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ORIGINAL ARTICLE

Insulin Plant (*Costus pictus*) Extract Restores Thyroid Hormone Levels in Experimental Hypothyroidism

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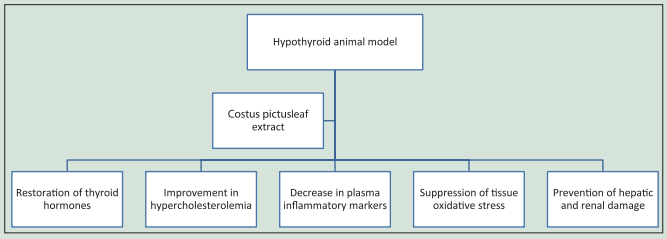
ABSTRACT

Background: The aim of the present study was to investigate the preventive effect of *Costus pictus* leaf extract in experimental hypothyroidism. **Materials and Methods:** Forty male Wistar rats were randomly divided into four groups with ten rats in each group: Control (C), hypothyroid (H), control+extract (C+E), and hypothyroid+extract (H+E). Rats in C group did not receive any intervention throughout the experimental period. The rats in the C+E and H+E groups received pretreatment with *C. pictus* leaf extract for 4 weeks. Subsequently, for the next 6 weeks, rats in the H group received 0.05% propylthiouracil in drinking water while C+E group received *C. pictus* leaf extract and H+E group received propyl thiouracil and *C. pictus* leaf extract. **Results:** Hypothyroid group rats exhibited dramatic increase in thyroid-stimulating hormone (TSH) levels with concomitant depletion in the levels of thyroid hormones. Treatment with the extract resulted in remarkable improvement in thyroid profile. Extract produced 10.59-fold increase in plasma free T3, 8.65-fold increase in free T4, and 3.59-fold decrease in TSH levels in H+E group in comparison with H group. Treatment with the extract ameliorated hypercholesterolemia, decreased levels of plasma C-reactive protein and tumor necrosis factor alpha, suppressed tissue oxidative stress and prevented hepatic and renal damage caused due to thyroid hormone depletion in the H+E group. Pentacyclic triterpenes alpha and beta amyrins were identified and quantified in the extract. **Conclusions:** This is the first study to reveal that *C. pictus* extract has therapeutic potential to restore thyroid hormone levels and prevent the biochemical complications due to thyroid hormone insufficiency in the animal model of experimental hypothyroidism.

Key words: *Costus pictus*, hypothyroidism, insulin plant, propylthiouracil, thyroid hormones

SUMMARY

- The preventive effect of *Costus pictus* leaf extract in experimental hypothyroidism was evaluated in the present study.
- Hypothyroidism was induced in the experimental animals by giving 0.05% propylthiouracil in drinking water.
- Hypothyroid rats exhibited dramatic increase in thyroid-stimulating hormone (TSH) levels with concomitant depletion in the levels of thyroid hormones.
- Treatment with *Costus pictus* leaf extract in hypothyroid rats significantly improved the thyroid profile. It also ameliorated hypercholesterolemia, decreased the levels of plasma inflammatory markers, suppressed tissue oxidative stress and prevented hepatic and renal damage caused due to thyroid hormone depletion.
- The possible active principles alpha and beta amyrins were identified and quantified in the extract through LC-MS.



Abbreviations Used: APCI: Atmospheric pressure chemical ionization; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; C group: Control group; C+E group: Control+extract group; *C. pictus*: *Costus pictus;* CRP: C-reactive protein; DPPH: 2,2-diphenyl-1-picrylhydrazyl; FRAP: Ferric reducing antioxidant power; HDL: High-density lipoprotein; H group: Hypothyroid group; H+E group: Hypothyroid+extract group; LDL: Low-density lipoprotein; LC-MS: Liquid chromatography–mass spectrometry; MDA: Malondialdehyde; PTU: 6-Propyl-2-thiouracil; SRM: Single reaction monitoring; TSH: Thyroid-stimulating hormone; TPTZ: 2,4,6-tri-(2-pyridyl)-5-triazine; TBA: 2–Thiobarbituric acid; TG: Triglyceride; TNFα: Tumor necrosis factor alpha; TAS: Total antioxidant status

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INTRODUCTION

Hypothyroidism is one of the most common endocrine diseases. In the general population, the major cause for hypothyroidism is autoimmune thyroiditis. It is characterized by decreased serum levels of thyroid hormones (T3 and T4) and elevated thyroid-stimulating hormone (TSH).[1] The current mode of treatment for hypothyroidism is levothyroxine replacement therapy. However, there are certain limitations associated with levothyroxine replacement therapy. Recent studies have reported that a significant number of hypothyroid patients on levothyroxine replacement therapy experience decreased neurocognitive function and lead poorer quality of life despite being biochemically euthyroid.[2,3] Clinically, it has been observed that since levothyroxine replacement therapy requires lifelong treatment, it associated with poor compliance in some patients.[4] Hence, there is an urgent need for more effective therapeutic strategies to treat hypothyroidism.

Recently, there has been renewed interest in the use of medicinal plants and their bioactive constituents in the treatment of endocrine diseases.[5] *Costus pictus* is one such medicinal plant belonging to the family of *Costaceae*. It is commonly known as insulin plant. It is grown in various parts of India.[6,7] The leaf of this plant is being consumed by diabetic patients to control their blood glucose levels. Leaf extract

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of *C. pictus* can stimulate insulin secretion,[8] can regenerate beta cells of the pancreas,[9] and has potent antidiabetic activity as evident from experiments carried out in animal models.[9-12]

Although the antidiabetic effect of *C. pictus* has been well documented, the effect of *C. pictus* extract on thyroid function has not been explored so far. It has been reported that pentacyclic triterpenes such as betulinic acid ameliorate experimental hypothyroidism.[13] We hypothesized that *C. pictus* extract containing pentacyclic triterpenes could exert beneficial effect in alleviating hypothyroidism. To the best of our knowledge, this is the first study to investigate the effect of *C. pictus* extract on hypothyroidism. In the present study, the ability of this plant extract to ameliorate hypothyroidism was studied in propyl thiouracil (PTU)-induced hypothyroid rat model.

MATERIALS AND METHODS

Chemicals

PTU, 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,4,6-tri-(2-pyridyl)-5-triazine, 2–thiobarbituric acid, alpha-amyrin, and beta-amyrin were of molecular grade purchased from Sigma-Aldrich (USA). All other chemicals used were of analytical grade obtained from SRL (India).

Plant material

Fresh leaves of *C. pictus* were obtained from plants cultivated by the Department of Horticulture, Jawaharlal Institute of Postgraduate Medical Education and Research (JIPMER), Puducherry. The identity of the plant was confirmed by the Botanical Survey of India, Coimbatore (Authentication Certificate No. BSI/SRC/5/23/2011-12/Tech/630 dated July 25, 2011).

Preparation of *Costus pictus* extract

Fresh leaves of *C. pictus* were shade-dried, powdered and extracted overnight with 80% methanol as solvent in a shaker. The solvent was evaporated to dryness using rotational vacuum concentrator (Martin Christ, Germany) and the final residue was lyophilized using lyophilizer (Martin Christ, Germany).

Ferric reducing antioxidant power assay

Ferric reducing antioxidant power (FRAP) assay was carried out based on the method described by Benzie and Strain.[14] The antioxidant capacity of *C. pictus* extract was measured based on the ability to reduce Fe(III)-tripyridyl triazine compound to Fe(II)-tripyridyl triazine compound. Ten microliters of *C. pictus* extract at different concentrations was added to 300 µl of FRAP reagent and thoroughly mixed. The reaction mixture was incubated at 37°C for 4 min. The increase in absorbance at 593 nm was measured. A standard curve was generated using different concentrations of $FeSO_4$ solutions. The antioxidant capacity of *C. pictus* extract was expressed as mmol of ferrous equivalent Fe(II) per gram of the sample.

2,2-Diphenyl-1-picrylhydrazyl scavenging assay

DPPH assay was carried out based on the method described by Brand-Williams *et al*.[15] The free radical scavenging activity of *C. pictus* extract was determined from the bleaching of purple-colored methanolic solution DDPH. Hundred microliters of 0.5 mM freshly prepared DPPH ethanol solution was added to 100 µL of sample solution in 50% ethanol at different concentrations. The mixture was shaken vigorously and incubated for 30 min in the dark at room temperature. The absorbance of each reaction mixture was measured at 517 nm. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity. The concentration of the extract that scavenges 50% of DPPH (IC_{50}) was calculated.

Liquid chromatography–mass spectrometry analysis of *Costus pictus* extract

The experiment was carried out using an Agilent 1290 Infinity ultrahigh-performance liquid chromatography (HPLC). Shim-pack XR-ODSIII C18 column was used (dimension: 150 mm × 2 mm internal diameter; particle size: 2 µm). The column oven temperature was 40°C. The mobile Phase A was 10 mM ammonium acetate in water and mobile Phase B was methanol. The mobile phase was used in gradient mode as follows: 0–3 min: 50% B, 3–5 min: 50–100% B, 5–25 min: 100% B, 25–25.1 min: 100–50% B, 25.1–30 min: 50% B. The flow rate was 0.2 ml/min. The run time was 30 min. For detection and quantification, HPLC system was coupled with a Thermo Fisher TSQ Vantage triple quadrupole mass spectrometer, operated with atmospheric pressure chemical ionization positive. The discharge current was 4 µA. The vaporizer temperature was 300°C. The sheath gas and auxiliary (Aux) gas used were nitrogen. Sheath gas flow rate was 25 Arb. Aux gas flow rate was 15 Arb. The analysis mode was single reaction monitoring mode.

Animal experiments

The study was conducted in the Department of Biochemistry, JIPMER, Puducherry, India, after obtaining approval from the institutional animal ethics and scientific advisory committees.

Five-month-old male Wistar rats were obtained from the institute central animal house and maintained in polycarbonate cages under a 12-h light/12-h dark cycle with the standard laboratory rat chow (35% carbohydrate, 25% proteins, 7% lipids, and 3% vitamins and minerals) and water available *ad libitum*. Totally, forty rats were randomized into four groups with ten rats in each group. To induce experimental hypothyroidism, 0.05% propylthiouracil was given in drinking water for 6 weeks, which is a well-accepted method for induction of hypothyroidism in experimental animals.[16-18] *C. pictus* extract was given at a dose of 150 mg/kg body weight/day through oral gavage.[9] This dose was fixed based on our pilot study conducted with different doses and results of previously reported toxicity studies on this plant extract.[19,20] The total duration of the study was 10 weeks – first 4 weeks was considered to be Phase 1 and next 6 weeks was considered to be Phase 2. The disease condition was induced in Phase 2 (Group 2 and Group 4). In the groups that received *C. pictus* extract treatment (Group 3 and Group 4), this extract was given in Phase 1 (pretreatment) and Phase 2 (co-treatment) as our aim was to see the preventive effect of this extract.

The four groups are as follows:

- Group 1: Control (C) – no intervention in both Phases 1 and 2
- Group 2: Hypothyroid (H) – no intervention in Phase 1 and 0.05% propylthiouracil (PTU) in drinking water in Phase 2
- Group 3: Control+extract (C+E) – intervention of *C. pictus* extract was given in both Phases 1 and 2
- Group 4: Hypothyroid+extract (H+E) – *C. pictus* extract was given in Phase 1 and 0.05% propylthiouracil (PTU) in drinking water + *C. pictus* extract in Phase 2.

Sample collection

Blood samples were collected at the beginning and end of the study. Plasma was separated and used for the estimation of thyroid profile, biochemical parameters, and inflammatory markers. Body weight, food intake, and water intake were measured periodically. At the end of the experimental period of 10 weeks, the animals were sacrificed under anesthesia; liver and kidney tissues were excised, snap frozen in liquid nitrogen and stored at −80°C for subsequent analysis.

Thyroid profile

Plasma free T3, free T4 and TSH were measured using rat-specific ELISA kits from CUSABIO, Japan. The intra-assay precision and inter-assay precision for these kits were <15% (precision details were provided by the manufacturer in the kit).

Measurement of free T3 and free T4 was based on competitive inhibition enzyme immunoassay technique. For estimation of FT3, standards and samples were added to microtiter plates precoated with antibody specific to FT3. Then, biotin-conjugated FT3 was added to the microtiter plates. FT3 present in the sample or standard competed with biotin-conjugated FT3 to bind to the antibody present in the microtiter plates. After a washing step, avidin-conjugated horseradish peroxidase (HRP) was added to the wells. This was followed by addition of substrate. Finally, the color developed was inversely proportional to the amount of FT3 in the sample/standard. Measurement of FT4 was similar to FT3 except that microtiter plates were precoated with antibody specific to FT4 and biotin-conjugated FT4 was added to the microtiter plates.

Measurement of TSH was based on quantitative sandwich enzyme immunoassay technique. Here, antibody specific to TSH was precoated onto microtiter plates. Then, standards and samples were added to the wells with HRP-conjugated antibody specific for TSH. Following a wash step, substrate was added to the wells. The color developed was directly proportional to the amount of TSH present in the sample/standard.

Lipid profile

The following biochemical parameters were analyzed in fasting plasma sample using a fully automated clinical chemistry analyzer (AU-400, OLYMPUS, Essex, UK). Total cholesterol was measured using cholesterol oxidase–peroxidase method (Genuine Bio-systems, Chennai, India), triglycerides (TGs) using an enzymatic glycerol phosphate oxidase–peroxidase method (Agappe Diagnostics, Kerala, India), and high-density lipoprotein (HDL) cholesterol by the cholesterol oxidase–peroxidase method (Lab-Care Diagnostics, Mumbai, India). Low-density lipoprotein (LDL)-cholesterol in plasma was calculated using Friedwald formula.[21]

Glucose tolerance

Glucose tolerance was assessed by method described by Yuan *et al*.[22] Blood samples were collected from the rats after they were maintained in a fasting state overnight (15 h). Rats were then injected 2.0 g/kg body weight of glucose (in 1.5 ml saline) intraperitoneally. Blood samples were collected 2 h after glucose injection. Plasma glucose was estimated by the glucose oxidase–peroxidase method (Reckon Diagnostics, Vadodara, India) in a fully automated clinical chemistry analyzer (AU-400, OLYMPUS, Essex, UK).

Liver and kidney function tests

The following parameters were analyzed in plasma sample in a fully automated clinical chemistry analyzer (AU-400, OLYMPUS, Essex, UK) using diagnostic kits purchased from Pathozyme, Kolhapur, India. Total protein was estimated using biuret method, albumin by modified bromocresol green method, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) by modified International Federation of Clinical Chemistry method. Urea was estimated by urease-glutamate dehydrogenase method and creatinine by modified Jaffe's method.

Assessment of inflammatory markers

Inflammatory response was assessed by measuring plasma levels of high-sensitivity C-reactive protein (CRP) and tumor necrosis factor alpha (TNFα). CRP was measured using sandwich ELISA (Immunology Consultants Laboratory, Newberg, OR, USA). The microtiter plates were precoated with anti-CRP antibodies. The samples and standards were added to these microtiter plates. CRP present in the sample was bound to the anti-CRP antibody in the microtiter plates. The unbound proteins were removed by washing. Then, HRP-conjugated anti-CRP antibodies were added. This was followed by addition of the substrate 3,3'5,5'-tetramethylbenzidine (TMB) to detect the bound HRP conjugate. Finally, the concentration of CRP was determined from the standard curve.

TNFα was measured using solid phase sandwich ELISA (Diaclone SAS, Besançon, France). Samples and standards were added to antibody-coated microtiter wells. Biotinylated polyclonal antibody specific for rat TNFα was simultaneously added and incubated. After washing, the enzyme–streptavidin–peroxidase was added and finally bound enzyme was detected using the chromogenic substrate TMB. The TNFα in each sample was determined from the standard curve.

Assessment of hepatic and renal oxidative stress

Liver and kidney tissues were homogenized using 0.1 M ice-cold Tris-HCI buffer (pH 7.5). The homogenate was centrifuged at 14,000 ×*g* for 15 min at 4°C. The supernatant was used for the estimation of malondialdehyde (MDA) and total antioxidant status (TAS) levels. MDA was measured according to the method described by Ohkawa *et al*.[23] TAS was estimated through FRAP assay.[14] Protein content in the liver and kidney homogenate was estimated by the method of Lowry *et al*.[24]

Statistical analyses

All values were expressed as mean ± standard deviation. The difference in mean values among the groups was analyzed using one-way analysis of variance (ANOVA) with Bonferroni *post hoc* test for anthropometric parameters, tissue oxidative stress markers and plasma inflammatory markers. Two-way repeated measures ANOVA with Bonferroni *post hoc* test was used for the analyses of rest of the parameters. All the analyses were carried out using PRISM 5 software (GraphPad Software, San Diego, CA, USA, [http://www.graphpad.com]). A $P < 0.05$ was considered statistically significant.

RESULTS

Liquid chromatography–mass spectrometry analysis of Costus pictus extract

We explored for the presence of pentacyclic triterpenes in *C. pictus* extract. It was found that *C. pictus* extract contains pentacyclic triterpenes, namely alpha and beta amyrins [Table 1 and Figures 1,2]. It contains 0.45 ng of alpha-amyrin/mg of extract and 1.29 ng of beta-amyrin/mg of extract.

Table 1: Liquid chromatography-mass spectrometry analysis of triterpenes in *Costus* pictus extract

	Molar mass (g/mol)	Retension time (min)	Mode of ionization	Parent ion (m/z)	Detection ion (m/z)	Detection mode	Amount present (ng/mg of extract)
Alpha amyrin	426.72	23.5	APCI+ve	409	271.246	SRM	0.45
Beta amyrin	426.72	22.3	APCI+ve	409	271.246	SRM	1.29
Estrone (internal standard for terpenes)	270.36	7.02	APCI+ve	271.2	159.1	SRM	

APCI: Atmospheric pressure chemical ionization; SRM: Single reaction monitoring

2,2-diphenyl-1-picrylhydrazyl assay

The IC_{50} value of *C. pictus* extract for DPPH assay was found to be 38.82 ± 1.26 μg/ml. The IC_{50} value of positive control ascorbic acid was found to be 6.73 ± 0.15 μg/ml.

Ferric reducing antioxidant power assay

Antioxidant capacity of *C. pictus* extract as assessed by FRAP assay was found to be 2.98 ± 0.03 mmol Fe^{2+}/g and that of positive control ascorbic acid was found to be 18.76 ± 0.38 mmol Fe^{2+}/g.

Body weight, food intake and water intake

The results of body weight, food intake and water intake is shown in [Table 2]. In comparison with control, PTU-induced hypothyroid rats exhibited a significant decrease in body weight. In comparison with hypothyroid group, the decrease in body weight was partially prevented in hypothyroid+extract group. In control+extract group, the body weight was similar to control group.

The amount of food and water intake was markedly reduced in the hypothyroid group in comparison with control. It was significantly improved in hypothyroid+extract group. The food intake and water intake were found to be normal in control+extract group.

Thyroid profile

At the beginning of the study, all the groups showed normal thyroid profile. At the end of the study in hypothyroid group, plasma free T3 and free T4 levels were decreased 19.92-fold and 15.43-fold, respectively, and TSH was increased 15.64-fold in comparison with control [Table 3]. This indicates that a state of severe hypothyroidism was successfully induced in the experimental animals. Hypothyroid rats treated with *C. pictus* extract (hypothyroid+extract group) exhibited a remarkable improvement in thyroid profile. In hypothyroid+extract group, plasma free T3 and free T4 levels were elevated by 10.59-fold and 8.65-fold, respectively, and further plasma TSH was decreased by 3.59-fold in comparison with hypothyroid group. These results clearly demonstrate that *C. pictus* extract has therapeutic potential to restore thyroid hormone levels in experimental hypothyroidism.

Control rats administered with *C. pictus* extract (control+extract group) showed a small increase in plasma free T3 and free T4, and this increase was statistically significant in comparison with control. However, no significant difference was seen in plasma TSH levels between control and control+extract groups.

Glucose tolerance

At the end of the study, there was no significant difference in fasting plasma glucose in hypothyroid group; however, 2 h postglucose load value was significantly elevated in this group in comparison with control group [Table 4]. Treatment with the extract brought back the elevated 2 h postglucose load value to normal levels in hypothyroid+extract group. Administration of the extract to control rats (control+extract group) resulted in normal fasting and 2 h postglucose load levels. Unlike currently used hypoglycemic drugs, administration of *C. pictus* extract

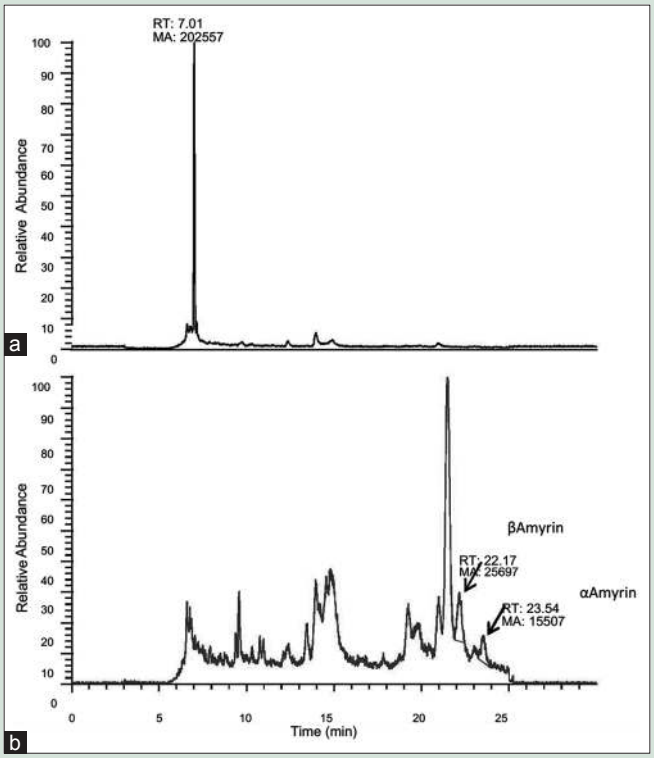

Figure 1: High-performance liquid chromatography chromatogram of (a) estrone (Internal standard for analysis of terpenes) (b) *Costus pictus* extract

Table 2: Body weight, food intake and fluid intake of the experimental groups

Groups	Final body weight (g)	Food intake (g/rat/day)	Fluid intake (ml/rat/day)
Control	328.8±4.34	16.48±1.19	31.96±1.47
Hypothyroid	270.4±5.08[a]	12.37±1.26[a]	18.6±3.12[a]
Control + extract	324.9±4.68[b]	16.22±1.17[b]	32.2±1.34[b]
Hypothyroid + extract	297.7±7.32[a,b]	14.92±1.15[a,b]	25.16±1.94[a,b]

Values are expressed as mean±SD. *n*=10/group. Differences between the groups were analyzed using one-way ANOVA with Bonferroni *post hoc* test. [a]*P*<0.05 in comparison to control group; [b]*P*<0.05 in comparison to hypothyroid group. SD: Standard deviation; ANOVA: Analysis of variance

Table 3: Effect of *Costus pictus* extract on thyroid profile in the experimental groups

Parameter	Time period	Control	Hypothyroid	Control + extract	Hypothyroid + extract
Free T3 (pMol/L)	End	4.78±0.49	0.25±0.05[a,x]	5.18±0.23[a,b,x]	2.55±0.21[a,b,x]
	Basal	4.75±0.42	4.77±0.40	4.73±0.47	4.71±0.49
Free T4 (pMol/L)	End	18.85±2.20	1.29±0.31[a,x]	21.06±2.34[a,b,x]	10.53±0.82[a,b,x]
	Basal	18.76±2.27	19.09±2.18	18.88±2.14	18.71±2.09
TSH (μIU/ml)	End	1.75±0.27	26.83±1.38[a,x]	1.69±0.24[b]	7.52±0.53[a,b,x]
	Basal	1.76±0.30	1.72±0.25	1.77±0.26	1.75±0.26

Values are expressed as mean±SD. *n*=10/group. Differences between the groups were analyzed using two-way repeated measures ANOVA with Bonferroni *post hoc* test. [a]*P*<0.05 in comparison to control group of the same period; [b]*P*<0.05 in comparison to hypothyroid group of the same period; [x]*P*<0.05 in comparison to basal values of the same group. SD: Standard deviation; ANOVA: Analysis of variance; TSH: Thyroid stimulating hormone; Free T3: Free triiodothyronine; Free T4: Free thyroxin

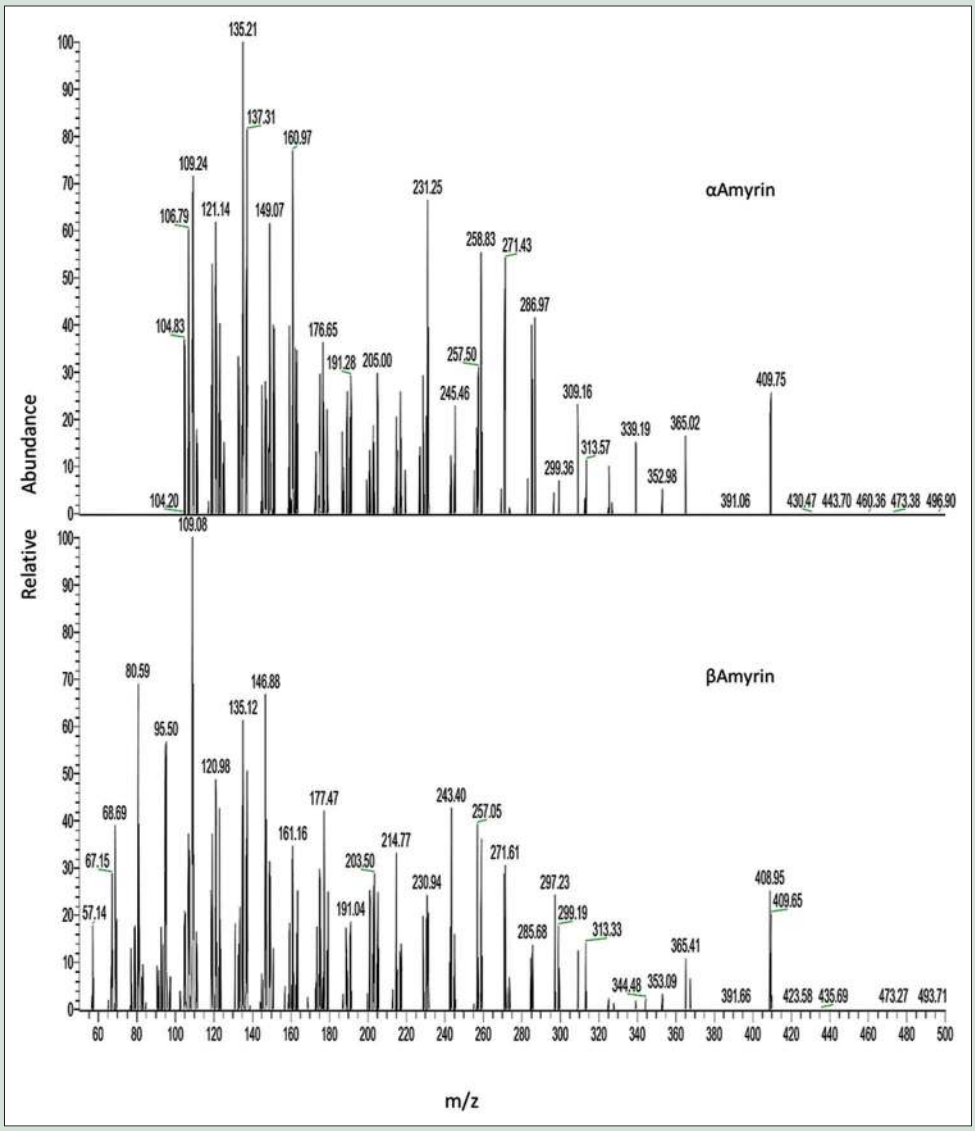

Figure 2: Liquid chromatography–mass spectrometry spectrum of alpha-amyrin and beta-amyrin

Table 4: Effect of *Costus pictus* extract on glucose and lipid profile in the experimental groups

Parameter	Time period	Control	Hypothyroid	Control + extract	Hypothyroid + extract
Fasting glucose (mg/dl)	End	76.2±7.93	81.2±15.77	74.3±7.01	78.4±8.88
	Basal	73.4±7.88	72.3±7.83	75.6±5.93	74.5±6.13
2 h postglucose load values during IPGTT (mg/dl)	End	113.8±12.09	148.9±19.68^{a,x}	110.3±8.96^{b}	119.5±11.37^{b}
	Basal	115.8±13.64	119.8±11.18	116.2±14.61	114.2±11.41
Total cholesterol (mg/dl)	End	61.6±5.66	88.2±4.69^{a,x}	63.2±4.96^{b}	68.8±5.90^{a,b}
	Basal	61.2±5.41	62.1±4.48	61.6±4.06	63.2±6.49
LDL cholesterol (mg/dL)	End	17.5±4.44	47.9±5.36^{a,x}	19.2±7.87^{b}	26.9±7.78^{a,b,x}
	Basal	18.2±6.11	18.7±5.14	18.9±6.10	18.6±4.75
HDL cholesterol (mg/dL)	End	27.1±3.84	24.1±4.56	27.5±4.06	26.1±4.63
	Basal	26.7±3.53	27.0±4.67	26.2±4.26	28.1±4.65
TG (mg/dl)	End	84.8±6.80	79.1±7.06	82.6±6.29	79.7±6.11
	Basal	81.5±7.60	82.2±6.56	82.4±7.55	82.3±5.42

Values are expressed as mean±SD. *n*=10/group. Differences between the groups were analyzed using two-way repeated measures ANOVA with Bonferroni *post hoc* test. $^{a}P<0.05$ in comparison to control group of the same period; $^{b}P<0.05$ in comparison to hypothyroid group of the same period; $^{x}P<0.05$ in comparison to basal values of the same group. TG: Triglyceride; LDL: Low-density lipoprotein; HDL: High-density lipoprotein; IPGTT: Intraperitoneal glucose tolerance test; SD: Standard deviation; ANOVA: Analysis of variance

to normal control rats did not result in hypoglycemia, but it maintained the glucose levels.

Lipid profile

Hypothyroid group showed a profound elevation in both total cholesterol (1.4-fold increase) and LDL-cholesterol (2.8-fold increase) in comparison with control at the end of the study [Table 4]. In comparison with hypothyroid group, hypothyroid+extract group exhibited a marked reduction in total cholesterol and LDL cholesterol by 1.3-fold and 1.9-fold, respectively. However, there was no significant difference seen in plasma TG and HDL cholesterol levels in hypothyroid group at the end of the study.

Liver and kidney function tests

The results of liver and kidney function tests is shown in [Table 5]. At the beginning of the study, all the experimental groups exhibited normal liver and kidney functions. At the end of the study, plasma AST levels were found to be significantly increased in hypothyroid rats in comparison with control and treatment with extract prevented the increase in AST levels. There was no significant difference seen in plasma ALT, total protein, and albumin levels between the experimental groups.

Plasma urea and creatinine levels were elevated in the hypothyroid group in comparison with control. In hypothyroid+extract group, plasma urea and creatinine levels were significantly decreased in comparison to hypothyroid group.

These results demonstrate that hypothyroid rats exhibited impairment in hepatic and renal function; treatment with *C. pictus* extract partially prevented hepatic and renal damage as indicated by AST, urea, and creatinine levels.

Inflammatory markers

In comparison with control, hypothyroid group showed a drastic increase in plasma TNFα and CRP levels by 4.21-fold and 1.46-fold, respectively. Treatment with the extract significantly attenuated the increase in TNFα and CRP in hypothyroid+extract group in comparison with hypothyroid group [Figures 3 and 4].

Hepatic and renal oxidative stress markers

In both liver and kidney tissues, hypothyroid rats displayed significant elevation in MDA levels and significant reduction in TAS levels in comparison with control [Figures 5 and 6]. In hypothyroid+extract group, the lipid peroxidation was found to be suppressed as indicated by decrease in MDA levels; in addition, TAS levels were enhanced in comparison with hypothyroid group. MDA and TAS levels were found to be similar between control group and control+extract group. To summarize the results, treatment with extract ameliorated hepatic and renal oxidative stress seen in hypothyroid rats.

DISCUSSION

The present study was conducted to evaluate the effect of *C. pictus* extract in experimental hypothyroidism. Severe hypothyroidism was induced in rats by administration of 0.05% PTU in drinking water for 6 weeks. Plasma levels of free T3 and free T4 were significantly decreased and TSH levels were dramatically increased in hypothyroid group. This confirmed the successful induction of hypothyroidism in the experimental groups under study. PTU is a reversible goitrogen. It induces hypothyroidism by inhibiting crucial enzymes required for thyroid hormone synthesis, namely thyroperoxidase and peripheral deiodinase.[25] Inhibition of these enzymes impairs the iodination of tyrosyl residues and coupling of iodotyrosyl residues to form iodothyronine.[26,27] Treatment with the extract in hypothyroid+extract group produced 10.59-fold increase in plasma free T3, 8.65-fold increase in free T4, and 3.59-fold decrease in TSH in comparison with hypothyroid group. Since PTU is a reversible goitrogen, withdrawal of PTU can restore the levels of thyroid hormones. Hence, the experiment was designed such that in H+E group after Phase

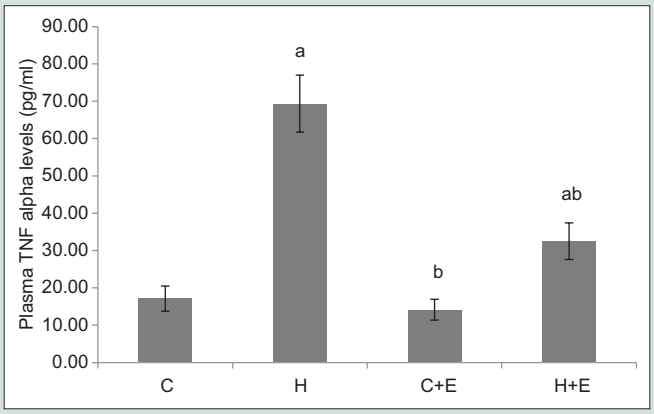

Figure 3: Effect of *Costus pictus* extract on plasma tumor necrosis factor alpha levels measured at the end of the study. Differences between the groups were analyzed using one-way analysis of variance with Bonferroni *post hoc* test. a = $P < 0.05$ in comparison to control group, b = $P < 0.05$ in comparison to hypothyroid group; C: Control group; H: Hypothyroid group; C+E: Control+extract group; H+E: Hypothyroid+extract group

Table 5: Effect of *Costus pictus* extract on parameters of liver and kidney function in the experimental groups

Parameter	Time period	Control	Hypothyroid	Control + extract	Hypothyroid + extract
AST (U/L)	End	85.4±2.76	102.8±4.66[a,x]	87.1±2.81[b]	89.4±3.75[a,b,x]
	Basal	86.7±3.06	83.2±3.49	85.2±3.58	84.8±2.90
ALT (U/L)	End	50.5±3.31	54.2±4.49	52.2±3.77	52.1±3.48
	Basal	49.7±4.27	51.8±4.49	51.5±4.14	50.1±4.75
Total protein (g/dl)	End	7.3±0.32	7.21±0.28	7.42±0.35	7.33±0.36
	Basal	7.32±0.25	7.26±0.30	7.34±0.33	7.29±0.24
Albumin (g/dl)	End	3.26±0.27	3.23±0.20	3.36±0.32	3.3±0.30
	Basal	3.23±0.22	3.24±0.24	3.32±0.23	3.41±0.33
Urea (mg/dL)	End	24.2±3.82	43.5±7[a,x]	25.3±4.14[b]	31.5±3.72[a,b,x]
	Basal	24.6±3.34	25.1±3.21	26±4.16	24.6±3.86
Creatinine (mg/dL)	End	0.52±0.10	1.03±0.25[a,x]	0.50±0.11[b]	0.68±0.12[a,b,x]
	Basal	0.52±0.09	0.51±0.12	0.49±0.10	0.51±0.10

Values are expressed as mean±SD. n=10/group. Differences between the groups were analyzed using two-way repeated measures ANOVA with Bonferroni *post hoc* test. [a]$P<0.05$ in comparison to control group of the same period; [b]$P<0.05$ in comparison to hypothyroid group of the same period; [x]$P<0.05$ in comparison to basal values of the same group. AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; SD: Standard deviation; ANOVA: Analysis of variance

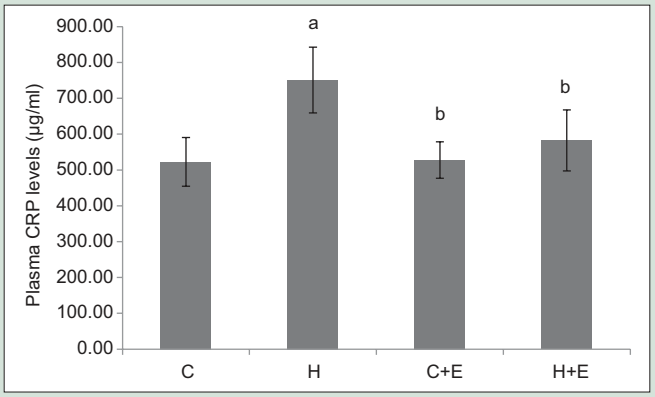

Figure 4: Effect of *Costus pictus* extract on plasma C-reactive protein levels measured at the end of the study. Differences between the groups were analyzed using one-way analysis of variance with Bonferroni *post hoc* test. a = $P < 0.05$ in comparison to control group, b = $P < 0.05$ in comparison to hypothyroid group; C: Control group; H: Hypothyroid group; C+E: Control+extract group; H+E: Hypothyroid+extract group

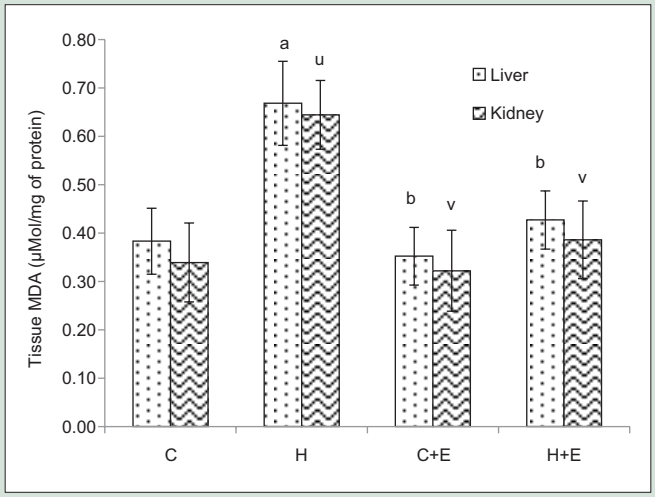

Figure 5: Effect of *Costus pictus* extract on tissue malondialdehyde levels measured at the end of the study. Differences between the groups were analyzed using one-way analysis of variance with Bonferroni *post hoc* test. a = $P < 0.05$ in comparison to control group of liver tissue, b = $P < 0.05$ in comparison to hypothyroid group of liver tissue, u = $P < 0.05$ in comparison to control group of kidney tissue, v = $P < 0.05$ in comparison to hypothyroid group of kidney tissue; C: Control group; H: Hypothyroid group; C+E: Control+extract group; H+E: Hypothyroid+extract group

1 (period of pretreatment with *C. pictus* extract), in Phase 2 along with the *C. pictus* extract PTU administration was continued until the end. Animals in hypothyroid+extract group showed a significant improvement in thyroid profile despite continued PTU administration in Phase 2. These results prove that *C. pictus* extract has therapeutic potential in improving the thyroid hormone levels in experimental hypothyroidism.

This is the first study to reveal the effect of *C. pictus* extract in ameliorating hypothyroidism. It is possible that *C. pictus* extract upregulates the expression of key enzymes involved in thyroid hormone synthesis, namely thyroperoxidase and 5'deiodinase, or increases the activity of these enzymes, thereby stimulating the thyroid gland to secrete thyroid hormones. However, the possibility that the reason for the improvement in thyroid profile in *C. pictus* extract treated hypothyroid rats due to antagonizing effect of *C. pictus* extract on PTU cannot be excluded. To conclusively prove the aforementioned mechanism, further investigations on expression and activity of thyroperoxidase and 5'deiodinase in the thyroid gland and metabolism of PTU on treatment with *C. pictus* extract needs to be carried out.

Previous studies have shown that *C. pictus* extract has good antioxidant property.[28] Our findings are in agreement with these studies as indicated by the results of DDPH assay, FRAP assay, tissue MDA and TAS. *C. pictus* extract also showed good anti-inflammatory effect. We found that administration of *C. pictus* extract significantly reduced plasma TNF-α and CRP levels in hypothyroid rats. *C. pictus* extract, being a good antioxidant and anti-inflammatory agent, could help to repair the damage caused by PTU in the thyroid gland.

It has been reported in previous studies that pentacyclic triterpenes such as betulinic acid alleviates experimental hypothyroidism. It reduces TSH levels and improves T3 and T4 levels in PTU-induced hypothyroid rats.[13] Hence, we explored for the presence of similar pentacyclic triterpenes in *C. pictus* extract. We found that *C. pictus* extract contains alpha and beta amyrins both of which belong to the family of pentacyclic triterpenes. Alpha and beta amyrins possess potent anti-inflammatory, antioxidant, hepatoprotective, and anti-nociceptive effects.[29-32] Further, it has been reported that alpha and beta amyrins inhibit nuclear factor-kappa B (NF-kβ) activation.[33] Betulinic acid alleviates experimental hypothyroidism by preventing the activation of NF-kβ. Activation of NF-kβ interferes with T3-dependent induction of 5'-Deiodinase gene expression, leading to impairment in the production of thyroid hormones.[34,35] Since alpha and beta amyrins in *C. pictus extract* are also pentacyclic triterpenes, structurally similar to betulinic acid and inhibit NF-kβ activation, it is possible that amyrins ameliorate experimental hypothyroidism through similar mechanism.

Plethora of human and animal studies have proved that hypothyroidism is associated with elevated plasma total cholesterol levels.[36-39] It has been reported that PTU-induced hypothyroid rats exhibited hypercholesterolemia with no significant increase in plasma TG levels.[40,41] Our results are in agreement with these studies. In the present study, treatment with *C. pictus* extract resulted in remarkable reduction in total cholesterol as well as LDL-cholesterol levels in hypothyroid rats. Hypocholesterolemic effect of *C. pictus* extract could be attributed to the presence of pentacyclic triterpenes such as alpha and beta amyrins in the extract. Santos *et al*. have shown that alpha and beta amyrins exert potential antihyperglycemic and antihyperlipidemic effects.[42] It has been reported that pentacyclic triterpenes possess hypolipidemic effect by downregulation of lipogenic genes such as acety l-CoA carboxylase, stearoyl-CoA desaturase 2, glycerol-3-phosphate acyltransferase, and acyl-CoA cholesterol acyltransferase.[43,44] *C. pictus* extract could alleviate hypercholesterolemia in PTU-induced hypothyroid rats through similar mechanism. We have reported in our earlier studies with *C. pictus* extract that it contains significant amount of phenolic compounds and flavonoids.[45] Apart from amyrins, phenolic compounds and flavonoids could also be responsible for the hypolipidemic activity exhibited by *C. pictus* extract in hypothyroid rats.

Liver and kidney are important target organs of thyroid hormones. Hypothyroidism leads to perturbed liver and kidney function.[46,47] In the present study, plasma levels of AST, urea and creatinine were significantly elevated in hypothyroid rats. Plasma ALT level showed an increase, but it was not significant. The increase in levels of transaminases in hypothyroidism is due to inadequate levels of thyroid hormones and decreased hepatic clearance.[47] The raise in plasma urea and creatinine levels in hypothyroid rats is due to reduced glomerular filtration rate and decreased tubular secretion of creatinine.[46,48] In the present study,

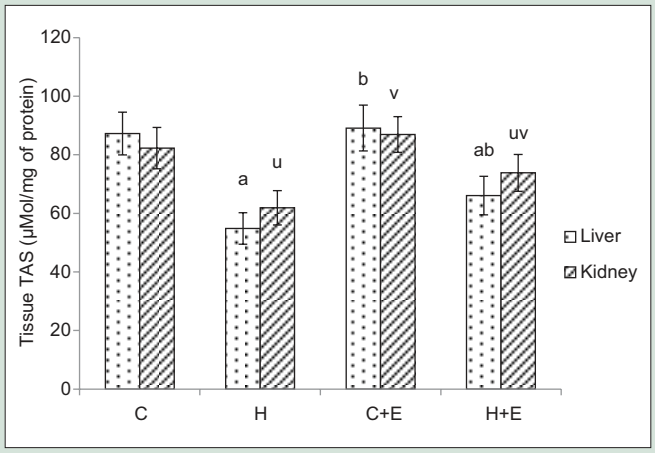

Figure 6: Effect of *Costus pictus* extract on tissue total antioxidant status levels measured at the end of the study. Differences between the groups were analyzed using one-way analysis of variance with Bonferroni *post hoc* test. a = $P < 0.05$ in comparison to control group of liver tissue, b = $P < 0.05$ in comparison to hypothyroid group of liver tissue, u = $P < 0.05$ in comparison to control group of kidney tissue, v = $P < 0.05$ in comparison to hypothyroid group of kidney tissue; C: Control group; H: Hypothyroid group; C+E: Control+extract group; H+E: Hypothyroid+extract group

it was observed that oxidative stress was seen in the liver and kidney tissues of hypothyroid rats as indicated by increased MDA and decreased TAS in these rats. The presence of oxidative stress further augments the hepatic and renal damage caused due to thyroid hormone insufficiency. Treatment with *C. pictus* extract significantly decreased the levels of AST, urea and creatinine in hypothyroid rats compared to untreated group, further hepatic and renal oxidative stress were also found to be decreased on treatment with extract. Previous studies have suggested that exogenous antioxidants can alleviate hepatic and renal damage caused in experimental hypothyroidism.[49-52] The protective effect of *C. pictus* extract against hypothyroidism-induced kidney and liver damage could be attributed to its antioxidant effect and its ability to restore thyroid hormone levels.

In the present study, we found that body weight, food intake and water intake were significantly reduced in hypothyroid rats. These results are in line with earlier studies.[16,17,51] The reduction in water intake is due to impaired ability to excrete water load in hypothyroidism.[53] The reduction in food intake could be due to impairment in energy metabolic process and decrease in basal metabolic rate as reported by previous studies.[54,55] Treatment with *C. pictus* extract improved both food and water intake. The beneficial effect exhibited by the extract on the aforementioned anthropometric parameters is mainly due to improvement in thyroid profile caused by the extract. Although induction of hypothyroidism by PTU is a well-accepted method of creating hypothyroidism in experimental animals, it is still associated with certain limitations. This model fails to produce increase in body weight as seen in human hypothyroidism. This has been reported in earlier studies.[16,17,51] We have also observed the same in our study. Despite this limitation, this model is still used worldwide till date because PTU-induced hypothyroid rat model mimics most of the main features of human hypothyroidism. Thyroidectomized hypothyroid model was not used because in thyroidectomized rats there is danger of removal of associated parathyroid glands resulting in tetany. Although this is the first study to report the beneficial effect of *C. pictus* on PTU-induced hypothyroidism, the exact mechanism at molecular level has not been explored in the same. Further investigations at molecular level are needed to conclusively prove the exact mechanism by which *C. pictus* extract exerts its beneficial effect with respect to thyroid profile in experimental hypothyroidism.

CONCLUSIONS

The present study has revealed that *C. pictus* extract ameliorates experimental hypothyroidism. Treatment with *C. pictus* extract in PTU-induced hypothyroid rats increased plasma free T3, free T4 levels and decreased TSH levels. Further, the extract exhibited antioxidant and anti-inflammatory effects, improved plasma lipid profile and partially prevented hepatic and renal damage in hypothyroid rats. Pentacyclic triterpenes alpha and beta amyrins were identified and quantified in the extract. Amyrins could be responsible for the aforementioned beneficial effects exhibited by *C. pictus* extract in experimental hypothyroidism. However, the exact molecular mechanism through which *C. pictus* extract ameliorates hypothyroidism warrants further investigations. *C. pictus* extract has the potential to emerge as a novel therapeutic agent in the treatment of hypothyroidism.

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Conflicts of interest

There are no conflicts of interest.

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