Saraca asoca Alleviates PTU-induced Hypothyroidism in Rats: LC-MS Profiling and Identification of Betulinic acid and Lupeol as Key Components for Its Thyroid Stimulatory Activity: In Vivo and In Silico Analyses

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Abstract

Hypothyroidism (HT) is the most common endocrine disorder in women and female reproduction is adversely affected by hypothyroidism. *Saraca asoca* (Roxb.) de Wilde (SA) is traditionally used as a remedy for gynaecological disorders. Although thyroid hormones are essential for reproductive function no data are available so far on the impact of SA on thyroid function. Therefore, the current study attempted to evaluate the effects of SA in regulating HT through *in vivo* and *in silico* approaches. HT was induced in rats by administering 0.05% 6-propyl-thiouracil (PTU) in their drinking water for 4 weeks and the effect of standardized dose of SA extract (100 mg/kg body weight) examined in HT-induced rats for 28 days. LC–MS/MS analysis identified the active constituents in SA. Molecular docking was used to predict the interaction between active component and key targets. Oral administration of SA effectively ameliorated HT initiated with PTU as revealed by decreased TSH, increased thyroid hormones,5'D1 activity, antioxidants and decreased inflammatory marker, TNF-α with improved liver histology. LC-MS analysis identified the notable presence of betulinic acid (BA) and lupeol (LPL) in extract. Molecular docking study predicted strong binding energies between BA and LPL and thyroid receptors, TRβ and TSHR, supporting BA and LPL as the active principles of SA for the management of hypothyroidism. Our study proposed for the first time that BA and LPL as potential thyroid agonist playing a crucial role in the thyroid stimulatory role of SA. However, further mechanistic study is needed to solidify our findings.

Introduction

Hypothyroidism is characterized by low levels of serum thyroid hormones and common symptoms include weight gain, intolerance to cold, dry skin, fatigue, constipation, and hoarse voice [1]. It is commonly treated with Levothyroxine, the standard drug for hypothyroidism. However, this conventional treatment is often accompanied by side effects and limitations such as increased risk of heart disease, osteoporosis, and fractures [2]. Therefore, the people are in search of alternatives, primarily a plant-based drug.

Saraca asoca (SA) De. Wilde (Family, Fabaceae) has been widely used in traditional Indian medicine especially for the treatment of various types of reproductive disorders. The bark of SA is known to ameliorate reproductive abnormalities and for its antioxidant, cardioprotective, antidepressant, anti-inflammatory and hypolipidemic activities [3]. However, no systematic study has been carried out on the plant to establish the scientific basis on thyroid related activity yet. Therefore, the potential of this plant in thyroid function has been explored in PTU-induced hypothyroid rat model. Besides, estimations of thyroid hormones, parameters measured include TSH estimations, lipid profile, oxidative stress markers, inflammatory cytokine, liver enzymes, and histological alterations. Compounds in the active extract of *S. asoca* were analysed by LC-ESI-MS/MS to correlate the biological activity to the phytochemical profile. However, efforts in understanding the role of triterpenes in treating thyroid problems are very limited. Therefore, special attention is imperative to explore the therapeutic potential of these compounds in thyroid function. Bark of *Saraca asoca* reported to contains many effective bioactive compounds such as tannin, catechol, flavonoids, sterol, glucosides, and alkaloids leaving aside the triterpenoids. For the first time we identified the presence of a naturally occurring two pentacyclic triterpenoids, betulinic acid (BA) and lupeol (LPL) in methanolic SA bark extract using LC-MS/ MS. Previously some preliminary studies suggest that pentacyclic triterpenes, such as betulinic acid, lupeol, betuline, oleanolic acid, alpha, and beta amyrins, can help alleviate hypothyroidism [6] and thus, the proposed study focused on triterpenoids in SA extract in thyroid function .

To evaluate the impact of SA on thyroid hormones, changes in biochemical indices such as lipid peroxidation, antioxidant enzymes, serum liver and inflammatory markers, serum lipids along with determining the histological changes in the liver of PTU-induced rats were performed. Thyroid-stimulating hormone (TSH) is the primary regulator of thyroid hormone synthesis and secretion and thyroid hormone receptors β (TRβ) are the most important regulators of the production of thyroid stimulating hormone (TSH). Thyroid hormones are known to act via TRβ in the hypothalamus and pituitary to inhibit TRH and TSH production and secretion and thus complete a negative feedback loop that maintains systemic thyroid status within a normal range [7]. Thyroid-stimulating hormone receptor (TSHR), plays pivotal role in the regulation of thyroid function and it is the main target of TSH in thyroid follicular cells responsible for the synthesis of thyroid hormones. [8]. For the first time, the potential binding mode for BA and LPL at TRβ and TSHR active site provided the insights into the potential mechanisms of its action in thyroid function.

It is expected that the findings of this study would be a future explorer for designing a promising new drug development for hypothyroidism treatment. At present, the exact mechanism of BA in treating hypothyroidism remains to be fully understood. Thus, further research is required to clarify its mechanism of action, associated signalling pathways and potential clinical applications in treating thyroid dysfunction.

Materials and Methods

Chemicals and reagents

ELISA kits to estimate triiodothyronine (T_3), thyroxine (T_4) and thyroid stimulating hormone (TSH) were purchased from Life Technologies Pvt. Ltd, India; whereas tumour necrosis factor-α (TNF- α) ELISA kit was purchased from Ray Biotech, Inc., Norcross, GA, USA.PTU, L-T_4, betulinic acid, Ferulic acid, chlorogenic acid, sinapic acid, gallic acid, syringic acid were purchased from Sigma (St. Louis, MO, USA). Triglycerides, (TG); and total cholesterol, (TC), alanine transaminase, (ALT); aspartate transaminase (AST) assay kits were obtained from *Erba* diagnostics Mannheim GmbH, Mannheim, Germany.

Plant material and preparation of the extract

The bark of *S. asoca* was collected from Indore, India, and was identified and authenticated by a local taxonomist. The plant name has been checked in http://www.worldfloraonline.orgon1/3/2025.WFO (2025), that was published on the internet; http://www.worldfloraonline.org/taxon/ wfo-0000193380. Accessed on: 01 Feb. 2026. Shade-dried bark pieces were powdered coarsely and was extracted with methanol by the hot percolation method using Soxhlet extraction process. The extract was concentrated to dryness under reduced pressure and controlled temperature (50–55°C) using a rotavapor [9]. The extract was evaporated under vacuum at 40 ◦C until complete dryness was achieved. The extraction yield was 10.2% (W/W).

Experimental animals

Sixty female rats (weight of 180 ± 10 g) were obtained from the DAVV, animal centre, Indore, M.P. for the use in this study. The rats were housed under standard conditions with a temperature at 25 ± 2 ◦C and 12/12 h light/dark cycle. Animals were allowed free access to food and drinking water ad libitum throughout the experiment. The experiments were initiated following acclimatization to these conditions for at least one week. Experimental

protocols were approved by the Institutional Animal Ethics Committee with a registration number 779/Po/Ere/S/03/CPCSEA and followed the Guidelines for the Use and Care of Experimental Animals in Research.

Dose standardization

To determine the effective dose of the bark extract on the alteration of thyroid hormones in euthyroid adult female albino Wistar rats, a total of 28 rats were randomly divided into four groups of seven each. While gr. 1 served as control, group 2, 3, and 4 received 50 ,100 and 200 mg/kg/day of SA extract respectively per oral for 28 days and thyroid hormones were estimated using ELISA kits. As 100 mg/kg of the bark extract maximally and significantly increased the level of thyroid hormones, this dose was selected as the effective dose and used for further evaluation (Table-S1).

Induction of hypothyroidism

Propyl thiouracil (PTU, Sigma Aldrich, USA) was used to induce hypothyroidism. The hypothyroid rat model was developed using 0.05% PTU in the drinking water for 30 days [10, 11].

Experiment:2

In this experiment a total of 35 female Wistar rats weighing 170.54 ± 10g were used. Rats were divided into five groups of seven each (7 normal, 7 only SA extract treated and 21 PTU-induced hypothyroid rats).

The groupings were:

Group 1: Normal rats + distilled water

Group 2: PTU-induced rats (administered with 0.05% PTU in their drinking water for 30 days, [11].

Group 3: SA extract treated (100 mg/kg for 2-weeks, orally)

Group 4: Hypothyroidic rats + SA at 100 mg/kg (for 2 weeks)

Group 5: PTU-induced rats + L-T_4 at 100 µg/kg (Rats were administered PTU for the first 30 days and then levothyroxine (0.1 mg/kg, p.o) [12], daily from 31st to the 45th day of the study.

Rats of Groups I and II received distilled water (the vehicle for the drug preparation) daily for the entire experimental period. All the treatments were given between 9.00 and 10.00 h of the day to avoid circadian variation. At the end, the animals were sacrificed, by decapitation and trunk blood was collected. Liver tissues were removed and used for biochemical estimations and histological evaluation.

Serum biochemical analysis

The collected blood was subjected to centrifugation for 20 min at a speed of 3000 rpm in a refrigerated centrifuge set at a temperature of 4°C to extract the serum. The levels of T_4, T_3, TSH, TNF-α, ALT, AST, TG; and TC were measured.

Preparation of tissue homogenates

Liver tissues were washed with cold normal saline and they were homogenized in phosphate-buffered solution (pH 7.0 and homogenate was centrifuged at 10,000x g at 4°C for 20 min. The supernatant was separated and stored at − 70°C for measuring oxidative stress biomarkers (LPO, lipid peroxidation) and antioxidant enzymes (CAT, SOD, GPx and GSH). Total protein levels and 5'D1 activity was also estimated in liver tissues.

Assay of thyroid hormones, TSH and TNF- α

T_3, T_4, TSH and TNF- α levels were estimated using the procedure mentioned in ELISA kits, as routinely done in our laboratory [11].

Assessment of Oxidative stress and antioxidant biomarkers in hepatic tissues

LPO was estimated spectrophotometrically through the thiobarbituric acid reactive substances (TBARS) assay which is based on the reaction of malondialdehyde (MDA;1,1,3,3-tetramethoxypropane) with thiobarbituric acid (TBA) using the method described by Okhawa et al. [13]. Briefly, liver supernatant was reacted with 20% trichloroacetic acid and 1% TBA. The reaction mixture consisted of 0.2 ml supernatant, 8.1% sodium dodecyl sulfate, 1.5 ml of 30% acetic acid (pH 3.5) and 1.5 ml of 0.8% TBA. Samples were mixed well and heated at 95°C for one hour, then it was cooled and 1.0 ml of distilled water and 5.0 ml of n-butanol: pyridine (15:1, v/v) were added and centrifuged at 5000 rpm for 10 min. Finally, the absorbance of TBARS was measured at 532 nm and Results were expressed as nM MDA formed/h/mg protein.

Estimation of GSH

The GSH level was measured by the method of Ellman [14]. Briefly, 1.5 ml of precipitating reagent (3.34 g meta-phosphoric acid, 0.4 g EDTA and 60.0 g sodium chloride) and 0.9 ml distilled water added to 0.1 ml of sample. Tubes were shaken and allowed to stand for 5 min at the room temperature. The mixture was centrifuged for 15 min at 4000 rpm at 4°C. To 1.0 ml supernatant, 4.0 ml of phosphate solution (0.3 M disodium hydrogen phosphate) and 0.5 ml DTNB were added. The development of yellow colour complex was read on a spectrophotometer at 412 nm.

Estimation of SOD

The activity of superoxide dismutase (SOD) was determined by the method of Marklund and Marklund [15], using inhibition of pyrogallol autoxidation at pH 8. The activity of SOD is expressed as units per mg protein per minute. In brief, to 20 µL of liver tissue supernatant 1 mL of Tris-HCl buffer, containing diethylenetriaminepentaacetic acid and pyrogallol was mixed and the absorbance of the samples at 420 nm was noted at an interval of 30 s. One unit of total SOD activity was calculated that caused 50% pyrogallol autooxidation inhibition.

Estimation of CAT

Catalase (CAT) activity in the liver was measured using the method of Aebi [16]. Briefly, 1 ml of 50 mM phosphate buffer (pH 7) and 0.1 ml of 30 mM H_2O_2 were added to 50 µl liver tissue supernatant and then the decline in absorbance at 240 nm was measured. One unit of CAT activity corresponded to the amount of the enzyme that degraded 1 µ mol H_2O_2 per minute at 25°C.

Estimation of GPx

Glutathione peroxidase (GPx) activity was determined by the method of Rotruck et al [17]. Briefly, the reaction mixture consists of 0.2 mL of 0.4 M of Tris-HCl buffer (pH7.0), 0.1 mL of 10 mM of sodium azide, 0.2 mL of glutathione and 0.1 mL of 0.2 mM of H2O2 were added to 0.5 mL supernatant and were incubated at 37^0 C for 3 minutes The reaction was terminated by addition 0.5 mL of 10% trichloroacetic acid (TCA). The tubes were incubated at 37^0 C for 3 minutes, and the reaction was terminated by the addition of 0.5 mL of 10% trichloroacetic acid (TCA) and centrifuged at 3000 rpm and the supernatant used to determine the residual glutathione content. 1 mL of DTNB reagent and 3 mL of disodium hydrogen phosphate was added to the supernatant, and the colour that developed was read at 412 nm. Results were expressed as µg of GSH consumed/mg protein.

Protein estimation

Protein was estimated in the liver by the method of lowry et al [18] using bovine serum albumin (BSA) as standard.

Assessment of liver toxicity biomarkers

Assessment of liver injury biomarkers was conducted by estimating the levels of ALT and AST using commercial kits following the manufacturer's instructions.

Histological analysis

Liver tissues obtained from each group were fixed at 10% formalin for 18 h and embedded in paraffin. Afterward, the tissues were cut into sections (5 µm) and stained with haematoxylin and eosin (H and E) for examination under light microscope. The samples rinsed with saline and were then preserved in a 10% formalin solution. Afterward, the tissues were cut into sections and subjected to haematoxylin and eosin (H&E) staining. The stained sections were then examined under a microscope at a 10x magnification and images of the slides were captured.

HPLC-ESI-QTOF-MS analysis of the methanolic extract of SA

HPLC-ESI-QTOF-MS analysis, used for the determination of bioactive compounds in the bark extracts was carried out at Sophisticated Analytical Instrument Facility, CSIR-Central Drug Research Institute, Lucknow, India following the method of Pai et al [19] with little modification. The methanolic extract was centrifuged at 12,000 rpm for 10 min and filtrated through a 0.45 µm membrane filter, and then injected in a volume of 10 µL into an Agilent 1200 HPLC system equipped with an online degasser, a quaternary solvent delivery system, an autosampler and a diode-array detector. Separation was made on a C-18 column (Agilent Eclipse, 5 µ, 15 cm, 4.6 mm id) with a flow rate of 1.0 mL/min and a column oven temperature of 35°C. Two mobile phases were used: A-0.1% formic acid in water and B- 90% of acetonitrile in water. The LC conditions were 5% at 0 – 2 min in B, a linear increase from 5 to 20% between 3 and 10 min, 20 to 50% during 15–20 min, and finally from 50 to 95% at 25–30min. The diode array detection (DAD) was set at 254 nm for real time monitoring of the peak intensities. UV spectra were recorded from 190–650 nm and were available after completion of the chromatographic run for identification of plant constituents. High-resolution mass spectra were acquired in positive ion mode using a micro-TOF-Q mass, Mass spectrometer equipped with an Apollo electrospray ion (ESI) source. The MS data were recorded over a range of m/z 50–1000. The tentative identification of 16 compounds presented in (Table 3).

Table 3
Phytochemicals in *Saraca asoca bark* extract, identified from LC–MS/MS data in negative ion mode with retention time and molecular weight.

Peak no	RT (min)	Empirical formula	MS/MS Fragment ions	Parent ion (m/z)	Identification
1	7.46	$C_{27}H_{30}O_{16}$	287,304 ,463	610.4	Rutin
2	8.43	$C_{21}H_{20}O_{11}$	304.2	447.42	luteolin 5-O-glucoside
3	11.10	$C_{15}H_{10}O_7$	173	302.1	quercetin
4	13.42	$C_{30}H_{48}O_3$	207.1,393.6,411.4	455.3	Betulinic acid
5	16.23	$C_{13}H_{12}O_9$	179.0	311.3	Caftaric acid
6	16.97	$C_{15}H_{18}O_8$	178	325.3	*p*-Coumaric acid glucoside
7	18.10	$C_{15}H_{12}O_7$	153.1	304.2	Taxifolin
8	21.37	$C_{28}H_{32}O_{16}$	300.4	622.3	isorhamnetin-3-O-rutinoside
9	27.32	$C_{30}H_{48}O_2$	158,279	452.5	oleanane triterpene derivative

HPLC analysis

The HPLC analysis of methanol extract of *S. asoca* was performed with Jasco chromatographic system (Model no. LC-2000 Plus) having a delivery system consisting of binary solvent (LC-20AD), an injector of Rheodyne type possessing 20 µL sample loop and DAD detector (SPD-M 20 A). Chromatographic separation was carried out using ODS C18 (5 µm) column (250x4.6 mm) with an extended guard column. The mobile phase composed of acetonitrile (99.99%) and 1% trifluoroacetic acid (0.01%). Lupeol and betulinic acid were quantified by UV detector at λ = 210 nm [20]. The rate of mobile phase flow was 1mL/min, while the column temperature was 25^{o}C.

Solvent system composed of acetonitrile (99.99%) and 1% trifluoroacetic acid (0.01%) run with isocratic elution. The optimal mobile phase flow rate was 1mL/min and the volume of samples and standard solutions were taken as 20 µL. The column temperature was maintained at 25°C. Jasco Borwin software was applied for acquiring the data and processing. On the basis of retention time and analysing UV spectra the peaks were identified by comparing them with reference standards confirming them by running the samples with a small amount of the standards. The chromatographic peaks of lupeol and betulinic acid in extract were confirmed by comparing their retention time and UV spectra with lupeol and betulinic acid standards. Quantification was done by the integration of the peak using external standard method. Peak areas of the standard and the sample solutions injected into the chromatograph were recorded. From the peak area of betulinic acid and lupeol the amounts in extract were computed by an external standard method.

In silico docking

The crystal structure of Thyroid hormone receptor β protein (PDB code: 3GWS) and TSHR (2XWT) were retrieved from Research Collaboratory for Structural Bioinformatics (RCSB) (https://www.rcsb.org.). The Phyto ligand with the PubChem CID Betulinic acid (64971), Lupeol (637775), Triiodothyronine (5920) standard for 3GWS and 2XWT;

the PubChem CID Betulinic acid (64971) and Lupeol (259846) were retrieved from the PubChem database. The target protein preparation including the removal of complexed inhibitor, heteroatoms and water molecules were performed in the discovery studio visualizer while the minimization of energy was carried out in the Autodock tool. The Ligand energy minimization was performed in the Chemdraw ultra tool software. The Phyto ligand betulinic acid, Lupeol were docked with the target protein TSHR and TRβ receptor which are crucial targets for developing thyroid agonists. We compared the results with triiodothyronine as positive control which acts as a stronger agonist for identifying potential new agonists.

Results

Experiment 1.

Dose standardization

When a preliminary experiment was done to determine the effective dose of the bark extract on the alteration of thyroid hormones in euthyroid adult female albino Wistar rats, out pf 3 different doses, 50,100 and 200 mg/kg/day of SA extract administered orally for 28 days, the moderate dose, 100 mg/kg significantly ($P < 0.001$) increased the level of thyroid hormones. Therefore, this dose was selected as the effective dose and used for further evaluation (Table S-1).

Experiment 2.

Body weight

Body weight of rats of control and experimental groups are presented in Table S-2. The final body weight of PTU-induced rats was significantly ($P < 0.001$) reduced in comparison with that of control. When 100 mg/kg of test plant extract was administered along with PTU no significant changes in final body weight were observed in SA extract or L-T_4 in PTU-induced rats. SA alone also not showed significant changes in body weight was observed.

Thyroid hormones and 5'D1 activity

Results are depicted on Table 1. Serum T_3 and T_4 levels decreased and TSH level increased significantly ($P < 0.001$) in PTU-induced rats, as compared to that of control group. A marked reduction in 5'D1 activity ($P < 0.001$) was also observed in these hypothyroid animals. On the other hand, SA extract in euthyroid rats significantly ($P < 0.001$) increased T_4 levels with a decrease in TSH ($P < 0.05$) as compared to that of control group. Significant increase in T_3, T_4 and hepatic 5'D1 activity with decrease in TSH level ($P < 0.001$ for all three) was also observed in PTU-induced SA-treated animals. In PTU+T_4-treated group also, significant increases in T_3, T_4 and hepatic 5'D1 activity with decrease in TSH level ($P < 0.001$ for all three) were observed as compared to that of PTU treated group.

Table.1. Effects of *Saraca asoca* methanolic extract (SA) at 100 mg/kg, for 20 days in euthyroid or in PTU-induced hypothyroid rats on the different biochemical parameters in comparison to that of a standard drug, T_4 in rats.

	Control	PTU	SA	PTU+SA	PTU+T_4
T_3(ng/ml)	0.75±0.06	0.21±0.04***	0.86±0.09	0.78±0.06###	0.69±0.04###
T_4(ng/ml)	52.23±3.29	21.8±2.88***	86.12±4.19***	66.18±3.88###	54.16±4.14###
TSH (μIU/ml)	1.21±0.06	9.45±1.33***	0.92±0.08*	2.64±0.08###	2.99±0.09###
5'D1(ngT_3 formed/hr/ mg protein	2.26±0.31	0.89±0.18***	2.97±0.39	3.17±0.21###	2.29±0.34###
LPO (nM MDA formed/mg protein)	0.45±0.03	1.8±0.04***	0.27±0.04**	0.51±0.04###	0.58±0.06###
SOD (Units/mg protein)	7.4±0.71	3.1±0.23***	8.1±0.68	7.76±0.52###	6.12±0.57
CAT (μM of H_2O_2 decomposed /min/mg protein)	48.79±3.04	22.08±3.87***	67.04±4.59***	46.67±2.99###	32.49±2.37###
GSH (μM/mg protein)	5.65±0.38	1.98±0.14***	6.17±0.31	5.19±0.26###	4.41±0.43###
TNF-α (pg/ml)	18.25±2.99	90.76±6.31***	12.88±1.87**	20.08±3.11###	38.77±4.16###
ALT(IU/L)	31.25±3.11	98.66±3.79***	28.66±2.73	42.43±4.10###	52.73±4.29###
AST (IU/L)	101.64±5.91	193.56±5.78***	98.99±4.29	107.88±4.22###	122.88±5.10###
CHOL (mg/dl)	84.34±5.66	180.34±3.88***	52.11±2.14***	96.56±4.23###	100.21±5.98###
TG (mg/dl)	76.12±2.79	201±6.11***	63.22±2.38*	88.98±4.88###	92.98±3.66###

Data are in means ± SEM, n=7. ***, P< 0.001,**P< 0.01 as compared to the respective control value; whereas ###, P< 0.001 as compared to the respective value of PTU-induced animals. PTU+ SA and PTU+T_4 treated rats were compared with that of PTU alone treated group, while values of PTU-induced and SA treated rats were compared with respective value of control/euthyroid rats.

Abbreviations; Triiodothyronine (T_3); thyroxine (T_4); thyroid stimulating hormone (TSH); 5'D1 (5'-deiodinase); superoxide dismutase (SOD), catalase (CAT), glutathione (GSH); tumour necrosis factor-α (TNF-α); Alanine aminotransferase (ALT);Aspartate aminotransferase (AST);Total cholesterol (TC);Triglycerides (TG).

Anti-inflammatory and liver function

In PTU-treated animals TNF-α level in serum increased significantly as compared to that of control group (P < 0.001) (Table 1). Administration of SA or T_4 to PTU-induced rats resulted in significant decline (P < 0.001) in this as compared to the PTU-alone treated animals. SA treatment to euthyroid rats also significantly decreased (P < 0.05) TNFα level as compared to the value of control animals.

Activities of ALT and AST in PTU alone treated group increased significantly (P < 0.001) as compared to the respective value of control rats (Table 1). But administration of SA orT_4 to PTU-induced hypothyroid rats resulted

in significant ($P < 0.001$) decrease in the activities of both the enzymes.

Lipid peroxidation and antioxidant levels

While a significant increase in hepatic lipid peroxidation (LPO) was observed in PTU treated group ($P < 0.001$), administration of SA or T_4 to PTU-induced rats significantly decreased ($P < 0.001$) the same (Table 1.).SA treatment to euthyroid animals also decreased hepatic LPO significantly ($P < 0.01$) as compared to the value of control animals.

A significant ($P < 0.05$) decrease in SOD, CAT activity and GSH levels in the PTU-induced rats was observed as compared to that of control group. However, SA or T_4 treatment in PTU-induced animals significantly increased ($P < 0.001$) the levels of SOD, CAT and GSH as compared to PTU alone treated animals (Table 1). SA in euthyroid animals also significantly increased SOD, CAT activity and GSH levels as compared to control animals ($P < 0.001$ for all).

Serum lipids

Table 1. shows the effects of SA extract on the levels of TC and TG in experimental rats. The PTU-induced rats had significantly higher ($P < 0.001$) levels of TC and TG as compared to that of control group. However, SA or T_4 treatment in PTU-induced hypothyroid animals, decreased their levels significantly ($P < 0.001$ for both) exhibiting its hypolipidemic effects. Also, a significant reduction in cholesterol ($P < 0.001$) and triglycerides ($P < 0.05$) was observed in the SA treated animals supported its antilipidemic property.

Liver histology

Results are depicted in Fig. 1. In liver histology of euthyroid animals, hepatocytes, their cord pattern, and centrilobular region appeared normal. In PTU-treated rat liver, severe damage is observed in the liver sections showing infiltration of inflammatory cells, improperly arranged hepatocytes around the central vein, irregular orientation of hepatic cords, and centrilobular necrosis. However, SA or T_4 treatment to PTU-induced animals led to marked improvement of liver histology as most hepatocytes showed a normal appearance with regular arrangement, and the cellular injury was reduced.

Phytochemical profiling of the SA bark extract.

Liquid chromatography-mass spectrometry (LC-ESI-MS/MS) was done to determine the constituents of SA. Total ion chromatograms of SA extract shown in Fig. 2a (a,b) displayed numerous peaks and the important peaks were studied using positive and negative electrospray ionization mode. The main phenolic compounds of SA (gallic acid, caffeic acid, sinapic acid, syringic acid and ferulic acid) and the pentacyclic triterpenoids, betulinic acid and lupeol were identified by comparing retention time, accurate mass, and MS/MS fragments with authentic standard through LC-ESI- MS analysis. Mass spectra of identified triterpenoid compound betulinic acid and lupeol is displayed in Fig. 2b (a, b) and other 14 compounds were tentatively identified with available literature by comparing their MS and MS/MS data and are shown in (Table 2). BA was detected with a precursor ion at m/z 455.3, corresponding to the deprotonated molecular ion $[M–H]^-$. The Mass fragmentation pattern of betulinic acid revealed key diagnostic fragment ions at m/z 175,207.1 and 409.3. Lupeol was identified at m/z 426.4 and the characteristic mass fragments were observed at m/z 411.2,218.1 and 207.1. in positive ion mode. All of the phytochemicals detected in SA are listed in Tables 2 and 3.

Table 2
Phytochemicals in *Saraca asoca bark* extract, identified from LC–MS/MS data in positive ion mode with retention time and molecular weight.

Peak no	RT (min)	Empirical formula	MS/MS Fragment ions	Parent ion (m/z)	Identification
1	0.16	$C_{10}H_{10}O_4$	-	194.18	Ferulic acid
2	1.10	$C_{16}H_{18}O_9$	-	170.1	Gallic acid
3	5.81	$C_{27}H_{30}O_{15}$	284.1 ,255.3,343	593.2	kaempferol-3-O-rutinoside
4	6.27	$C_9H_8O_4$	163.1	181.1	Caffeic acid
5	8.08	$C_9H_{10}O_5$	---	197.1	Syringic acid
6	8.43	$C_{15}H_{12}O_7$	153.1	304.2	Taxifolin
7	9.40	$C_{15}H_{10}O_6$	229.2	287.2	Kaempferol
8	15.32	$C_{30}H_{26}O_{12}$	177	559.3	proanthocyanidin B
9	16.90	$C_{16}H_{18}O_8$	211,227.2	337	5-Coumaroyl quinic acid
10	19.78	$C_{24}H_{26}N_2O_{13}$	m/z 393 ,m/z 343	551.4	Betanin
11	20.33	$C_{15}H_{10}O_7$	151,279	301.2	Quercetin
12	20.97	$C_{28}H_{34}O_{16}$	302.2,317.2	634.7	isorhamnetin-3-O-rutinoside
13	22.55	$C_{11}H_{12}O_5$	205	224.2	Sinapic acid
14	23.72	$C_{30}H_{50}O$	207.1,218.1,409.3	426.72	Lupeol
15	31.84	$C_{29}H_{50}O.$	---	413.4	Beta-sitosterol
16	32.10	$C_{16}H_{18}O_9$	------	354.3	Chlorogenic acid

Quantification of betulinic acid and lupeol in SA extract through HPLC analysis

The HPLC chromatogram is shown in Fig. 2c (a, b). The retention time for lupeol (peak-1) and betulinic acid (peak-2) was 8.11 and 12.56 min respectively, and 8.19 and 12. 85 in *S. asoca* extract which were both very close to the standard values. Quantification was done by the integration of the peak using external standard method. Methanol extract of SA was found to have 18.8 mg/g of betulinic acid and 12.2 mg/g of lupeol.

Molecular docking analysis

Molecular docking analyses of lupeol and betulinic acid revealed that BA and LPL had the most stabilized interactions with TRβ and TSHR (Table 4). Betulinic acid and Lupeol showed excellent docking with TSHR (-8.4 kcal/mol and – 8.2 kcal/mol) and TRβ (-8.3 kcal/mol and – 8.1 kcal/mol) respectively as compared to positive control T_3 (-6.5 and – 8.2 kcal/mol for TSHR and TRβ respectively), suggesting its receptor-mediated mechanism for its thyroid stimulatory potential. Docking studies of BA and LPL were displayed in Fig. 3(a, b).

Table 4 Molecular docking of Betulinic acid, Lupeol and Triiodothyronine with TRβ and TSHR receptor and their binding energy.

TSHR	Binding ligand	Free energy of binding (kcal/mol)
TRβ	**Betulinic acid**	**-8.4**
	Lupeol	**-8.2**
	Triiodothyronine	**-6.5**
	Betulinic acid	**-8.3**
	Lupeol	**-8.1**
	Triiodothyronine	**-8.2**

Discussion

The present results clearly show that administration of SA extract has a thyroid stimulatory effect. To the best of our knowledge, no data have been published so far on SA extract in the regulation of thyroid function. In the present study, the effect of SA extract on thyroid hormones levels, oxidative stress markers, inflammatory markers, lipid levels have been examined in PTU-induced hypothyroid rats.

PTU is the well-established thionamide drug to induce hypothyroidism in animal model. It inhibits thyroid hormone synthesis by interfering with thyroid peroxidase–mediated iodination of tyrosine residues in the thyroid gland at the steps of iodine organification and also inhibit extra thyroidal conversion of T_4 to T_3 in liver and kidney.

Thyroid hormones are known to regulate various metabolism in the body and their insufficient release can induce hypothyroidism leading to inhibition of basic body metabolism. In this study the final body weight in PTU-treated rats of hypothyroid rats was less than the control group and this may be attributed to the decrease in basal metabolism is in agreement with our previous study [11].

The results of this study indicated that PTU administration induced hypothyroidism in rats, as evidenced by reduced thyroid hormone concentrations and high TSH levels, similar to the observation made earlier in hypothyroid humans [21]. However, when 100 mg/kg of the test extract, SA was administered to the PTU-induced hypothyroidic rats, nearly normal TSH, T_3, T_4 levels were restored revealing the ameliorating role of SA in hypothyroidism. The bark extract enhanced the thyroxine concentration significantly in euthyroid animals also again showing the thyroid stimulatory nature of this extract. In fact, this is the first report on the thyroid regulating property of bark extract of *S. asoca*. Of course, bark of another plant was reported by us to exhibit pro-thyroidal role in animal model [22].

Liver plays an important role in thyroid hormone metabolism as major amount of T_3 is generated in this organ due to the extra thyroidal conversion of T_4 to T_3 [23]. In the current study, the decreased 5'D1 activity in PTU-induced

rats. It has been found that the increased 5'D1 activity in SA + PTU treated animals is partly attributed to enhanced conversion of T_4 to T_3 in the liver further suggesting the thyroid stimulatory potential of SA extract in PTU-induced hypothyroidism.

Receptors for TNF-alpha, present in thyroid follicular cells have been implicated in the cytotoxic mechanisms that leads to the thyroidal destruction in autoimmune thyroid disease [24]. It was also demonstrated that an increase in serum level of TNF-α inhibit the peripheral generation of T_3 from T_4 decreasing formation and release of T_3 [25]. Therefore, a decrease in TNF-alpha levels not only indicates the anti-inflammatory property of the test extract, but also is related to the increase in 5'D1 activity by SA bark extract in PTU-induced animals supporting, its anti-inflammatory and organ/tissue protecting activities of SA extract as suggested by Ahmad et al [26]. The clinical diagnosis of liver dysfunction is usually based on the estimations of blood AST and ALT activity levels [27]. An increase in ALT and AST levels for PTU treated groups of animals was observed compared to the control group indicating the hepatotoxic effects of PTU. Interestingly, SA extracts demonstrated hepatoprotective effects manifested by normalization of serum liver enzymes AST and ALT level compared with the group given PTU only. We emphasize for the first time that SA extract can alleviate the serum levels of ALT and AST in hypothyroid rats.

It has been previously reported that hypothyroidism have been associated with oxidative stress [10]. Our study revealed higher levels of TSH, increased lipid levels, decrease in antioxidants resulted elevation of lipid peroxides in PTU intoxicated rats. These results are in line with the previous report [28]. Interestingly, treatment with SA suppressed LPO and enhanced antioxidant defences in liver tissues in PTU-induced animals. These observations indicate the antiperoxidative effects of the test plant extract. Similar to our findings, previous studies have also shown that SA bark extract possesses potent antioxidant activities [29].

Hypothyroidism is frequently associated with dyslipidaemia. Earlier studies on hypothyroid animals demonstrated a direct relationship between serum TSH, total cholesterol and triglyceride levels [30]. We found reduction in the level of serum cholesterol and triglycerides in PTU-induced rats by SA, suggesting that SA can ameliorate the abnormal lipid metabolism in hypothyroidism and these findings are consistent with previous report [31].

Histological examination of the liver from the present study revealed treatment with SA restored normal histological appearance in PTU-induced hypothyroid animals. The reduction of ALT and AST activities following SA administration, observed in this study, can be considered as evidence for the prevention of hepatic damage by SA extract. Thus, these results showed that SA possess hepatoprotective activity and the possible mechanism for its hepatoprotective activity may be due to the antioxidant property of phytoconstituents present in bark [32].

Five phenolic compounds such as gallic acid, caffeic acid, sinapic acid, syringic acid and ferulic acid and two pentacyclic triterpenoids, such as betulinic acid and lupeol were identified by comparing retention time, accurate mass, and MS/MS fragments with authentic standards through LC-ESI- MS analysis. Earlier research has demonstrated that aforesaid phenolic compounds exhibit thyroid stimulatory activity through enhancing the increasing the secretions of thyroid hormones [33–36]. Previously, the two pentacyclic triterpenoids, such as betulinic acid and lupeol were reported to be thyroid stimulatory [5, 37].

Molecular docking analyses of lupeol and betulinic acid revealed that BA and LPL had the most stabilized interactions with TRβ and TSHR. TSHR is an important thyrocyte membrane protein in the thyroid gland. Its main function is to regulate thyroid hormone synthesis and release. TSH binds with high affinity to the TSHR and stimulates thyroid gland to produce the thyroid hormones. Our findings revealed that in case of TSHR, betulinic

acid exhibited superior docking scores (-8.4 kcal/mol) as compared to standard T_3 (-6.5) suggesting its receptor-mediated mechanism for its thyroid stimulatory potential. TRβ receptors are the most potent regulators of the production of TSH and most effects of thyroid hormones on the liver and other tissues are related to the β-forms of the receptor [38].TRβ the predominant isoform of TR in the liver and is primarily responsible for the reduction of cholesterol levels, thus in this study better binding mode of BA and LPL with TRβ highlighted its therapeutic potential in lipid disorders associated in thyroid function. Earlier study also showed that TRβ has the major role in regulating D1 expression in liver and kidney, that helps in T_3 generation [39]. Thus, our findings suggest that TRβ might have played an important role in increasing the thyroid hormone levels, particularly T_3, through 5'D1 activity. In fact. We, here demonstrate for the first time the thyroid stimulatory role of *Saraca asoca* bark in PTU-induced hypothyroid rats. Our results of molecular docking showed that both the compounds, BA and LPL in SA extract, both possess thyroid-enhancing potential with a better energy score as compared to the conventional drug, T_3.

In conclusion, the present findings reveal, for the first time provides overall evidence for the thyroid stimulatory activity of SA bark extract in PTU-induced hypothyroid animal model. However, animals treated with SA extract or the reference drug thyroxine resulted in a significant improvement in thyroid function as compared to group of PTU-induced hypothyroidism.

Results also suggest its possible use in regulating hyperlipidaemia at least in hypothyroid-induced hyperlipidaemia. We further suggest that the possible mechanism of action of the test drug is related to the thyroid stimulatory action of betulinic acid and other biologically active phenolic compounds such as gallic acid, caffeic acid, sinapic acid, syringic acid and ferulic acid, as identified in the extract through LC-MS analysis. To our knowledge, for the first time, molecular docking of these two triterpenoids was carried out, targeting thyroid hormone receptors TRβ and TSHR proteins. This is the first study that shows the presence of BA and LPL in SA bark extract which act as thyroid hormone receptor β agonist and these two compounds may be exploited for their therapeutic potential to treat hypothyroidism and its associated lipid disorders.

Declarations

Author's contributions

The author, Anand Kar was involved in the conceptualization, design the experiments; reviewing, and editing the manuscript and over all supervision. Meenakshi Singh, Sagarika Biswas and Debolina Chakraborty performed the molecular docking study. Sunanda Panda involved in performing the in vivo experiments, in silico and in vivo data analyses, and writing the manuscript. All authors reviewed the manuscript.

Competing Interests

The authors declare no competing interests.

Ethics Approval and Consent to Participate

Experimental protocols were approved by the Institutional Animal Ethics Committee with a registration number 779/Po/Ere/S/03/CPCSEA and followed the Guidelines for the Use and Care of Experimental Animals in Research.

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Data Availability

All relevant data are within the paper.

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Figures

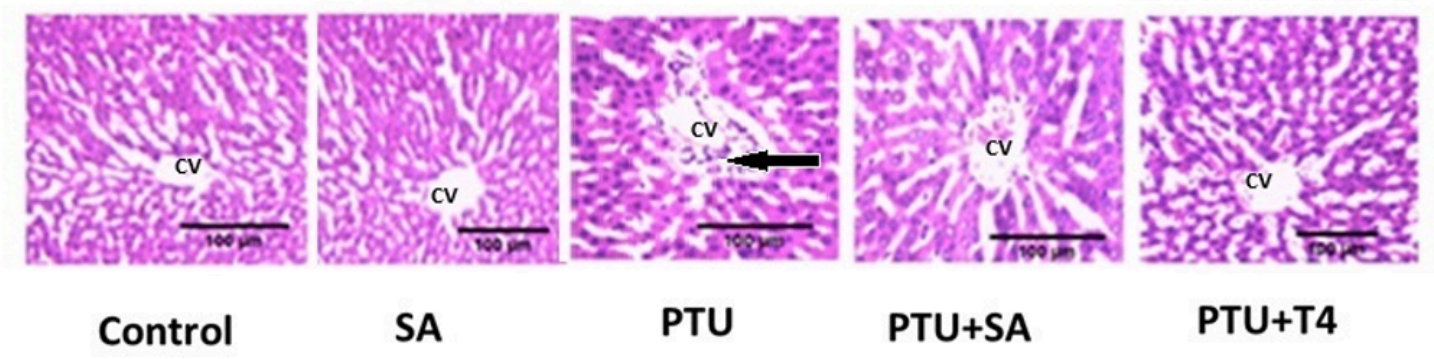
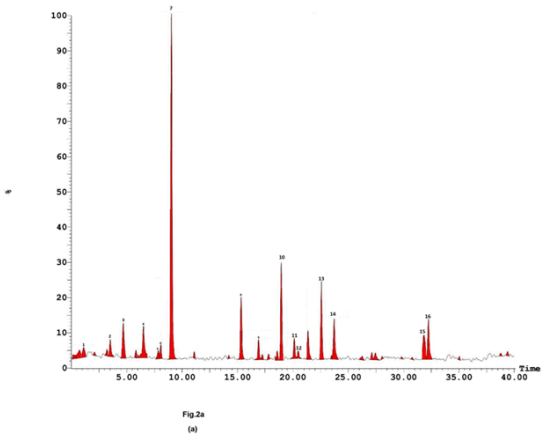
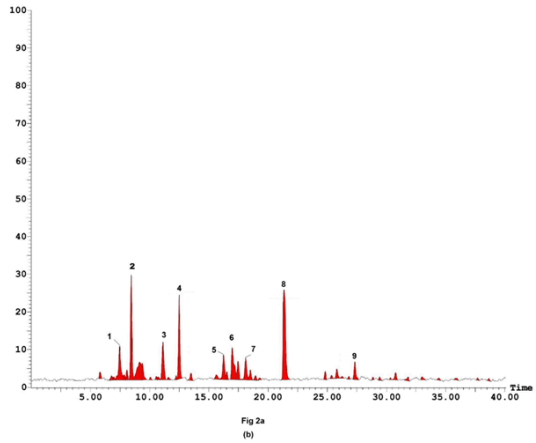


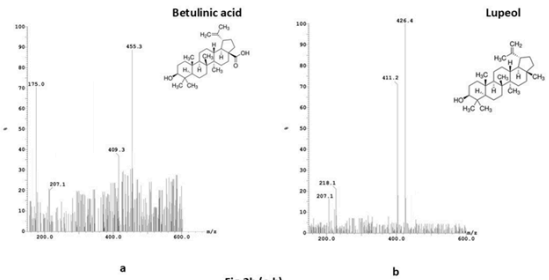
Fig.1

Figure 1

Control group rats showing the normal histological structure with normal central vein (CV) and normal hepatocytes. Treated with PTU showing massive centrilobular hepatocellular necrosis associated with inflammatory cells infiltration (arrow). SA, SA+PTU and SA+T_4 group shows well-organized hepatic structure. (H& E stain) X100; X400; (H&E, scale bar =100µm, X400) (inserted images X 400).







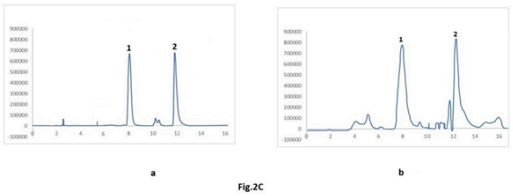


Figure 2

a. Chromatogram of the methanolic extract of *Saraca asoca* bark, using HPLC–Q-TOF/MS. (**a**) Positive ionization mode; (**b**) Negative ionization mode.

Fig.2b (a, b). a, Mass spectra of Betulinic acid and b, mass spectra of Lupeol.

Fig.2c. Representative HPLC chromatograms of Lupeol and betulinic acid detected at 210 nm. (a) Standard chromatogram; Lupeol (1), betulinic acid (2) (b) *Saraca asoca* bark extract; Lupeol (1), betulinic acid (2).

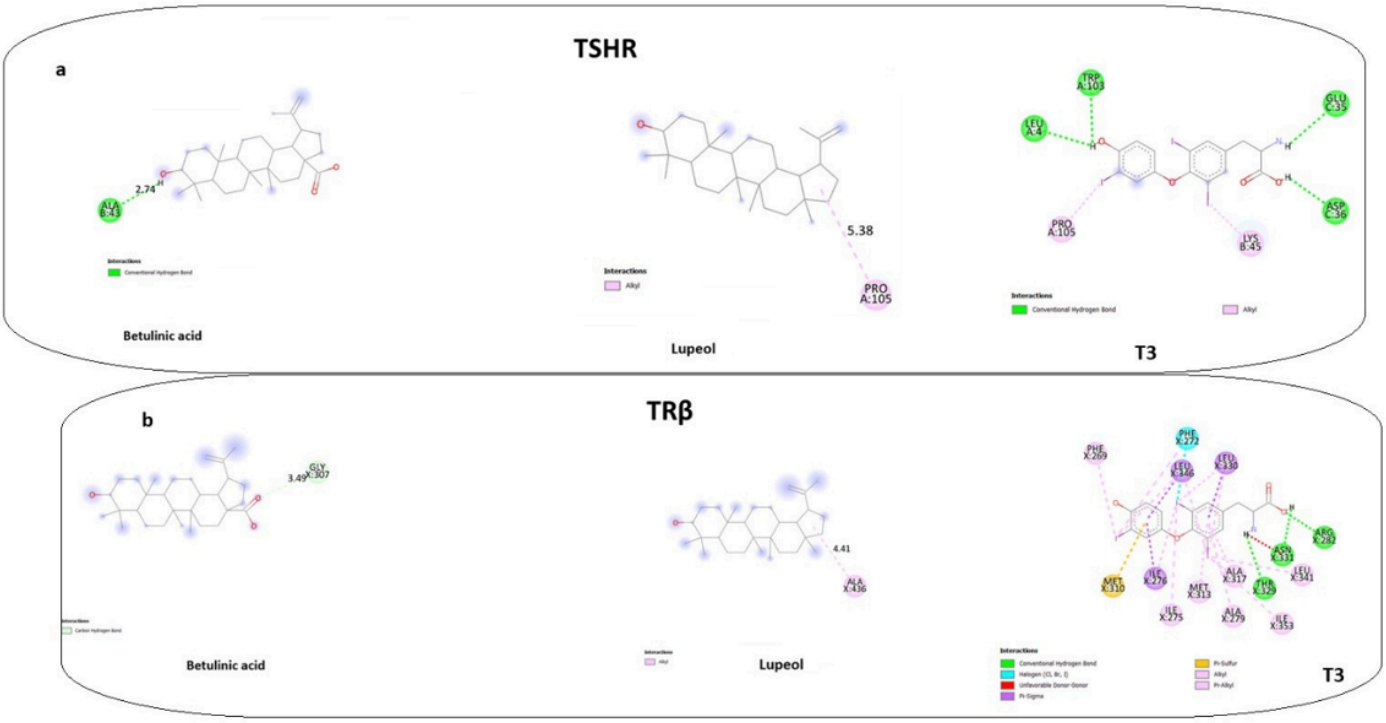


Fig.3(a,b)

Figure 3

(a, b). The interactions of betulinic acid and lupeol in the active site of the TSHR and TRβ receptors in molecular docking.2D view of the best docked ligands against thyroid disease targets; **a**, TSHR interaction with betulinic acid and lupeol, **b**, THRβ interaction with betulinic acid and lupeol.

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