring was performed on the basis of C-cell hyperplasia. The scoring criteria was considered as follows: Normal presence of C-Cell – 0; Mild C-cell hyperplasia – 1; Moderate C-cell hyperplasia – 2; Severe C-cell hyperplasia – 3.

Data representation and statistical analysis

The data is expressed as mean ± standard error of the mean (SEM) for each group. Statistical analysis was done using Graph Pad Prism version 7.04 software using one-way Analysis of Variance (ANOVA) followed by Dunnett's multiple comparison post-hoc test. A P value < 0.05 was considered to be statistically significant.

Results

Compositional analysis of Thyrogrit (THY)

Ultra-High-Performance Liquid Chromatography (UHPLC) using a Photodiode Array (PDA) detector (UHPLC-PDA analysis) identified seven phyto-compounds. Each milligram of THY powder contained gallic acid (5.297 µg), protocatechuic acid (0.070 µg), corilagin (0.345 µg), ellagic acid (1.530 µg), piperine (3.361 µg), guggulsterone E (0.116 µg) and guggulsterone Z (0.213 µg) (Table 2; Fig. 2).

THY restored PTU-induced reduction in serum T3 and T4 levels

Blood was withdrawn from the animals on day 0, day 15, and day 79 (the last day of the experiment) and subjected to quantitative estimation of T3 and T4. On day 0, no significant change was observed in serum T3 and serum T4 levels in different groups when compared to normal control animals (Fig. 3A and D respectively).

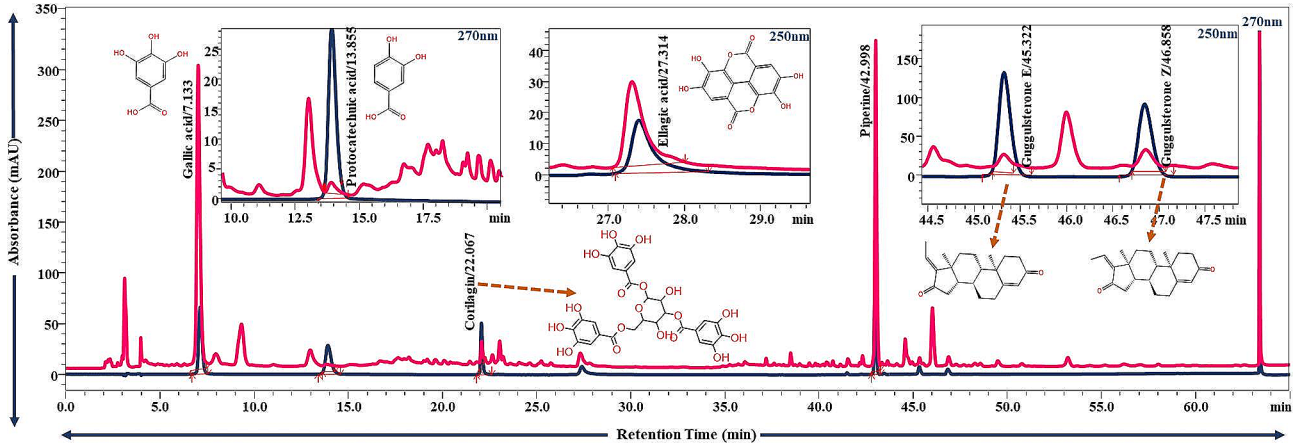


Fig. 2 Phytoconstituents detected in Thyrogrit. Thyrogrit powder was analyzed for marker compounds (chromatogram depicted in pink) by utilizing UHPLC-PDA and by employing reference standards (chromatogram represented in blue). Seven compounds were identified and quantified in Thyrogrit as outlined in Table 2

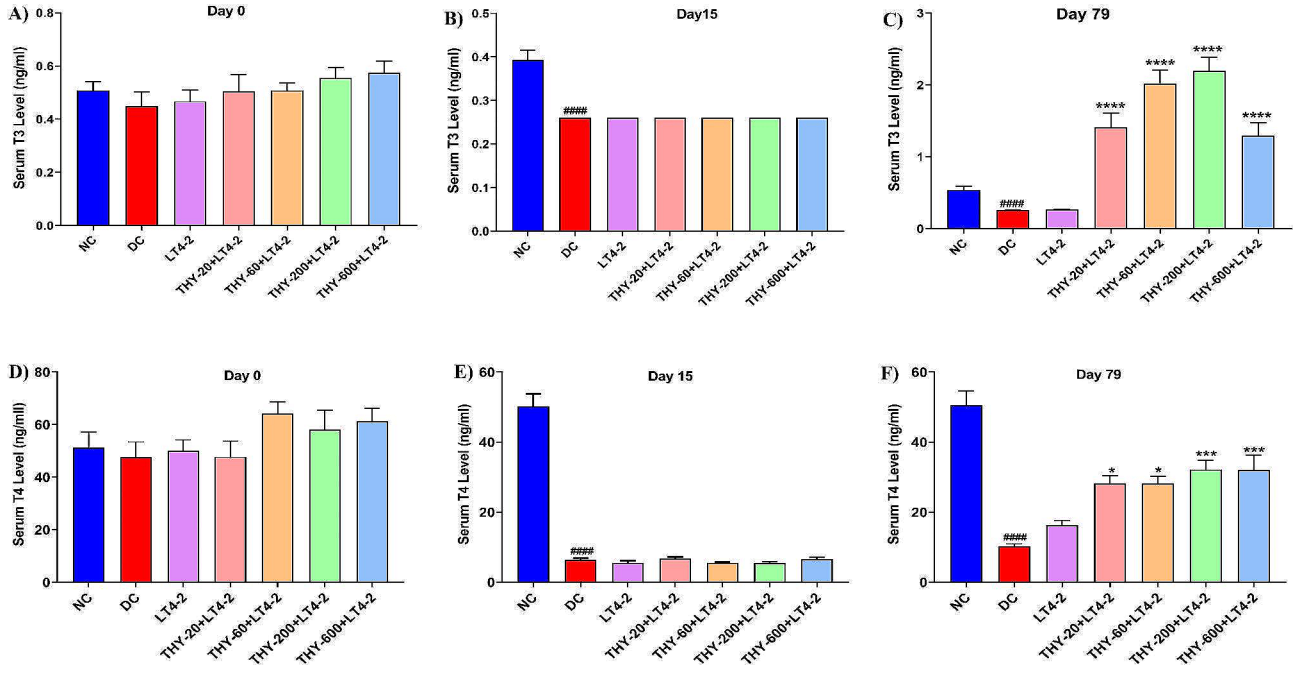


Fig. 3 Thyrogrit restores PTU-induced decrease in Serum T3 and T4 levels. Serum T3 and T4 were measured prior to PTU-administration (**A** and **D** respectively), Fifteen-days post-disease induction (**B** and **E** respectively) and at study termination (**C** and **F** respectively) as outlined in the Materials and Methods section. All data is presented as Mean ± SEM ($N = 6–7$ animals per group). Data was statistically analyzed by employing one-way ANOVA followed by Dunnett's multiple comparison test. #### designates significant difference with respect to NC ($p < 0.0001$); * $P < 0.05$, *** $P < 0.001$ and **** depicts $P < 0.0001$ when compared to DC

On day 15 of PTU administration, serum T3 and T4 levels were decreased significantly ($P < 0.0001$, Fig. 3B and E respectively) in all the groups when compared to normal control animals. Administration of THY along with suboptimal dose of LT4 (2 µg/kg) for 78 days, lead to significant increase in serum T3 ($P < 0.0001$ at all the evaluated doses of THY, Fig. 3C) and T4 levels ($P < 0.001$ for THY-200 + LT4-2 & THY-600 + LT4-2; $P < 0.05$ for THY-20 + LT4-2 & THY-60 + LT4-2 groups, Fig. 3F) in all the treatment groups when compared to disease control group. However, administration of LT4 (2 µg/kg) alone did not significantly increase serum T3 and T4 levels (Fig. 3C and F respectively).

THY modulated the PTU- induced changes in the relative organ weight

Thyroid, liver and kidney were harvested and their weights were recorded subsequent to necropsy.

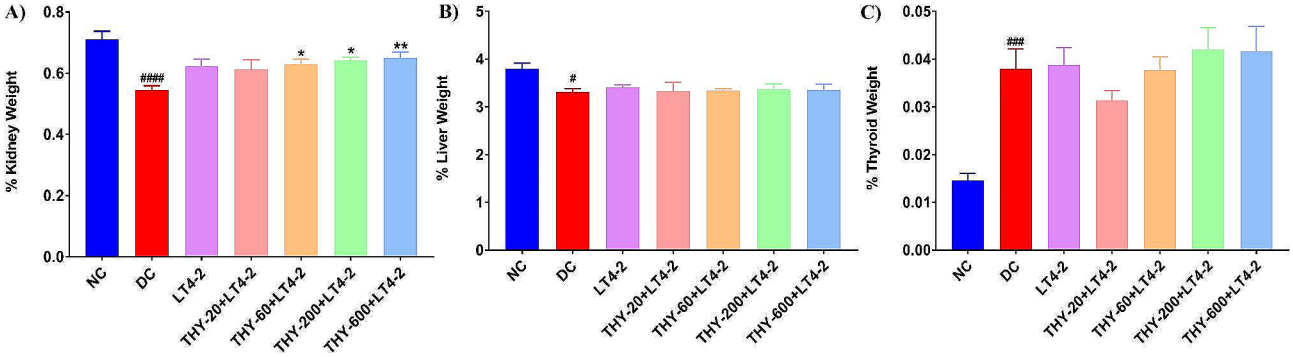


Fig. 4 Thyrogrit modulates PTU-induced changes in the relative organ weights. Subsequent to necropsy, the kidney, liver and thyroid were excised, weighed and the organ weights were expressed as a percentage of the body weight as elaborated in the Materials and Methods section. All data is presented as Mean ± SEM ($N = 6–7$ animals per group). Data was statistically analyzed by employing one-way ANOVA followed by Dunnett's multiple comparison test. # $P < 0.05$, ### $P < 0.001$ and #### $P < 0.0001$ vs. NC; * $p < 0.05$, ** $P < 0.01$ when compared to DC

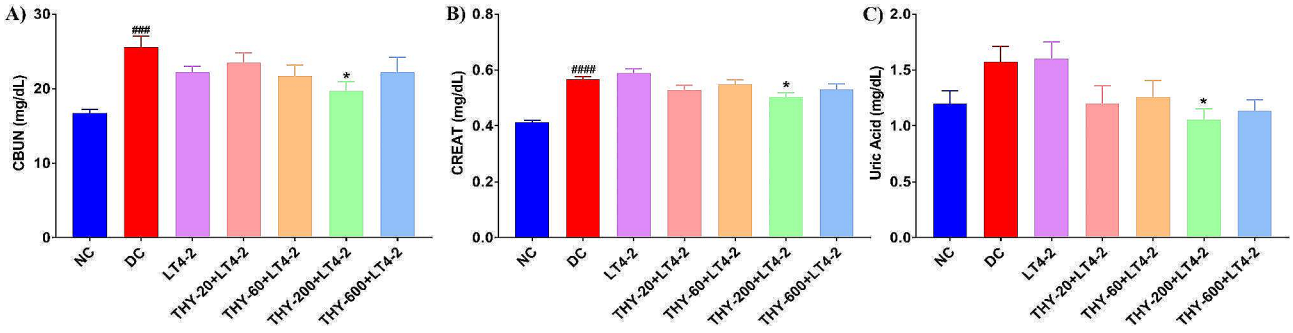


Fig. 5 Thyrogrit reduced PTU-induced impairments in biochemical markers related to kidney function. Prior to necropsy, blood was withdrawn from the animals and the serum was subjected to clinical chemistry analysis as described in the Material and Methods section. All data is presented as Mean ± SEM ($N = 6–7$ animals per group). Data was statistically analyzed by employing one-way ANOVA followed by Dunnett's multiple comparison test. ### $P < 0.001$ and #### $P < 0.0001$ vs. NC; * $P < 0.05$ when compared to DC

The relative organ weight of the kidney was significantly reduced in animals assigned to the DC group when compared to the NC group animals ($P < 0.0001$, Fig. 4A). Oral administration of THY with LT4-2 ameliorated the PTU-induced reduction in the relative organ weight of the kidney in a dose-related manner ($P < 0.05$ for THY-60 + LT4-2 and THY-200 + LT4-2 treated groups and $P < 0.01$ for THY-600 + LT4-2 treated groups, Fig. 4A). The relative organ weight of the liver was significantly decreased ($P < 0.05$, Fig. 4B) whereas that of the thyroid gland was significantly increased ($P < 0.0001$, Fig. 4C) in the DC group animals as compared to the NC group animals. However, no significant effect on the altered organ weights were evident in other treatment groups when compared to the DC group.

THY reduced PTU-induced impairments in kidney function

Continuous administration of PTU for 78 days, lead to a significant increase in serum CBUN ($P < 0.001$, Fig. 5A) and CREAT ($P < 0.0001$, Fig. 5B) levels in disease control animals as compared to the animals assigned to the normal control group. Co-administration of THY-200 with LT4-2 significantly reduced the PTU-induced increase in serum CBUN and CREAT levels ($P < 0.05$, Fig. 5A and B respectively) when compared to the disease control group. Further, the serum uric acid values in animals allocated to the disease control group also tended to be elevated as compared to the animals assigned to the normal control group and a significant reduction was elicited by oral administration of THY-200 + LT4-2 ($P < 0.05$, Fig. 5C).

THY exhibited an acceptable non-clinical safety profile on selected clinical chemistry parameters even after its repeated long-term administration

Since in the present study, THY was orally administered repeatedly for more than 90 days, the present study was also assessed the basic safety of THY formulation. Therefore, the impact of repeated administration of THY on selected basic clinical chemistry parameters such as the serum levels of cholesterol, AST, ALT and blood glucose was analyzed. No significant difference was observed in the selected clinical parameters as compared to both normal and disease

control groups (Fig. 6A-D), which signifies that THY did not alter the normal body physiology and hence it demonstrates an acceptable safety profile.

THY restored the PTU-induced abnormal architecture of the thyroid tissue in a dose-dependent manner

H&E staining: Thyroid glands of animals of different study groups were examined for any histopathological changes after staining with H&E stain. It was observed that in the normal control group, the architecture of the thyroid gland was normal, exhibiting eosinophilic, homogenous colloid in the follicles with well-formed interfollicular connective tissue. On the contrary, PTU administration in rats revealed closely packed thyroid follicles, one of the key characteristics associated with follicular hyperplasia and displayed abundant granular cytoplasm with reduced lumen size, severely reduced interfollicular connective tissue and complete absence of eosinophilic colloid ($P<0.0001$, Fig. 7A). Administration of the test compound along with LT4-2 µg/kg exhibited gradual restoration of thyroid gland architecture in a dose-dependent manner. With the increase in the dose of the THY in different treatment groups, there was an increase in the restoration of thyroid gland architecture. As per the histopathological descriptions of the thyroid gland, the high dose group of the test compound i.e. THY-600 + LT4-2 exhibited normal histo-architecture with uniform thyroid follicles lined by cuboidal cells containing a moderate amount of colloid in the lumen. No evidence of fibrosis or inflammation was noted. A significant increase of the colloid score ($P<0.01$, Fig. 7B), when compared to the disease control group was evident by the appearance of colloid in the follicular cells.

Immunohistochemistry: Positive immunoreactive C-cells exhibit brown coloration with different intensity as proportional to the presence of calcitonin. Normal control animals demonstrated normal scattered distribution of C-cell with brown colour of low intensity (Fig. 8A and B). Contrastingly, in animals allocated to the DC group, diffuse C-cell hyperplasia was evident and was characterized by abundant highly intense calcitonin-positive cells, which were significantly greater as compared to the NC group ($P<0.01$, Fig. 8A and B). LT4-2 treated group also revealed similar type of pathological changes as observed in the DC group. Animals treated with THY-600 + LT4-2 (600 mg/kg) exhibited significant reduction ($P<0.01$ vs. DC) in number of C-cells along with intensity (Fig. 8A and B).

Immunofluorescence: After detailed IHC analysis, immunofluorescence measurements were also taken. Positive immunofluorescent labelled C-cells emit characteristic apple-green fluorescent signals. Normal control animals exhibited normal scattered distribution of

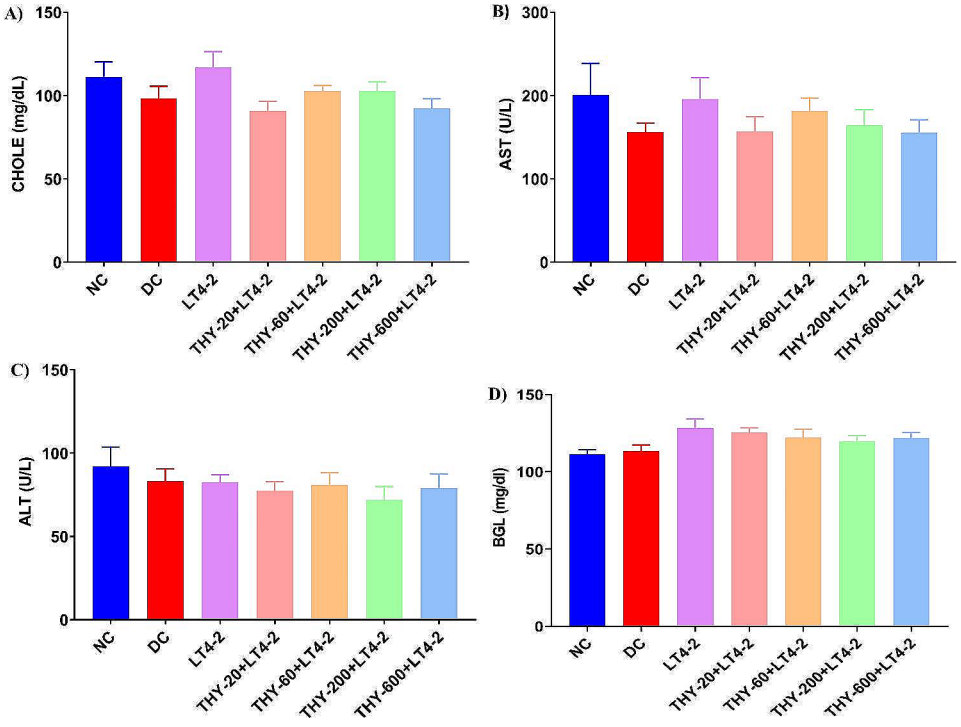
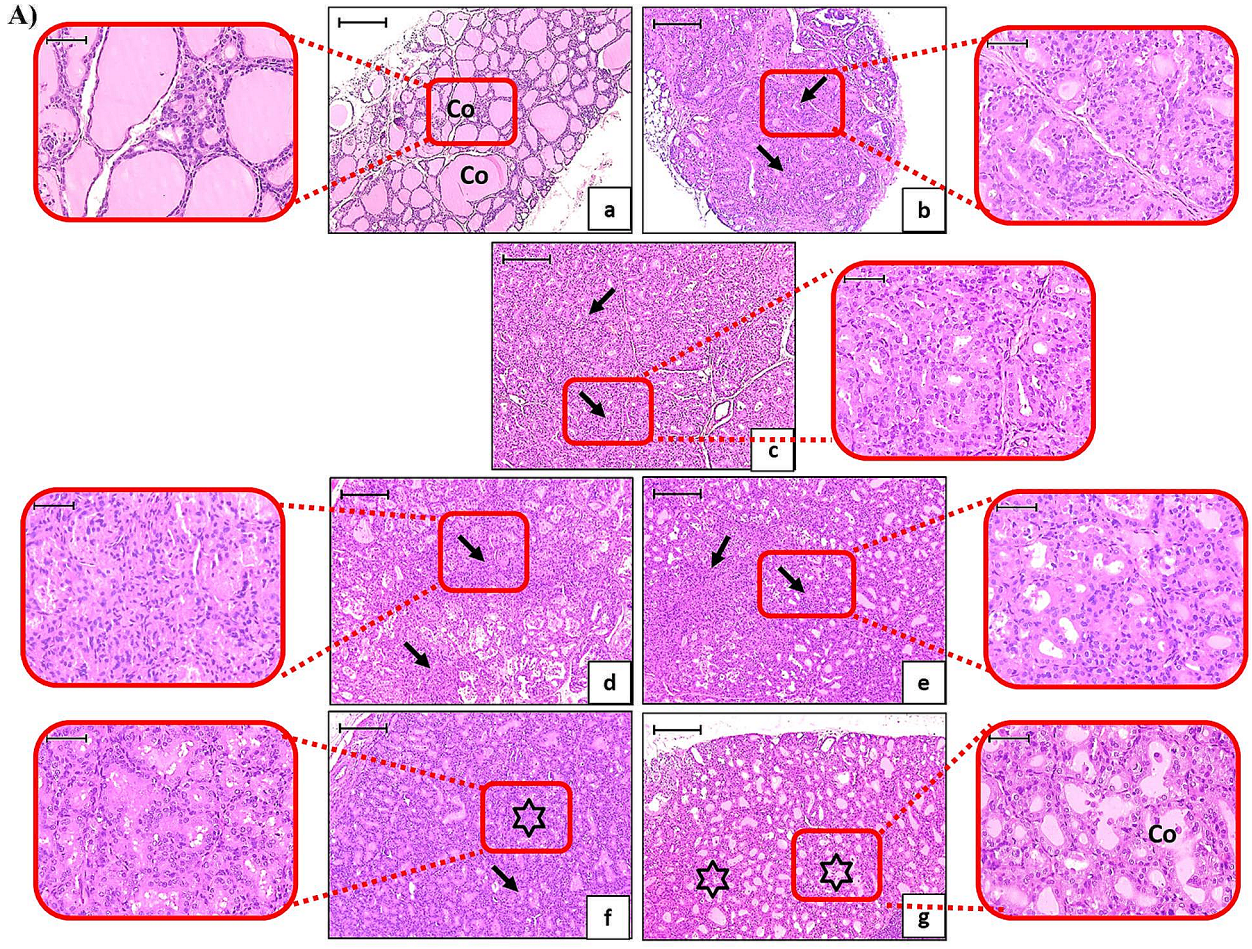


Fig. 6 Thyrogrit did not alter selected clinical chemistry parameters upon long-term administration thereby demonstrating an adequate safety profile. Prior to necropsy, blood was withdrawn from the animals and the serum was subjected to clinical chemistry analysis as described in the Material and Methods section. All data is presented as Mean ± SEM ($N=6–7$ animals per group). Data was statistically analyzed by employing one-way ANOVA followed by Dunnett's multiple comparison test and was compared with the NC group



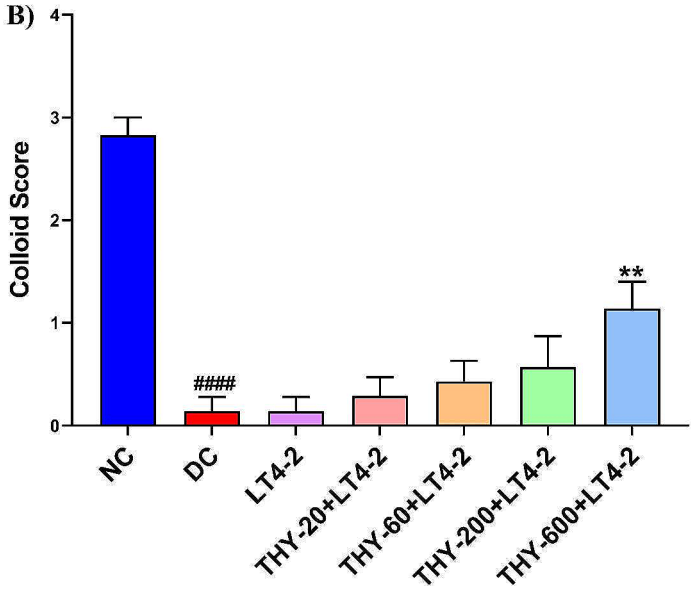


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Fig. 7 THY + LT4-2 restores the amount of colloid in follicles of thyroid associated with hypothyroidism. (**A**) Photomicrographic images (H&E staining, × 100 and × 400, Scale = 100 µm) of the thyroid gland of (**a**) NC group rat showing normal amount of colloid (Co), appearing as homogenous eosinophilic substance in the follicular lumen, uniformly distributed throughout the section; (**b**) DC group rat exhibiting diffuse follicular hyperplasia along with absence or very scanty amount of colloid (arrow) in the follicles; (**c**) Levothyroxine (2 µg/kg) treated rat showing no significant ameliorating effect on pathological changes observed in DC rats; (**d**, **e**) Rats treated with THY + LT4-2 (20 and 60 mg/kg respectively) exhibiting absence of colloid in most follicles and very scanty amount of colloid (arrow) in a few follicles; (**f**) Rats treated with THY + LT4-2 (200 mg/kg) exhibiting restoration of minimal amount of colloid (star) in most follicles in a scattered orientation; (**g**) Rats treated with THY + LT4-2 (600 mg/kg) exhibiting minimal amount of colloid (star) in follicles in approximately uniform distribution. (**B**) Depicts Colloid score. All data is presented as Mean ± SEM ($N = 6$ animals per group) and was statistically analyzed by employing one-way ANOVA followed by Dunnett's multiple comparison test. ## designates significant difference with respect to NC ($P < 0.01$) whereas ** depicts a statistically significant effect when compared to DC ($P < 0.01$)

C-cell with apple-green fluorescence, whereas disease control animals were characterized by diffuse C-cell hyperplasia and by a multiple number of highly intense calcitonin-positive C-cells $P < 0.0001$, Fig. 9A and B). LT4-2 treated group animals revealed no significant effects on pathological changes as observed in DC. Animals of groups THY-20 + LT4-2, THY-60 + LT4-2 and THY-200 + LT4-2 revealed reduction in the number of immunofluorescent C-cells and animals treated with THY-600 + LT4-2 exhibited a significant reduction C-cell hyperplasia ($P < 0.05$, Fig. 9A and B), in comparison to disease control animals.

Discussion

Primary hypothyroidism, the main category of clinical hypothyroidism occurs due to varying etiologies ranging from iodine deficiency, autoimmune or iatrogenic reasons, with a greater prevalence in females [21]. Dysfunctional thyroid secretes inadequate amount of T3 and T4 and therefore the only therapeutic modality, which currently is the standard of care, is lifelong replacement with synthetic levothyroxine (LT4). Nevertheless, the medical fraternity is of the view that employing LT4 alone does not completely restore the damaged tissue structure and the cellular milieu observed in healthy euthyroid individuals. Moreover, deciding on the optimal dose of LT4 can be quite a challenging task for a clinician as the ideal dose can be influenced by a host of patient-related factors such as pregnancy, age, gender, other comorbidities and concomitant pharmaceuticals consumed by the patient. Consequently, LT4 monotherapy is fraught with the risk of development of iatrogenic hyperthyroidism due to overtreatment and iatrogenic hypothyroidism on account of under treatment. It has been reported from older as well as newer studies that up to 20% patients develop iatrogenic hyperthyroidism whereas another 20% are rendered hypothyroid [22]. It naturally follows that overtreatment will be definitely associated with the risk of life-threatening cardiac arrhythmias and reduced bone mineral density, which may pre-dispose the patient to develop osteoporosis. Another issue with LT4 monotherapy is patient dissatisfaction, as 34–48% patients report persistence of the symptoms associated with hypothyroidism, in spite of a euthyroid state achieved by the replacement therapy [22]. Furthermore, LT4 monotherapy is also associated with diminished homeostasis of the T3 hormone reflected by lower T3 levels as compared to free T4 [22]. T3 has a greater biological activity as compared to T4; the latter acting as a prohormone, which needs to be converted to T3 [23]. Finally, to make the clinical management of hypothyroidism a further daunting task, resistance to supplementation with exogenous LT4 has also been reported [24].

The problems associated with LT4 monotherapy spurs the need for combination therapies for a comprehensive management of the disease, which on one hand can complement LT4 in restoration of a euthyroid state and on the other address the comorbidities arising due to LT4-related iatrogenic hyper- or hypothyroidism. In this light, herbal-based phyto-pharmaceuticals hold promise for an all-inclusive approach towards the treatment of hypothyroidism. Thyrogrit (THY) has been uniquely formulated by encompassing the traditional wisdom of Ayurveda and possesses herbal constituents which have a potential utility in the management of hypothyroidism. Oxidative stress due to low anti-oxidant reserves are frequently associated with the pathogenesis of the disease [25]. Consequently, the resultant oxidative stress elicited due to lack of neutralization of reactive oxygen species, ultimately translates to the generation of an inflammatory state, which additionally contributes to thyroid dysfunction. Moreover, the chronic use of levothyroxine may itself lead to generation of reactive oxygen species, particularly with overtreatment, with possible detrimental outcomes on the overall health status of the patient [26]. THY is enriched with several phytoconstituents, which are proven to target both the oxidative stress as well as inflammatory states and in addition have a thyroid stimulant effect as well. Amongst these constituents, gallic acid, a polyphenol is known to possess antioxidant and anti-inflammatory activities [27]. To elaborate further, it has additionally demonstrated beneficial activity in potassium dichromate-induced thyroid dysfunction in rats by attenuating the levels of lipid peroxidation biomarkers and pro-inflammatory

cytokines, whilst upregulating the endogenous anti-oxidant enzymes [28]. Further, the alkaloid piperine, another constituent of THY, exhibited thyroid stimulating effect in rats that were fed with a high fat diet and subsequently rendered hypothyroid by administration of carbimazole [29]. The authors reported that, piperine increased the serum levels of T3 and T4. Piperine is also known to possess anti-oxidant and immunomodulatory properties [30]. In addition, it is well documented to enhance the bioavailability of the concomitantly administered medications and is hence expected to have a favourable outcome of the pharmacokinetics of the other components of THY [31]. Ellagic acid, a polyphenol present in THY has been shown to demonstrate efficacy in methimazole-induced subclinical hypothyroidism in both db/db and C57BL/6 mice [32]. THY also contains Guggulsterone Z, a plant steroid, which has demonstrated thyroid stimulating activities in rats by increasing iodine-uptake by the thyroid and enhancing the activities of thyroid peroxidase and protease [14]. The isomer of Guggulsterone Z, namely Guggulsterone E, which is also present in THY is known to possess anti-inflammatory activity [33], thereby founding the rationale for its probable efficacy in hypothyroidism. Protocatechuic acid [34] and corilagin [35], the other polyphenols present in THY, exert anti-inflammatory and anti-oxidant activities. Taken together, the rationale for positioning THY in the therapeutic armamentarium for hypothyroidism can be well deciphered on the proven biological activities of its phytoconstituents.

PTU, is chemically a thioamide and an anti-thyroid medicine, which is used to treat thyrotoxicosis associated with hyperthyroidism. Consequently, PTU administration leads to a decrease in the T3 and the T4 levels with a concomitant increase in the serum TSH levels by the pituitary gland [36]. Accordingly, with an aim to elicit a prolonged thyroid hormone deficiency state in rats, PTU was selected as the disease-inducing agent in the present study. In addition, the chosen animal model demonstrates hallmark histopathological changes associated with thyroid dysfunction [37]. Furthermore, this experimental hypothyroidism model, additionally exhibits the development of renal and hepatic dysfunction, often associated with hypothyroidism-related oxidative stress [38].

The present study was conducted with an aim to evaluate the in-vivo efficacy of THY in rat model of PTU-induced experimental hypothyroidism in combination with an ineffective dose of LT4. Previous studies evaluating the efficacy of test compounds in this model have employed LT4 as a method control at the dose of either 100 µg/kg/day [36, 39] or 20 µg/kg [37] administered by oral gavage. Both these doses have found to exhibit efficacy in the studied parameters. As a pilot study, we evaluated the effect of LT4 administered orally at the dose 2 µg/kg, 12.5 µg/kg and 100 µg/kg, wherein we came to the conclusion that the lowest dose was ineffective in restoring the PTU-induced decrease in serum T3 and T4, whereas 100 µg/kg produced a hyperthyroid state in terms of the serum levels of T3 and T4 (data not shown). Accordingly, for the present study LT4-2 µg/kg was selected for combination with graded doses of THY. The rationale for selecting the ineffective dose was based on the premise that any beneficial effect of THY on PTU-induced diminished thyroid function, should not be masked by the effect of LT4 per se. Various end-point parameters were recorded to evaluate the efficacy and safety of the THY, like its effect on the level of thyroid hormones, blood glucose, cholesterol, liver function tests, kidney function tests, and histopathological analysis as well as immunohistochemistry (IHC) and immunofluorescence (IF).

On analyzing the T3 and the T4 levels on day 0, day 15, and day 79 it could be deciphered that the treatment groups showed a significant increase in serum T3 and T4 levels compared to the disease control group. This signals the synergistic effect of LT4 and THY. As discussed previously, the major thyroid hormone responsible for the maintenance of basal metabolic rate is T3. The reduction in T3 in turn may lead to higher body mass index, weight gain, and obesity [40]. In our study, T3 levels showed a greater increase as compared to the T4 levels, which is in accordance with the study conducted to analyze the T3 and the T4 levels after the administration of *Commiphora mukul* Engl. (Shuddh Guggul) [15]. This finding is suggestive of the fact that THY might have stirred up the extra-thyroidal conversion of T4 to T3. Given, the LT4 monotherapy-associated impairment of T3 homeostasis, the positive observed effect in the present study on serum T3 levels is of particular significance and merits further investigation with regards to the availability of T3 hormone in the peripheral target organs in rodents. However, the observed preliminary preclinical finding does indicate the potential clinical utility of THY in restoring euthyroidism with respect to T3 levels, when administered with LT4.

Apart from increasing the serum T3 and T4, the present study also addressed hypothyroidism-associated impairment of renal function. Physiologically, it is well known that the glomerular filtration rate (GFR) and renal blood flow (RBF) are both increased by the pre-renal and intrinsic renal actions of thyroid hormones. Reduced GFR is a sign of hypothyroidism, and increased GFR is a sign of hyperthyroidism. Primary hypothyroidism and subclinical hypothyroidism

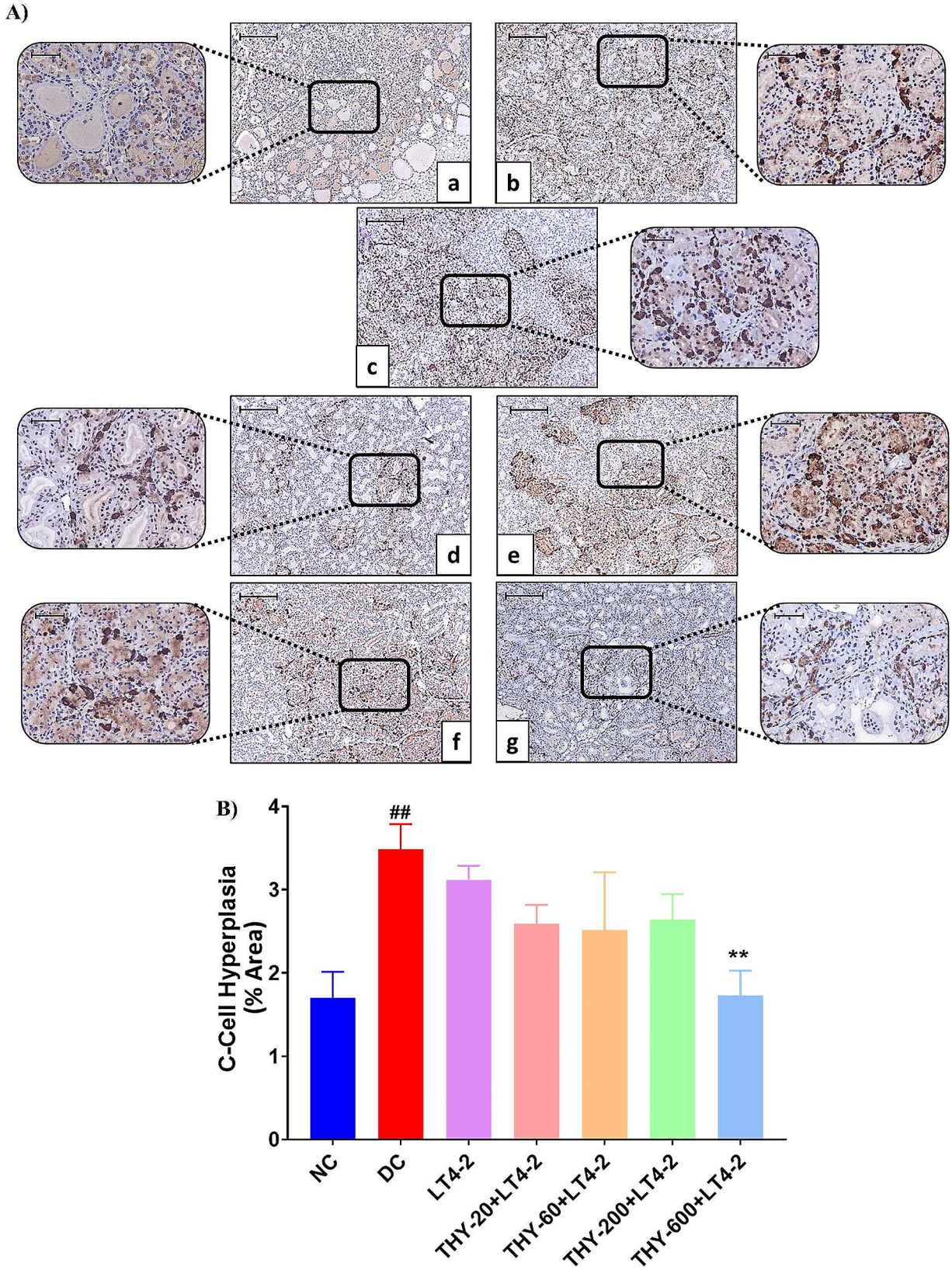

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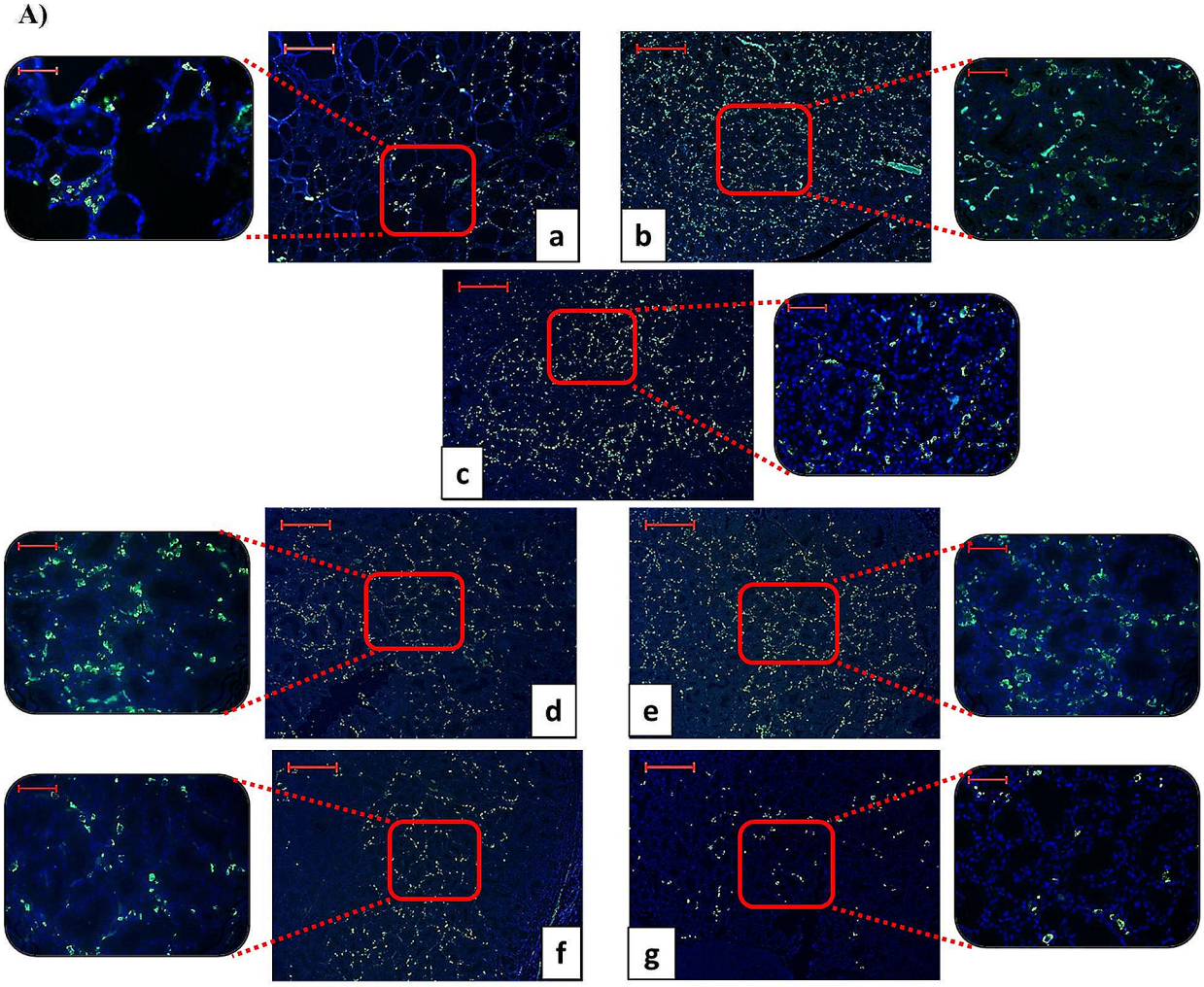
Fig. 8 Effects of THY + LT4-2 on the parafollicular or C-cell population around follicles of thyroid associated with hypothyroidism. (**A**) Photomicrographic images (Immunolabelling with antibody against calcitonin, × 100 and × 400, Scale = 100 µm) of the thyroid gland of (**a**) NC group rat showing normal population of C-cell appearing as brownish deposition scattered in the section; (**b**) DC group rat exhibiting diffuse C-cell hyperplasia; (**c**) Levothyroxine (2 µg/kg) treated rat showing no significant ameliorating effect on pathological changes observed in DC rats; (**d**, **e** and **f**) Rats treated with THY + LT4-2 (20, 60 and 200 mg/kg respectively) exhibiting minimal reduction in increased C-cell population; (**g**) Rats treated with THY + LT4-2 (600 mg/kg) exhibiting significant reduction in increased C-cell population. (**B**) Depicts percentage area of C-Cells. All data is presented as Mean ± SEM ($N = 6$ animals per group) and was statistically analyzed by employing one-way ANOVA followed by Dunnett's multiple comparison test. #### designates significant difference with respect to NC ($P < 0.0001$) whereas ** depicts a statistically significant effect when compared to DC ($P < 0.01$)

are closely associated in patients with chronic kidney disease (CKD) [41]. Hypothyroidism causes hyponatremia, an increase in serum creatinine and urinary protein content; whilst a decrease in serum cystatin C, water load excretion, GFR, RBF, sodium reabsorption, and renal ability to dilute urine [41, 42]. The defects in the renal function due to hypothyroidism are mainly due to a reduction in the cardiac output, intra-renal vasoconstriction, and increased peripheral vascular resistance [43, 44]. Also, it causes thickening of the basement membrane and expansion of the mesangial matrix leading to obstruction in the glomerular capillaries, and subsequently diminishing the renal blood flow and the glomerular filtration rate [45]. In the present study, clinical chemistry parameters related to kidney function were investigated. As anticipated, PTU-induced prolonged hypothyroidism lead to an increase in CBUN, and CREAT levels, reflecting development of renal dysfunction. THY + LT4 treatment could reverse the elevated levels of these pivotal parameters. Moreover, PTU-induced reduction in the relative kidney weight were abrogated by the co-administration of THY and LT4. These observed effects hereby generate preclinical evidence for the potentially beneficial effect of THY co-administered with LT4 on hypothyroidism-associated renal dysfunction.

Since, both clinical and experimental hypothyroidism leads to liver dysfunction [46], the present study additionally evaluated the possible efficacious effect of the combination therapy on the two important markers of liver function, namely AST and ALT. However, in this study PTU-administration alone did not lead to elevation of the either parameter, which is in agreement with a previously reported study [37]. Nevertheless, administration of THY for more than three months did not elevate the serum levels of AST and ALT suggesting its preclinical safety with respect to liver function. Similarly, THY did not alter the serum levels of blood glucose and total cholesterol, further signifying its safety with respect to glucose and lipid metabolism.

The thyroid produces the hormones, T3 and T4, which in turn is regulated by TSH. Accordingly, well differentiated thyroid architecture is crucial for each step in the synthesis of T3 and T4. Hypothyroidism leads to damage at the tissue and cellular level in the thyroid gland. Previous studies which employed PTU as the disease inducing agent have demonstrated that the histo-architecture in hypothyroid rats treated with PTU exhibit thyroid follicles which are closely packed and with a copious amount of granular cytoplasm. Furthermore, lumen size was shortened with no colloid indicating cellular degeneration [36]. These findings are very similar to the observation in H&E stained thyroid tissue, in the present study. Histopathological evaluation of the thyroid gland revealed that co-administration of graded doses of THY with LT4 demonstrated a tendency towards the restoration of the normal architecture of the thyroid gland with properly structured colloidal tissue. As discussed previously, restoration of the tissue architecture is the ultimate goal of any therapeutic intervention for hypothyroidism and may not be fully realized with LT4 monotherapy alone. The findings of the current study provide preclinical evidence that THY administered in combination with a sub-optimal dose of LT4 has the potential to reverse PTU-associated abnormal histological changes in the thyroid.

In addition to the follicular cells, the thyroid also contains parafollicular cells also referred to as C-cells. These cells are scarce and are responsible for the secretion of calcitonin, a hormone that is physiologically important for maintaining calcium homeostasis. In experimental hypothyroidism induced by PTU, it has been reported that the number of C-cells is increased along with the well-documented follicular cell hyperplasia [20, 47] and it has been postulated that C-cells are additionally involved in the intrathyroidal regulation of the follicular cells as well [47]. Accordingly, the present study also examined the effect of co-administration of THY with LT4 on probable C-cell hyperplasia by employing standard immunohistochemical and immunofluorescence techniques. Similar to the observations mentioned in the previously conducted study, parafollicular cell hyperplasia was noted [47]. The depletion of colloid in the follicles in the thyroid of rats rendered hypothyroid by PTU (as ascertained by evaluation of H&E stained thyroid) and the concomitant increase in the number of C-cells lend further support to the aforementioned hypothesis. The C-cell hyperplasia was quantified by immunohistochemistry



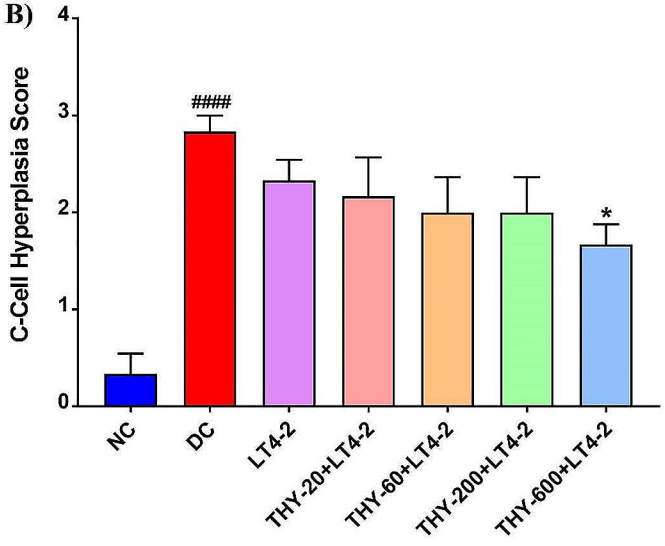


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Fig. 9 Effects of THY + LT4-2 on the parafollicular or C-cell population around follicles of thyroid associated with hypothyroidism. (**A**) Photomicrographic images (Immunolabelling with antibody against calcitonin, × 100 and × 400, Scale = 100 µm) of the thyroid gland of (**a**) NC group rat showing normal population of C-cell appearing as apple-green fluorescence in the section; (**b**) DC group rat exhibiting diffuse C-cell hyperplasia; (**c**) Levothyroxine (2 µg/kg) treated rat showing no significant ameliorating effect on pathological changes observed in DC rats; (**d**, **e** and **f**) Rats treated with THY + LT4-2 (20 mg/kg, 60 mg/kg and 200 mg/kg respectively) exhibiting no significant reduction in increased C-cell population; (**g**) Rats treated with THY + LT4-2 (600 mg/kg) exhibiting significant reduction in increased C-cell population. (**B**) Depicts C-Cell hyperplasia score. All data is presented as Mean ± SEM ($N = 6$ animals per group) and was statistically analyzed by employing one-way ANOVA followed by Dunnett's multiple comparison test. #### designates significant difference with respect to NC ($P < 0.0001$) whereas * depicts a statistically significant effect when compared to DC ($P < 0.05$)

as percentage of the area immunoreactive for anti-calcitonin antibody. It was further evaluated semi-quantitatively by immunofluorescence. Both employed methodologies concluded that combined administration of THY and LT4 abrogated the PTU-induced C-cell hyperplasia.

Conclusions

In conclusion, the present study demonstrated that the combination treatment regimen of THY and the sub-optimal dose of LT4 was able to restore thyroid function reflected by an observed increase in the levels of T3 and T4 hormones. Moreover, it was able to restore the hypothyroidism-associated aberrant histological architecture of the thyroid gland. Furthermore, THY + LT4 co-administration abrogated the impaired renal function induced by PTU. Finally, it demonstrated an acceptable safety profile with respect to liver function, glucose and cholesterol metabolism. Taken together, the findings of this preclinical study suggests a potential clinical utility of Thyrogrit in combination with levothyroxine, in patients afflicted with hypothyroidism and its associated co-morbidities.

Abbreviations

ALT	Alanine Transaminase
ANOVA	Analysis of variance
AST	Aspartate Transaminase
BGL	Blood glucose
CBUN	Calculated Blood Urea Nitrogen
CCSEA	Committee for Control and Supervision of Experiments on Animals
CHOLE	Total Cholesterol
CREAT	Creatinine
DAPI	4′,6-diamidino-2-phenylindole
DC	Disease control
DIT	Diiodotyrosine
GFR	Glomerular filtration rate
H&E	Hematoxylin and Eosin
HPLC	High-Performance Liquid Chromatography
IF	Immunofluorescence
IHC	Immunohistochemistry
LR	Laboratory Reagent
LT4	Levothyroxine
MC	Methylcellulose
MIT	Monoiodotyrosine
NC	Normal control
PDA	Photodiode Array
PTU	6-propyl-2-thiouracil
q.d.	Quaque die (once daily)
SEM	Standard error of the mean
SPF	Specific pathogen free
T3	Triiodothyronine
T4	Thyroxine
THY	Thyrogrit
TSH	Thyroid stimulating hormone
UHPLC	Ultra-High-Performance Liquid Chromatography

Acknowledgements

We express our deepest gratitude to Michael A Popp Nature Science Foundation for sponsoring the Article-processing charges. Further, we are grateful to Dr. Jyotish Srivastava and Dr. Seema Gujral for analytical chemistry support. We thank Ms. Deepika Mehra and Ms. Deepika Kumari for their technical supports in conducting clinical chemistry, routine histopathology as well as immunohistochemical and immunofluorescence studies. Further, we are also thankful to Dr. Aakanksha Tiwari Khanduri for her assistance in the arranging of the manuscript, and Mr. Devendra Kumawat and Dr. Sonam Sharma for their excellent help with graphics. We thank Mr. Deepak Rawat and Dr. Daya Shankar Singh for facilitating and conducting the evaluations of serum T3 and T4. Additionally, we also thank Mr. Ram Hari Sharma, Mr. Pusphendra Singh, Mr. Sonit Kumar and Mr. Rajat for providing technical assistance in animal handling and maintenance of the animal house. Finally, we express our gratitude to Mr. Tarun Rajput, Mr. Gagan Kumar, and Mr. Lalit Mohan for their swift administrative supports.

Author contributions

AB: Conceptualization, Planning, Visualization, Supervision, Writing - review & editing. RP: Conceptualization, Planning, Visualization, Methodology, Investigation, Data curation, Formal analysis, Writing - original draft. MM: Methodology, Investigation, Formal analysis. YV: Methodology, Investigation, Formal analysis. SS: Data curation, Writing - review & editing, Visualization, Project administration, Supervision. AV: Writing - review & editing, Project administration, Conceptualization, Visualization, Supervision.

Funding

This research work was supported by internal funds from Patanjali Research Foundation Trust, Haridwar, India, a non-profit organization.

Data availability

The data that support the findings of this study are not openly available due to reasons of sensitivity and are available from the corresponding author upon reasonable request. Data are located in controlled access data storage at Patanjali Research Foundation.

Declarations

Ethics approval and consent to participate

The experimental procedure on laboratory animals was conducted according guidelines prescribed by the Committee for Control and Supervision of Experiments on Animals (CCSEA), Ministry of Fisheries, Animal Husbandry and Dairying, Govt. of India [17]. and with the approval of the Institutional Animal Ethics Committee of Patanjali Research Foundation, vide protocol number PRIAS/LAF/IAEC-116.

Competing interests

Thyrogrit was sourced from Divya Pharmacy, Haridwar, Uttarakhand, India. AB is an honorary trustee in Divya Yog Mandir Trust, which governs Divya Pharmacy, Haridwar. In addition, he holds an honorary managerial position in Patanjali Ayurved Ltd., Haridwar, India. Other than supplying the test article, Divya Pharmacy was not involved in any aspect of the research stated in this study. The remaining authors declare no competing interests.

Author details

[1]Drug Discovery and Development Division, Patanjali Research Foundation, Haridwar, India

[2]Department of Allied and Applied Sciences, University of Patanjali, Haridwar, India
[3]Patanjali UK Trust, Glasgow, UK
[4]Vedic Acharya Samaj Foundation, Inc. NFP 21725 CR 33, Groveland, FL 34736, USA
[5]Special Centre for Systems Medicine, Jawaharlal Nehru University, New Delhi, India

Received: 26 December 2023 / Accepted: 12 June 2024
Published online: 20 June 2024

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