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ORIGINAL ARTICLE

Antithyroidic and Hepatoprotective Properties of High-Resolution Liquid Chromatography–Mass Spectroscopy-Standardized *Piper betle* Leaf Extract in Rats and Analysis of Its Main Bioactive Constituents

Sunanda Panda, Rajesh Sharma, Anand Kar[1]

School of Pharmacy, Devi Ahilya University, [1]School of Life Sciences, Devi Ahilya University, Indore, Madhya Pradesh, India

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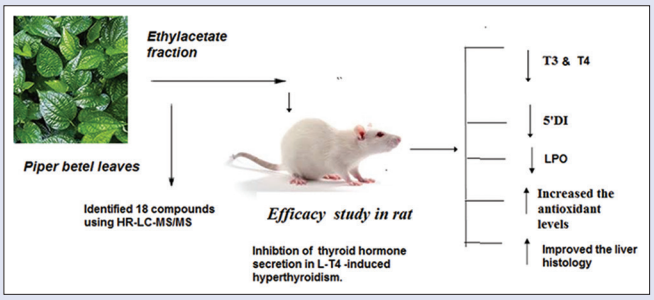
ABSTRACT

Background: Hyperthyroidism can be a serious health problem, if not treated properly. This investigation primarily aimed to evaluate the thyroid regulatory and hepatoprotective activities of ethyl acetate fraction (EPBL) of *Piper betle* leaf extract in L-thyroxine (L-T_4)-induced hyperthyroid rats. **Materials and Methods:** Effects of EPBL extract at a prestandardized dose of 50 mg/kg (p. o.) were studied in L-T_4 (500 µg/kg/d, i. p.)-administered rats to examine the alterations in the levels of serum triiodothyronine (T_3), thyroxine (T_4), thyrotropin, alanine transaminase, and aspartate aminotransferase; in the activities of hepatic 5′-monodeiodinase (5′DI), glucose-6-phosphatase (G-6-Pase), and Na^+-K^+-ATPase; in the level of tissue malondialdehyde (MDA) and lipid hydroperoxides (LOOHs); and in the activities of antioxidants. **Results:** Administration of the EPBL extract reversed the T_4-induced increase in serum thyroid hormones, the liver marker enzymes, MDA, and LOOH but enhanced the activities of antioxidative enzymes and reduced the glutathione content. Light microscopic findings of liver histology revealed distorted hepatic tissue architecture in hyperthyroid animals that were improved by the EPBL administration. High-resolution liquid chromatography–mass spectroscopy analysis revealed four main flavonoid glycosides such as quercetin, rutin, kaempferol, and luteolin. **Conclusion:** For the first time, our findings revealed the antithyroidic property of EPBL in T_4-induced hyperthyroidism, without any hepatotoxicity. The antithyroidic and antioxidative properties of EPBL in hyperthyroid animals could be due to the presence of flavonoid glycosides in the extract which might have inhibited the thyroid hormone secretion and conversion of T_4 to T_3 through an inhibition of 5′DI.

Key words: Antioxidants, high-resolution liquid chromatography–mass spectroscopy, hyperthyroidism, lipid peroxidation, *Piper betle*

SUMMARY

- Efficacy of the ethyl acetate fraction from *Piper betle* leaves (EPBL) was examined for its possible amelioration of L-T_4-induced hyperthyroidism in rats
- L-T_4 administration increased the levels of serum thyroid hormones and decreased the thyrotropin level and antioxidants
- However, the test extract, EPBL, decreased the T_3 and T_4 concentrations and 5′-monodeiodinase activity in hyperthyroid rats showing its antithyroidic potential
- It also reduced the lipid peroxidation and enhanced the antioxidants in the liver indicating its hepatoprotective effects
- The antithyroid activity of the extract is due to the presence of flavonoids, identified by high-resolution liquid chromatography–mass spectroscopy
- EPBL appears to inhibit thyroid function at glandular level as well as at the level of peripheral conversion of T_4 to T_3.



Abbreviations used: EPBL: Ethyl acetate *Piper betle*; HR-LC-MS: High-resolution liquid chromatography–mass spectroscopy; T_3: Triiodothyronine; T_4:Thyroxine; TSH: Thyrotropin; PTU: Propylthiouracil; ALT:Alaninetransaminase;AST:Aspartateaminotransferase;DTT:Dithiothreitol; G-6-Pase: Glucose-6-phosphatase; Na^+-K^+-ATPase: Sodium-potassium adenosine triphosphatase; 5′DI: 5′-monodeiodinase; MDA: Malondialdehyde; LOOHs: Lipid hydroperoxides; SOD: Superoxide dismutase; CAT: Catalase; GSH: Reduced glutathione; GPx: Glutathione peroxidase; CPCSEA: Committee for the Purpose of Control and Supervision of Experiments on Animals.

Correspondence:

Dr. Sunanda Panda,
School of Pharmacy, Devi Ahilya University,
Khandwa Road, Indore, Madhya Pradesh, India.
E-mail: spanda4@rediffmail.com
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INTRODUCTION

Leaves of *Piper betle* L. (Family, Piperaceae) are commonly used as a masticator in Asia and as a traditional medicine in different countries such as India, Malaysia, Indonesia, Philippines, Thailand, China, and many other western countries.[1] Its leaf extract has been reported to stimulate pancreatic lipase activity and to inhibit radiation-induced lipid peroxidation (LPO).[2] The extract also increases the activities of antioxidants.[3] Its hepatoprotective effect is well understood.[4] In fact, the *P. betel* phenolics were found to protect photosensitization-mediated lipid peroxidation in rat liver[5] and to enhance antioxidant

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activities.[6] Indomethacin-induced gastric ulceration was cured by betle leaf phenolics.[7] Further, its methanolic leaf extract was found to exhibit immunomodulatory activity.[8] Despite all these beneficial effects of betle leaf, at present, not a single report is available on its role in regulating hyperthyroidism-induced oxidative stress. The present study is an attempt to determine the thyroid inhibitory and hepatoprotective effects of *P. betle* leaves in hyperthyroid animals. Furthermore, for the first time, we attempted to find the bioactive compounds present in ethyl acetate fraction of *P. betle* (EPBL) using high-resolution liquid chromatography–mass spectroscopy studies.

MATERIALS AND METHODS

Chemicals

L-thyroxine (L-T_4), propylthiouracil (PTU), and dithiothreitol (DTT) were purchased from Sigma Chemical Co. Ltd., St. Louise, USA, while trichloroacetic acid, sodium dodecyl sulfate, Ellman's reagent, and tris buffer were from E. Merck Ltd., Mumbai, India. Thiobarbituric acid (TBA), xylenol orange, sodium azide, ethylenediaminetetraacetic acid (EDTA), and meta-phosphoric acid were obtained from Hi-Media, Mumbai, India, while alanine transaminase (ALT) and aspartate aminotransferase (AST) kits were purchased from ERBA Diagnostics, Germany. ELISA kits for thyroxine (T_4), triiodothyronine (T_3), and thyrotropin (TSH) were obtained from Life Technologies Pvt. Ltd, New Delhi, India.

Preparation of the extract and fractionation

Fresh *P. betle* leaves (Mysore variety) were purchased locally and were scientifically identified by a local taxonomist, Prof. A. Serwani. The voucher specimen was kept in the School of Pharmacy, Devi Ahilya University, with a number: BL 06102. The plant material was shade-dried and then grounded to powder, passed through a #40 sieve (mesh size, 0.425 μm), and stored in an air-tight container. The dried powder (500 g) was refluxed with MeOH for 3 h, and then, the total filtrate was concentrated in vacuum at 40°C to 120 g. The extract was subsequently suspended in distilled water and partitioned with chloroform ($CHCl_3$) and ethyl acetate (EtOAc) to obtain the $CHCl_3$ and EtOAc fractions (46 and 25 g, respectively) and the H_2O residue (49.0 g).

Liquid chromatography–tandem mass spectrometry

Liquid chromatography–mass spectrophotometry (LC-MS/MS) analysis was performed in Hewlett-Packard 1100 (Waldbronn, Germany), composed of a quaternary pump with an online degasser, a thermostatic-column compartment, a photo DAD, an autosampler, and Agilent 1100 ChemStation software. For High Performance Liquid Chromatography (HPLC) separation, Eclipse XDB C18 column (50 mm × 2.1 mm, 1.8 μm, Agilent Company, USA) was used. The elution solvent system was performed by gradient elution using two solvents, solvent A (water containing 0.1% formic acid) and solvent B (acetonitrile containing 0.1% formic acid). A linear gradient elution with a flow rate of 0.2 ml/min was carried out with 20%–30% B for 5 min, then with 30%–50% B for 20 min, 50%–65% B for 10 min, and with 65%–95% B for 10 min to reach 95% B until the run ended. The absorption spectra of eluted compounds were monitored at 280 nm.

The HPLC–MS system consisted of an Agilent HP1100 HPLC unit, equipped with an auto-sampler and a UV-visible absorbance detector, coupled with electrospray ionization (ESI) interfaced Bruker Daltonik Esquire-LC ion trap mass spectrometer (Bremen, Germany) coupled with an Agilent HP 1100 HPLC unit, equipped with an autosampler and an ultraviolet–visible absorbance detector. Eluted components were ionized by ESI, using N_2 for nebulization (1.0 bar) and drying (flow of 7 L/min, temperature of 250°C). The time-of-flight-mass spectrometry (TOF-MS/MS) scan mass spectra were recorded in the positive ion mode. The analysis was achieved in the positive ion mode in a mass range from m/z 50 to -2000.

The EPBL extract was tested in positive ESI-MS ion mode, and peaks were identified based on their T_R and MS fragmentation patterns. All the major compounds were detected with greater sensitivity.

Animals

The experiments were carried out in Wister albino female rats in accordance with the guidelines of our Institutional Animal Ethical Committee, registered with the Ministry of Social Justice and Empowerment, Government of India (reg. No. 779/Po/Ere/S/03/CPCSEA) including the maintenance/handling of animals and administration of drugs. Rats, weighing 160 ± 10 g, were housed in polypropylene cages in a standard light: dark (14 h light: 10 h dark) cycle and in temperature (27 ± 1°C)-controlled room with the provision of laboratory feed (Gold Mohur Feed, Hindustan Lever Limited, Mumbai, India) and water *ad libitum*.

Experimental design

A preliminary experiment was performed to establish the dose-dependent effects of EtOAc EPBL in L-T_4-induced hyperthyroid female rats. Out of 3 different doses (25, 50 and 100 mg/kg, suspended in 1% acacia gum) of EPBL, 50 mg/kg significantly decreased (data not shown) the levels of serum thyroid hormones and hepatic LPO. Therefore, we used 50 mg/kg as the test dose in the final experiment.

In the final experiment, 35 female rats were divided into 5 groups of 7 animals each. Initial body weight of each animal was recorded. Group I animals receiving the drug vehicle, acacia gum (0.1 ml/day/animal) served as control, while Groups II, IV, and V were administered with T_4 (500 μg/kg, i. p., daily) for 12 days to induce hyperthyroidism.[9] After 12 days of L-T_4 treatment, Group II animals, serving as hyperthyroidic control, were also administered with acacia gum (0.1 ml/day/animal), in which the test drug was suspended, while animals of Group III received only 50 mg/kg of EPBL and that of Group IV 25 mg/kg of EPBL and equivalent dose of T_4 as administered in other groups. Animals in Group V continued to receive equivalent dose of T_4, along with PTU at 10.0 mg/kg/day (i. p.) as used earlier.[10] All the treatments were made daily between 10 and 11 h to avoid circadian interference, and the experiment was continued for 15 days. On the last day of experiment, the final body weight of each animal was taken, and overnight-fasted rats were sacrificed under anesthesia. Serum was separated and stored for the estimation of thyroid hormones and liver marker enzymes. The liver was removed quickly, weighed, and homogenized in 10% (w/v) ice-cold phosphate-buffered saline (0.1 M, pH 7.4). Homogenates were centrifuged at 10,000 ×g at 0°C for 20 min, and the supernatant was used for the estimation of different biochemical indices.

Biochemical assays

Estimation of total circulating T_3 and T_4 levels and 5'-monodeiodinase (5'DI) activity were done using ELISA kits following the specific protocol mentioned in each kit.

Hepatic 5'DI activity was determined by the method, described earlier by us.[10] In brief, the liver was homogenized in 4 volumes (w/v) ice-cold phosphate buffer (0.15 M, pH 7.2) with 0.25-m sucrose and 5-mM EDTA. The homogenate was centrifuged at 2000 g for 30 min at 4°C, and then, the supernatant was incubated with T_4 (4 μM) and DTT (4 mM) at 37°C for 1 h. Finally, the incubation was terminated with the addition of absolute ethanol, and the amount of T_3 generated was measured by ELISA as mentioned earlier.

TSH estimation was based on the 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 horseradish peroxidase-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. The optical density of the color solution was measured with a microplate reader at 450 nm.

Serum ALT and AST enzyme activities were assessed using the specific assay kits and according to the manufacturer's instructions. LPO and endogenous antioxidants were determined using the methods routinely used in our laboratory.[11] Malondialdehyde (MDA) was measured with the TBA reaction,[12] while the estimation of tissue lipid hydroperoxides (LOOHs) was done by the method of Jiang *et al.*[13] Activities of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) were evaluated by the method of Marklund and Marklund,[14] Aebi[15] and Ellman[16], respectively. Reduced glutathione (GSH) was estimated by the method of Rotruck *et al.*[17] The activities of hepatic Na^+/K^+ ATPase and glucose-6-phosphatase (G-6-Pase) were measured following the method of Esmann[18] and Fiske and Subbarow[19], respectively. For phosphorus (Pi), the method of Baginske *et al.*[20] was followed. Protein was estimated by the method of Lowry *et al.*[21]

Histological changes

Liver was dissected out, trimmed to approximately 2-mm thickness, and was rapidly fixed in 10% neutral formalin. The fixed tissues were then embedded in paraffin wax, sectioned (5 μm) with a rotary microtome, and stained with hematoxylin and eosin. Liver sections were evaluated histologically with a camera attached to a light microscope (Nikon E400), and its pathology was scored as described by French *et al.*[22] Scores 0, 1, 2, and 3 indicate no visible cell damage, hepatocyte damage <25% of the tissue and mild inflammation, hepatocyte damage, 25%–50% of the tissue and extensive hepatocyte necrosis, respectively [Table 1].

Statistical analysis

Data are presented as mean ± standard error of the mean and were analyzed by one-way ANOVA, followed by *post hoc* Tukey–Kramer multiple comparison test using the GraphPad InStat software (LaJolla, CA, USA). $P < 0.05$ was considered as significant.

RESULTS

Liquid chromatography–electrospray ion source–mass spectrophotometry/mass spectrophotometry analysis

All the identified major compounds have been presented in Table 2. Several peaks were found. However, the major peaks were for quercetin, rutin, syringic acid, epicatechin-(4 β→8)-catechin, kaempferol 3-O-(4"-O-acetyl) rutinoside, piptocarphin B, and luteolin-7-*O*-rutinoside at m/z 301.16, 609.30, 197.12, 579.32, 637.33, 437.17, and 593.30, respectively [Figure 1a-g]. Phenolic acids such as trans-cinnamic acid, chlorogenic acid; allylpyrocatechol-3, 4-diacetate and anethole; the derivative of phenylpropene and a-sesquiterpene, respectively; β-caryophyllene, a bicyclic sesquiterpene and a-sesquiterpene lactone (cnicin) were also identified in leaf extracts of *P. betle.* These peaks were confirmed from the published literature.[23,24]

Effects in body weight, thyroid hormones, and hepatic 5'-monodeiodinase enzyme

While there was a decrease in body weight (b. wt.) of the animals following the administration of L-T_4, nearly normal b. wt. was found in T_4 + EPBL- and T_4 + PTU-treated animals [Figure 2a]. In hyperthyroid animals, serum T_4 and T_3 concentrations and the activity of hepatic 5'DI increased significantly with a parallel decrease in the TSH level, as compared to their respective control values, indicating hyperthyroid condition [Figure 2b]. On the other hand, EPBL extract administration to L-T_4-treated animals significantly decreased the serum T_3 and T_4 concentrations, hepatic 5'DI activity with an increase in serum TSH level, when compared to the respective value of T_4-induced hyperthyroid rats. EPBL alone increased only the concentration of TSH significantly.

Effects in lipid peroxidation and on the levels of antioxidants

Significant increase in hepatic LPO was observed in hyperthyroid animals, whereas EPBL significantly decreased LPO in T_4-induced animals. L-T_4 also decreased SOD, CAT, and GPx activities and GSH level, as compared to that of normal control rats. However, treatment with EPBL in T_4-induced animals significantly increased the aforesaid antioxidant levels as compared to T_4 alone treatment [Table 3]. PTU too decreased hepatic LPO and activity of one antioxidative enzyme, i.e., CAT in T_4-induced animals.

Effects in hepatic G-6-Pase and Na^+-K^+-ATPase activities

Activities of G-6-Pase and Na^+-K^+-ATPase were significantly increased in T_4-induced rats as compared to that of control group. However, administration of EPBL extract to the hyperthyroid rats resulted in a marked decrease in both the activities [Figure 2b]. In PTU + T_4-treated animals also, a significant decrease in the activities of these two enzymes was observed.

Effects in liver marker enzymes

L-thyroxine treatment resulted in a significant increase in activities of ALT and AST, whereas, EPBL at 25 mg/kg in T_4-induced rats reduced their levels significantly [Table 3]. A similar reduction in these marker enzymes was exhibited in PTU + T_4-treated animals.

Table 1: Scoring for histological alterations in the liver under different treatments

Group (s)	Necrosis	Degeneration of hepatocytes	Inflammation	Dilation of the central vein
Control	0	0	0	0
T_4	3	3	3	3
EPBL	0	0	0	0
EPBL + T_4	2	1	1	1
PTU + T_4	2	2	1	1

Liver from each group was scored for hepatic injury through light microscopy with Score 0: No visible cell damage; Score 1: Hepatocyte damage <25% of the tissue and mild inflammation; Score 2: Hepatocyte damage on 25%-50% of the tissue with moderate inflammation; Score 3: Extensive hepatocyte necrosis with severe inflammation. T_4: Thyroxine; PTU: Propylthiouracil; EPBL: Ethyl acetate fraction of *Piper betle*

Table 2: Phytoconstituents identified in the ethyl acetate fraction of *Piper betle* leaf (ethyl acetate fraction) by high-resolution liquid chromatography-mass spectroscopy in positive ion mode

Compounds	Retention time (min)	Molecular formula	Measured Mass (m/z)
1. Syringic acid	1.8	$C_9H_{10}O_5$	197.12
2. Betanin	1.8	$C_{24}H_{27}N_2O_{13}$	551.91
3. Cinnamic acid	53.2.	$C_9H_8O_2$	147.12
4. β-caryophyllene	53.2	$C_{15}H_{24}$	203.19
5. Myricetin	53.2	$C_{15}H_{10}O_8$	318.13
6. Cnicin	53.2	$C_{20}H_{26}O_7$	378.15
7. Allylpyrocatechol-3,4-diacetate	53.2	$C_{13}H_{14}O_4$	235.18
8. Quercetin-3-O-glucoside	53.2	$C_{21}H_{18}O_{12}$	463.29
9. Piptocarphin B	54.5	$C_{22}H_{28}O_9$	437.17
10. Kaempferol 7-O-glucoside	54.5	$C_{27}H_{31}O_{15}$	593.40
11. Rutin	55.6	$C_{27}H_{30}O_{16}$	609.30
12. Chlorogenic acid	55.6	$C_{16}H_{17}O_9$	353.28
13. *p*-hydroxybenzoic acid	55.6	$C_{17}H_{16}O_4$	282.29
14. Epicatechin-(4β→8)-catechin	53.6	$C_{26}H_{26}O_{15}$	579.32
15.Kaempferol-3-O (4”-*O*-acetyl) rutinoside	56.7	$C_{29}H_{32}O_{16}$	637.33
16. Quercetin	53.6	$C_{15}H_{10}O_7$	301.16
17. Anethole	53.6	$C_{10}H_{12}O$	149.03
18. Luteolin-7-*O*-rutinoside	55.8	$C_{27}H_{29}O_{15}$	593.30

TIC: Total ion chromatogram

Table 3: Effects of *Piper betle* leaf extract on the alterations in body weight (g), liver marker enzymes, hepatic lipid peroxidation (nM malondialdehyde/h/mg protein; lipid hydroperoxides, nM/mg protein), superoxide dismutase (units/mg/protein), catalase (μM of H_2O_2 decomposed/min/mg protein), glutathione peroxidase (μ moles of glutathione oxidized/mg protein), and glutathione content (μM/mg protein) in euthyroid- and in thyroxin-induced hyperthyroid rats

Parameters	Control	T_4	EPBL	T_4 + EPBL	T_4 + PTU
Body weight (g)					
Initial body weight	160±3.96	162±4.35	167±4.71	166±3.37	164±4.12
Final body weight	171±1.89	119±3.71[a]	179±3.31	172±3.02	168±3.11
Liver markers					
ALT (IU/L)	36.98±1.60	81.18±2.02***	29.22±1.07*	37.11±2.31###	46.19±3.90###
AST (IU/L)	62.14±2.46	96.58±2.10***	54.19±2.27	52.09±1.13###	73.18±3.98###
Lipid peroxidation and antioxidants					
LPO	0.46±0.05	1.11±0.09***	0.28±0.02*	0.23±0.03###	0.61±0.07##
LOOH	4.09±0.61	8.95±1.14***	3.09.±0.16*	4.12±0.31###	6.59±0.79#
SOD	5.94±0.27	1.17±0.60***	5.16±0.51***	5.92±0.31###	3.97±0.40
CAT	39.17±1.01	14.22±3.96***	71.21±5.39	46.58±3.79###	25.07±3.22#
GSH	4.76±0.31	1.31±0.13***	5.93±0.41	4.99±0.51##	2.01±0.52
GPx	7.17±2.15	2.04±0.28***	9.46±2.36	8.78±1.95###	4.14±1.56

Data are in mean±SEM, n=7. [a]P<0.001 as compared to the initial body weight, *P<0.05 and***P<0.001 as compared to the respective control value, whereas ###P<0.001, ##P<0.01, and #P<0.05 as compared to the respective value of T_4-treated hyperthyroid animals. T_4: Thyroxine; SEM: Standard error mean; ALT: Alanine transaminase; AST: Aspartate aminotransferase; LPO: Lipid peroxidation; SOD: Superoxide dismutase; CAT: Catalase; GSH: Reduced glutathione; GPx: Glutathione peroxidase; PTU: Propylthiouracil; EPBL: Ethyl acetate fraction of *Piper betle*; LOOH: Lipid hydroperoxide

Histopathological observations

While control rat liver showed a normal histological structure, T_4-induced tissues revealed necrosis around the central vein, inflammatory cell infiltration, and hepatocyte damage. In addition, congestion in sinusoidal spaces was observed in the liver of hyperthyroid rats. Administration of the EPBL extract to L-T_4-treated rats improved the liver architecture by reducing the necrosis and inflammation and by an increase in sinusoidal spaces with respect to that of hyperthyroid rats [Figure 3]. PTU treatment in T_4-administered animals also exhibited nearly normal histological features. Hepatocyte necrosis and inflammatory cells were prominent in the T_4-induced animals. On giving a different score to the histological features [Table 1], in hyperthyroid animals, tissue damage score found to be more which was reduced following the simultaneous administration of EPBL, reflecting the beneficial effects of the test extract.

DISCUSSION

The test plant extract was found to possess the potential to ameliorate hyperthyroidism and to protect the L-T_4-induced rats from hepatic LPO. When L-T_4 was administered to animals, it significantly decreased the body weight and increased the serum T_3 and T_4 levels and the activities of hepatic 5’DI, G-6-Pase, and $Na^{+/}K^+$ ATPase with a decreased TSH level, as also observed earlier.[10] However, treatment with EPBL extract decreased the levels of both the thyroid hormones and increased TSH level in the hyperthyroid animals, suggesting an inhibition in thyroid hormone synthesis. Interestingly, hepatic 5’DI activity was also decreased ascertaining that the test compound primarily inhibited extracellular conversion of T_4 to T_3, the main pathway of T_3 production. Thus, EPBL fraction appeared to have the potential to inhibit thyroid hormone synthesis. This finding is in line with an earlier observation with flavonoids that also exhibited thyroinhibitory action in rats.[25]

Similarly, the increase in Na^+-K^+-ATPase and G-6-Pase activities in the liver of hyperthyroid animals is in accordance with an earlier report.[26] However, EPBL decreased both Na^+-K^+-ATPase and G-6-Pase activities in the hyperthyroid rats, further supporting the thyroinhibitory role of the test extract.

Serum ALT and AST are sensitive markers of liver damage, and their high levels are commonly seen in response to oxidative stress induced

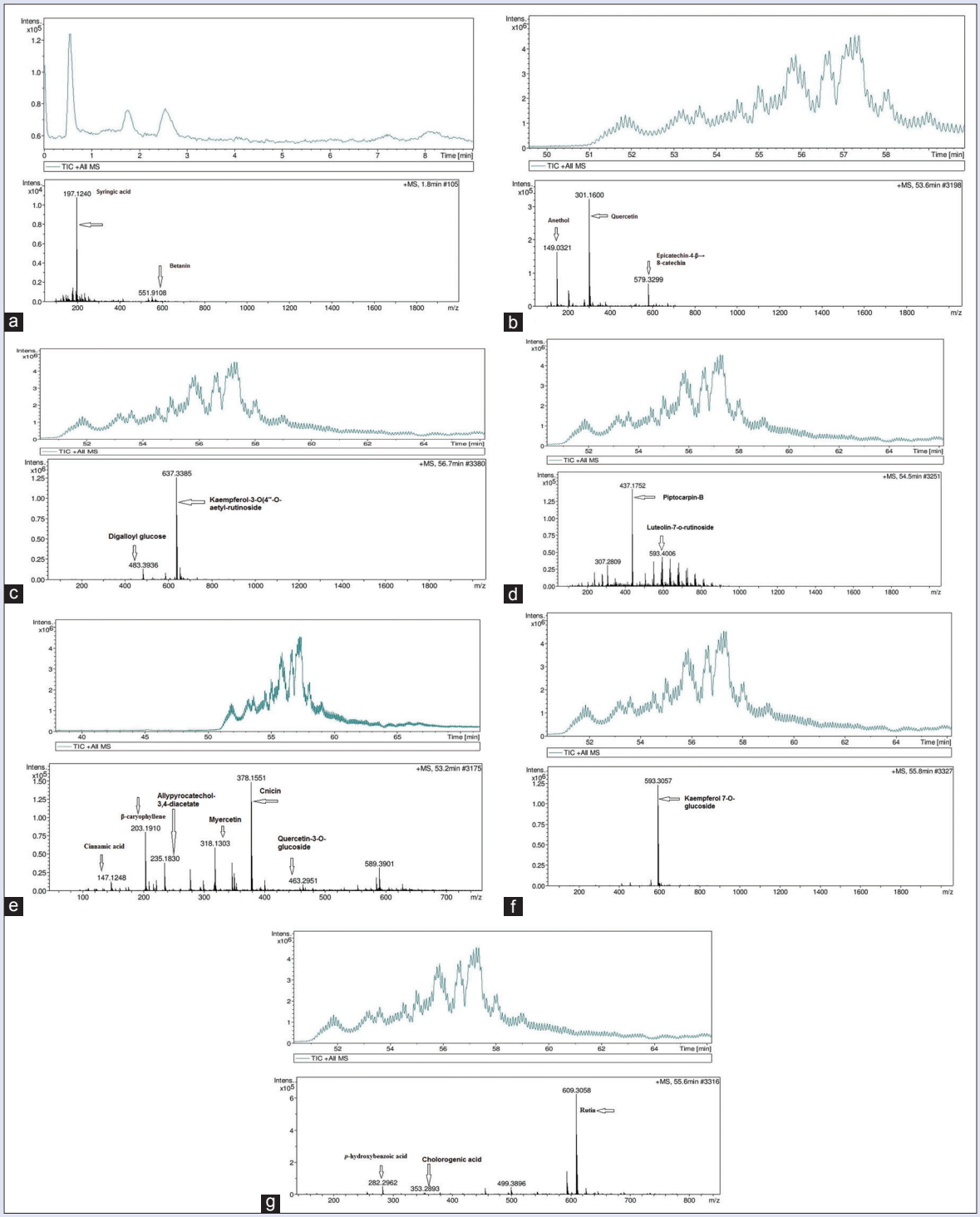

Figure 1: (a) Liquid chromatography–electrospray ion source–mass spectrophotometry/mass spectrophotometry spectra $(M + H)^+$ ions and the total ion chromatogram in full scan mode present in ethyl acetate extract fraction of *P. betle* leave showing syringic acid and betanin. (b) Liquid chromatography–electrospray ion source–mass spectrophotometry/mass spectrophotometry spectra $(M + H)^+$ ions and the TIC of ethyl acetate *Piper betle* fraction showing epicatechin-4-β-8 → catechin, quercetin, and anethole. (c) Liquid chromatography–electrospray ion source–mass spectrophotometry/mass spectrophotometry spectra $(M + H)^+$ ions and the TIC of ethyl acetate *Piper betle* fraction showing kaempferol-3-O (4″-O-acetyl-rutinoside and diallyl glucose. (d) Liquid chromatography–electrospray ion source–mass spectrophotometry/mass spectrophotometry spectra $(M + H)^+$ ions and the TIC of ethyl acetate *Piper betle* fraction showing piptocarphin B and luteolin-7-*O*-rutinoside. (e) Liquid chromatography–electrospray ion source–mass spectrophotometry/mass spectrophotometry spectra $(M + H)^+$ ions and the TIC of ethyl acetate *Piper betle* fraction showing quercetin-3-*O*-glucoside, cnicin, myricetin, allylpyrocatechol-3, 4-diacetate, β-caryophyllene, and cinnamic acid. (f) Liquid chromatography–electrospray ion source–mass spectrophotometry/mass spectrophotometry spectra $(M + H)^+$ ions and the TIC of ethyl acetate *Piper betle* fraction showing kaempferol 7-O-glucoside. (g) Liquid chromatography–electrospray ion source–mass spectrophotometry/mass spectrophotometry spectra $(M + H)^+$ ions and the TIC of ethyl acetate *Piper betle* fraction showing rutin, chlorogenic acid, and *p*-hydroxybenzoic acid

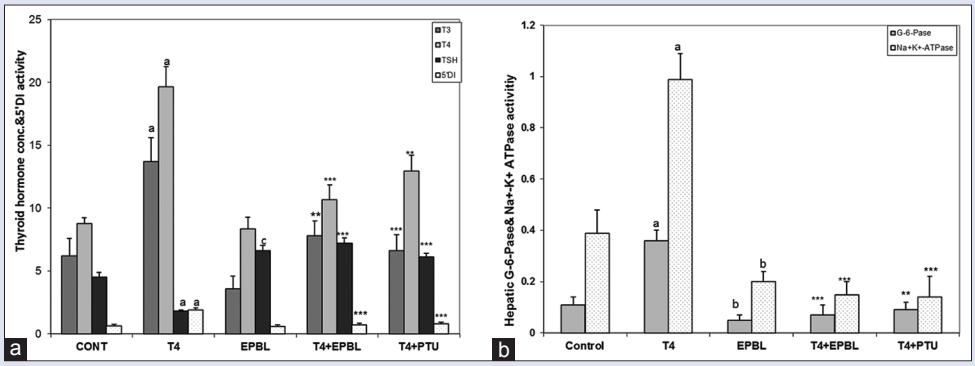

Figure 2: (a) Changes in concentrations of serum T_3 (ng/ml × 10^{-1}), T_4 (ng/ml × 10) and thyrotropin (μIU/ml) levels and in hepatic 5'DI (ng/ml/h) activity, following the administration of ethyl acetate *Piper betle* (50 mg/kg/d) to the L-T_4-induced animals. Each vertical bar represents the mean ± standard error of the mean. (n = 7). $^aP < 0.001$, $^bP < 0.01$, and $^cP < 0.01$ as compared to the respective control value, whereas ***$P < 0.001$ and **$P < 0.01$ as compared to the respective value of thyroxine-treated animals. One-way ANOVA, followed by Tukey's posttest. Ethyl acetate *Piper betle*-induced amelioration in thyroid indices in hyperthyroid rats is clearly observed. (b) Changes in hepatic G-6-Pase (μM of Pi liberated/h/mg protein) and Na^+-K^+-ATPase activity (μM of Pi liberated/h/mg of protein) following the administration of ethyl acetate *Piper betle* (50 mg/kg/d) to the L-T_4 -induced animals. Each vertical bar represents the mean ± standard error of the mean (n = 7); $^aP < 0.001$ and $^bP < 0.01$ as compared to the respective control value, whereas ***$P < 0.001$ and **$P < 0.01$ as compared to the respective value of thyroxine-treated animals. One-way ANOVA, followed by Tukey's post-test. Ethyl acetate *Piper betle* reverses the changes induced by T_4

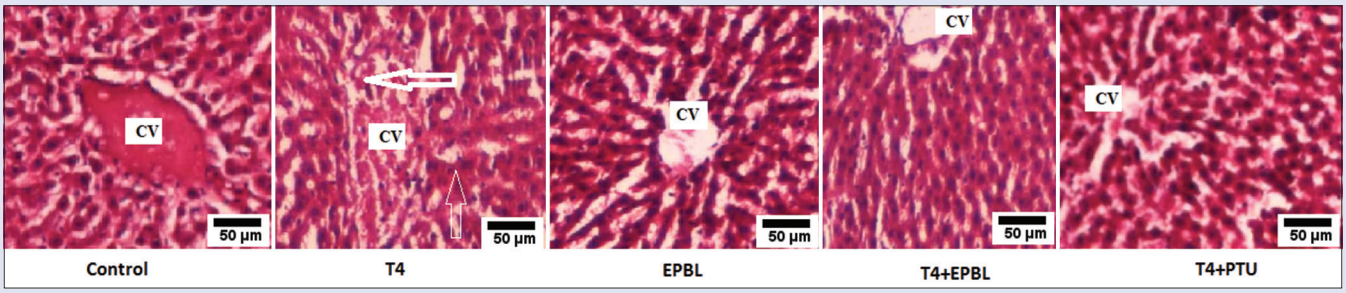

Figure 3: Histological photographs (H and E, 40) of rat liver cells (scale bar, 50 μm). Liver sections of control rat show normal architecture, whereas T_4-induced cells present necrosis around the central vein (arrow) and severe inflammation. While no change in ethyl acetate *Piper betle*-treated rat liver is observed, T_4-induced ethyl acetate *Piper betle*-treated rat shows marked decrease in necrotic and degenerative changes, milder inflammation, and protection from centrilobular necrosis. Liver sections of the animals administered with propylthiouracil in T_4 induced show only moderate degree of liver damage

by hyperthyroidism.[27] We too observed similar effects in thyrotoxic animals. However, following EPBL treatment, both the enzymes were decreased, suggesting the hepatoprotective nature of the test compound.

The process of LPO involves oxidative conversion of polyunsaturated fatty acids (PUFA) to a product known as MDA, which is usually measured as Thiobarbituric acid reactive substances (TBARS) or lipid peroxides, the most studied biologically relevant products of free radical reaction.[28] The present study also showed that hepatic LPO products such as TBARS and LOOH were increased after T_4 administration as also observed earlier.[29] However, EPBL treatment to L-T_4induced rats protected the liver against lipid peroxidative damage.

A significant decrease in the endogenous antioxidants such as SOD, CAT, GPx, and GSH in the liver was observed in hyperthyroid rats, which is in line with the earlier report.[30] This reduction in the activity of aforesaid enzymes could be the result of increased generation of superoxide and hydrogen peroxide radicals, which in turn lead to reduction in the activity of these enzymes. The observed decrease in GSH levels in T_4-induced rats could possibly be due to its conversion to oxidized glutathione (GSSG) or due to decreased synthesis under oxidative stress. Interestingly, in hyperthyroid rats, EPBL was able to reduce the level of LPO and normalized the antioxidant levels indicating its antioxidative potential/hepatoprotective effects. As all the antioxidants were not enhanced by PTU, but by the test extract, it seems that the EPBL extract is more antiperoxidative than the known antithyroid drug. The observed antioxidative potential of the test extract could be due to the presence of flavonoids in EPBL that are known to enhance endogenous antioxidants.[31]

As both the thyroid hormones were reduced by the test extract, it appears that the antithyrodic role of EPBL is mediated through inhibition in synthesis and/or release of T_4, the predominant hormone of thyroid gland, and also by inhibiting 5'DI activity in liver, the prevalent site of T_3 generation. This may be emphasized that till to date, the compounds in ethyl acetate fraction were not identified using LC-MS, and the role of the fraction in relation to regulation of thyroid function was not clear. Therefore, the present report appears to be the first one on this aspect suggesting the potential of the EPBL in the amelioration of hyperthyroidism and the oxidative stress induced by it. We believe that the antithyroid role of test extract is most likely due to the presence of flavonoids including quercetin, rutin, kaempferol, and luteolin that were identified as major peaks in LC-MS/MS study.

CONCLUSION

EPBL was helpful in minimizing the pathophysiology of T_4-induced hyperthyroidism in rats. Furthermore, it was found to be hepatoprotective

with a reduction of the oxidative stress, suggesting its ameliorative role in thyrotoxicosis. It appears that the antithyroidic action of EPBL extract is primarily mediated through inhibition of synthesis and/or release of thyroid hormones as well as by inhibiting 5'DI activity, the major process of T_3 generation.

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Conflicts of interest

There are no conflicts of interest.

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